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Probing The Adaptive Significance Of Circadian Rhythms Using *Drosophila melanogaster*

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A Thesis

Submitted for the Degree of

Doctor of Philosophy

By

SHEEBA V

To

MANIPAL ACADEMY OF HIGHER EDUCATION

Through

JAWAHARLAL NEHRU CENTRE FOR ADVANCED SCIENTIFIC RESEARCH, BANGALORE-560 064, INDIA

NOVEMBER 2001

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Dedicated to

My Parents

for their support and encouragement

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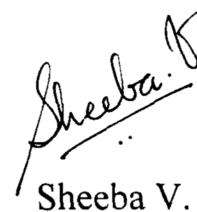
DECLARATION

I declare that the matter presented in my this thesis entitled "Probing The Adaptive Significance Of Circadian Rhythms Using *Drosophila melanogaster*" is the result of studies carried out by me at the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under the supervision of Prof. Amitabh Joshi and that this work has not been submitted elsewhere for any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described has been based on the findings of the other investigators. Any omission, which might have occurred by oversight or error of misjudgment, is regretted.



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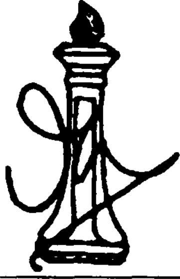

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19 November, 2001

CERTIFICATE

This is to certify that the work described in the thesis entitled "**PROBING THE ADAPTIVE SIGNIFICANCE OF CIRCADIAN RHYTHMS USING *DROSOPHILA MELANOGASTER***" is the result of investigations carried out by Ms. Sheeba V. in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560 064, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

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Summary

The ubiquity of circadian rhythms suggests that they are adaptive and, though there is some evidence supporting this assertion, there have not been conclusive and systematic empirical investigations of the adaptive significance of circadian organisation. Circadian rhythms could in principle, confer an adaptive advantage to organisms in either or both of two ways: (a) by enabling the organism to synchronise its activities to external periodic time cues of light, temperature, humidity, food availability, presence of predators or mates, and (b) by maintaining appropriate phase relationship among the different rhythms within the organism. While previous studies have focussed on the ecologically derived adaptive value accruing from being able to synchronise to external light/dark cycles, we addressed primarily the issue of intrinsic adaptive value of circadian rhythms, deriving from possessing biological clocks *per se*, rather than the ability to use those clocks to appropriately phase one's activities to environmental periodicities.

We studied four separate populations of fruit flies *Drosophila melanogaster* (henceforth, baseline populations), that had been maintained in the laboratory for more than 600 generations in an aperiodic environment with constant light, temperature and humidity. The results of our experiments showed that these flies have retained the ability to exhibit circadian rhythms in eclosion (which is a populational level rhythm), oviposition and locomotor activity (which are individual level rhythms) even after having been in an aperiodic environment for many hundred generations. Unlike populations sampled from the wild or maintained in the laboratory under periodic conditions, variation in free running period is quite large in these populations. It is now well established that mutation accumulation and random genetic drift noticeably affect traits that do not confer any fitness advantage to organisms under a given environment within 100-200 generations. Therefore, our observations strongly support the notion of an intrinsic adaptive

value to possessing biological clocks, as distinct from the necessity to possess biological clocks with periodicity of 24 h.

To assess what components of fitness may contribute to the intrinsic adaptive value, we assayed the effect of three different light regimes, constant light (LL), light/dark cycles of 12:12 h (LD), and constant darkness (DD), on pre-adult components of fitness: egg to adult development time and survivorship and the body weight at eclosion, and also adult components of fitness: lifetime fecundity, adult body weight profile and lifespan in these populations. Pre-adult development was significantly faster in LL as compared to LD, and DD without any attendent fitness cost in pre-adult survivorship or dry weight at eclosion. Adult lifespan of reproducing males and females was significantly shorter in LL as compared to LD and DD, as seen by previous workers, but lifetime fecundity was highest in LL. As reproductive output is far more important to fitness than lifespan, the previously observed reduction of lifespan in LL cannot necessarily be considered as deleterious, as it is offset by higher reproductive output, at least in populations reared for many generations in LL.

In order to establish whether circadian organisation undergoes adaptive evolution in LD regimes, we derived three sets of selected populations from the baseline populations. One derivative each from every baseline population was subjected to one of three light regimes, yielding three sets of four replicate populations subjected to LL, LD and DD regimes respectively. We then sought evidence for consistent patterns of genetic differentiation, in terms of circadian organisation, between the populations subjected to the LD regime and those that were maintained in aperiodic regimes (LL, DD). After about 30 generations of maintenance of the selected populations in their respective light regimes, we assayed the locomotor activity rhythm in the progeny of these twelve populations reared for one generation under a common

regime (LD 12:12 h) to equalise selection regime induced maternal effects. From the activity records, free running period in DD regime (τ_{DD}) and phase angle difference (ψ) in LD were estimated. Similar experiments were also done on the eclosion rhythm. Maintenance under LD for about 30 generations has resulted in a significant shift in the mean τ_{DD} of the locomotor activity rhythm towards 24 h, which is the periodicity of the maintenance regime. The mean ψ of locomotor activity rhythm in LD regime of the populations subjected to 30 generations of maintenance in LD was significantly greater than that of LL selected populations, In the case of the eclosion rhythm, too, ψ was seen to evolve in populations reared under LD for ~ 30 generations, although no statistically significant difference in τ was seen, probably because the evolutionary shift in τ is too small to be detected using a rhythm for which the resolution of sampling the time series is on the order of 2 h. These results on the selected populations clearly demonstrate for the first time that key components of circadian organisation, like τ and ψ, undergo adaptive evolution in a periodic LD regime.

Further studies on the evolutionary genetics of circadian organisation will require a more detailed understanding of both relationships among different rhythms at the genetic and clock organisational levels, and of the interaction of circadian organisation and environmental conditions experienced at different stages in the life history. Consequently, we also investigated the influence of light regimes experienced during pre-adult developmental stages on circadian parameters of the adult locomotor activity rhythms and found that the τ_{DD} was significantly shorter in flies exposed to DD regime during pre-adult stages, as compared to those that were exposed to LL and LD regimes. In a separate study, we assayed the circadian rhythms of eclosion, oviposition and locomotor activity in DD and LD and found that ψ in LD cycles and the τ_{DD} of all the three rhythms significantly differed from one another, suggesting that multiple

circadian pacemakers may be involved in the control of different circadian rhythms within an individual fly. We also observed that the mean τ_{DD} and ψ of oviposition rhythm in flies that eclosed within a narrow window of time in the LD cycle (referred to as "gate of eclosion") was significantly different from those that eclosed outside the gate, suggesting that though separate oscillators may regulate eclosion and oviposition rhythms in *D. melanogaster*, they are not entirely independent.

In the studies described here, we attempted to study the significance of circadian organisation using the tools of modern experimental evolutionary biology, an approach not hitherto used in chronobiology. Our results provide clear evidence for the adaptive evolution of circadian organisation in populations reared in LD for ~ 30 generations after having previously been reared in LL for more than 600 generations. The studies also point towards a complex control of different rhythms by separate, but not independent, oscillators that exhibit developmental plasticity with respect to light regimes experienced during pre-adult stages. The studies also underscore the importance of assaying multiple components of fitness when studying the adaptive significance of circadian organisation.

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1. Sheeba V, Sharma VK, Chandrashakaran MK, and Joshi A (**1999**): Effect of different light regimes on pre-adult fitness in *Drosophila melanogaster* populations reared in constant light for over six hundred generations. **Biol. Rhythm Res.** 30: 424-433.
2. Sheeba V, Sharma VK, Chandrashakaran MK, and Joshi A (**1999**): Persistence of eclosion rhythm in the fruitfly *Drosophila melanogaster* after 600 generations in an aperiodic environment. **Naturwissenschaften** 86: 448-449.
3. Sheeba V, Sharma VK, Shubha K, Chandrashekaran MK, Joshi A (**2000**): The effect of different light regimes on adult lifespan in *Drosophila melanogaster* is partly mediated through reproductive output. **J. Biol. Rhythms** 15:380-392.
4. Sheeba V, Nihal M, Mathew SJ, Swamy NM, Chandrashekaran MK, Joshi A, Sharma VK. (**2001**): Does the difference in the timing of eclosion of the fruit fly *Drosophila melanogaster* reflect differences in the circadian organisation? **Chronobiol. Internat.** 18: 601-612.
5. Sheeba V, Chandrashekaran MK, Joshi A, Sharma VK. (**2001**): Persistence of oviposition rhythm in individuals of *Drosophila melanogaster* reared in an aperiodic environment for several hundred generations. **J. Exp Zool.** 290: 541-549.
6. Sheeba V, Chandrashekaran MK, Joshi A, Sharma VK. (**2001**): A case for multiple oscillators controlling different circadian rhythms in *Drosophila melanogaster*. **J. Insect Physiol.** 47:1217-1225.
7. Sheeba V, Chandrashekaran MK, Joshi A, Sharma VK. (**2001**): Developmental plasticity of locomotor activity rhythm of *Drosophila melanogaster*. **J. Insect Physiol.** (in press)

Chapter 1

Introduction

1.1 The study of adaptive evolution

The phenomenon of adaptation is at the core of modern biology, and natural selection, the mechanism universally regarded as the primary causal influence on phenotypic evolutionary change, provides an explanation for adaptation (Amundson, 1996). From a historical perspective, Aristotle first asked the question of why organisms exhibit certain traits more than two thousand years ago. He was the first to hypothesise that all structures and activities exhibited by organisms have a biological meaning. During the 14th century, the dramatic increase in the awareness of the immense diversity of life forms on the earth resulted in efforts to classify living organisms and also to seek explanations for their relatedness and diversity. Ironically, the rise of natural theology, a philosophy which attempted to understand the various components of the natural environment as works of a divine creator, in the 18th century, helped to lay the foundations of evolutionary biology, as its followers attempted to understand how organisms adapted to one another and the natural environment (Mayr, 1982). Jean Baptiste Lamarck first proposed a theory of evolution that represented a marked departure from tradition. He recognised that organisms are adapted to their environment and concluded that they must change in order to maintain their adaptation to the changing environment. His Theory of Transformation postulated an intrinsic tendency of organisms to strive towards perfection. Although his explanatory endeavours were largely unsuccessful because he depended on the Theory of Acquired Inheritance of Characters, he laid the ground for one of the most profound theories of evolution that soon followed (Mayr, 1982). It was with Charles Darwin's epic "Origin of Species" published in the 1859 that the fundamental principles of evolution and adaptation were first laid out in a systematic and rigorous manner. Now, nearly 150 years later, although several arguments continue on the

intricacies of the mechanics of evolution, the overriding principles of Darwin's treatise continue to retain their validity.

The word "adaptation" has come to acquire several connotations (Pittendrigh, 1958). One sense, in which it is used mostly by physiologists, is the process of acquiring a trait *within* the lifetime of an individual. This process is also referred to as somatic adaptability or phenotypic plasticity and takes place without genetic change in the germline. A second meaning of "adaptation", which is the sense in which I will use the word in the rest of the thesis, implies a historical process in which genetic composition of a population which inhabits a certain environment is altered over generations through the process of natural selection. The word "adaptation" is also used as a noun, representing the product of adaptive evolutionary change, to refer to the characteristics that evolved during the process of adaptation.

The past two and a half decades of research in evolutionary genetics have underscored the fact that adaptive evolution is constrained by several forces (Loeschcke, 1987; Rose et al., 1996). The evolutionary trajectory of a population is a resolution of the forces of natural selection acting on it, its past selection history and ancestry (phylogenetic forces), and chance in the form of random genetic drift (Travisano et al., 1995; Joshi, 1997 a). Random genetic drift occurs when allele frequencies in a population change randomly from one generation to the next due to the sampling error associated with the formation of a finite and small number of zygotes from a very large number of gametes. Drift can act as a genetic constraint to adaptation, especially in small populations, because it tends to erode genetic variation, which is the raw material for adaptive evolutionary change. Another problem faced by small populations is that the actual subset of potential genotypes that is realised at each generation is small, thus acting as a constraint on the variation available for selection to act upon (Kirkpatrick, 1996). Another

genetic force that can constrain adaptive evolution is the presence of negative genetic correlations between traits, which can prevent two traits from being simultaneously maximised by selection. Other constraints to adaptive evolution may be phylogenetic, because certain lineages may not possess genetic variation in traits that could have given rise to the evolution of characteristics which would otherwise have been adaptive. Similarly, developmental constraints can greatly delimit pathways of evolutionary change and can prevent certain traits from evolving. This is because, almost invariably, developmental processes tend to be highly conserved, such that even small changes in these processes may lead to large reductions in fitness (Gould and Lewontin, 1979).

Many years of field and laboratory selection studies of evolutionary genetics in *Drosophila* have led to the growing realisation that correlated responses to selection are the result of myriad complex and subtle interactions between the nature of selection and the laboratory ecology of the populations being used (Rose et al., 1996; Ackermann et al., 2001; Prasad et al., 2001). For example, even the apparently well established notion of a positive association between body size and male mating success in *Drosophila* is now known to depend crucially on the factors responsible for the size variation in the population being studied, and on its genetic composition (Santos et al., 1994; Zamudio et al., 1995; Santos 1996; Joshi et al., 1999). Similarly, rates of larval weight gain actually decline in populations subjected to selection for faster development and early reproduction, even though on optimality principles one might expect increased rates to be very beneficial under such a selection regime (Prasad et al., 2000). These studies point out that one must be very careful when formulating broad principles of the kinds of correlated responses one expects under different selection regimes. Often seemingly trifling details of the ecology of the organism, and the specific behavioral or physiological traits

underlying changes in fitness components, can give rise to unexpected patterns of correlated responses to selection. Given that such complex and subtle patterns of evolution are seen in laboratory studies conducted in a very simple environment, it would not be surprising that the process of adaptation in natural populations is extremely complicated and far more subtle than often allowed for in much evolutionary speculation.

One biological phenomenon that is widely believed to be an adaptation to the environment is that of rhythmicity in several physiological and behavioural processes that occurs with a periodicity of ~ 24 hours (*circadian rhythms*: Latin: *circa* - about, *dies* - a day) even under constant conditions. This phenomenon is ubiquitous and occurs at several levels of organisation ranging from mRNA concentrations within a cell, to the patterns of eclosion in populations of fruit flies (Scully and Kay, 2000). This thesis presents the results of a series of experiments that attempted to empirically test the assertion that possessing circadian rhythms confers an adaptive value to organisms.

1.2 Introduction to circadian rhythms

Life on earth has been under the influence of several geophysical periodicities arising due to the rotation of the earth on its own axis, its revolution around the sun and the revolution of the moon around the earth. Hence, from the time of its origin, about 3.5 billion years ago (de Duve, 1995), life forms have been subjected to, and have had to cope with, pronounced daily and annual cycles of light and temperature. Almost all living organisms studied so far have shown an ability to either counteract or exploit these periodic variations in the external environment. It is now well known that many periodic phenomena exhibited by organisms are not merely reflections of the response of the organism to periodicity of the external environment due to the

daily cycles of day and night, but are in fact generated endogenously (Pittendrigh, 1960). These biological oscillations are referred to as circadian rhythms and are characterised by the following features: (1) they are endogenous in origin (are generated within the body of the organism and have a genetic basis), (2) they are self sustaining (under constant conditions where the periodic factors which could possibly impart information about local time are absent, they continue to oscillate) and exhibit a periodicity of ~ 24 hours in the absence of external time cues, (3) they are innate rather than learnt, (4) they can be synchronised by periodic light/dark cycles, and (5) they are temperature compensated (their periodicity remains unaltered to a large extent with increase or decrease of temperature, within a physiologically tolerable range) (Pittendrigh, 1960).

1.3 Adaptive significance of circadian rhythms

The ubiquity of circadian rhythms suggests that these rhythms confer some adaptive value to the organisms exhibiting them (Roenneberg and Foster, 1997; Ouyang et al., 1998). In principle, such advantages could stem from two different features of biological clocks: from their ability to (a) persist even in the absence of external time cues and synchronise several overt rhythms within the organism, and to (b) enable the organism to maintain a stable phase relationship with the environmental light/dark, temperature and humidity cycles or other cycles of resource availability or predator activity (Roenneberg and Foster, 1997). These two views suggest, respectively, an *intrinsic* adaptive value of clocks deriving from periodicity *per se*, versus an *extrinsic* adaptive value derived from being able to maintain an appropriate phase relationship with cyclic factors of the environment. The persistence of circadian rhythms in organisms which typically inhabit environments where abiotic and biotic factors do not vary periodically, as in subterranean caves and the deep sea, suggest that even in aperiodic

environments circadian rhythms contribute in some way to maintain the functional integrity of the internal milieu of organisms (Daan and Aschoff, 1982). Compared to studies on intrinsically derived adaptive value of circadian organisation, many more studies have sought evidence for the extrinsically derived adaptive value of circadian rhythms through phasing of activities to the environmental cycles (reviewed in Johnson and Golden, 1999). However, with the benefit of hindsight deriving from several decades of research in evolutionary genetics, a great majority of these studies can be seen to have several shortcomings in experimental design and interpretation (reviewed in Sharma and Joshi, 2002).

1.4 Studies on organisms living in aperiodic environments for several hundred generations

The results of a few studies on organisms living in aperiodic environments like the deep sea and subterranean caves suggested that these organisms either do not show any overt circadian rhythms, or, if they do, the periodicities of these rhythms are usually very deviant from 24 h (hours). For example, the eyeless crayfish *Niphargus puteanus* did not exhibit any clear circadian locomotor activity rhythm, although a few scattered components of activity with periods ranging from 10 to 57 h were seen in a few individuals (Blume et al., 1962). However, several other studies have shown that organisms living in aperiodic environments do exhibit circadian rhythms in some behavioural and physiological functions. Although Amblyopsid fish did not exhibit a circadian rhythm in locomotor activity, oxygen consumption in these fish showed a circadian rhythm that could not be entrained to light/dark (LD) cycles (Poulson and White, 1969). Rhythm in locomotor activity showing brief rhythmic sequences with periodicities in the range of 12 h to 24 h, was also reported in a blind and depigmented cave millipede, *Blaniulus lichentsteini* under

constant darkness (DD) (Mead and Gilhodes, 1974). In another study on cave dwelling millipedes *Glyphiulus cavernicolus* 57% of the individuals assayed showed circadian rhythm in locomotor activity (Koilraj et al., 2000). These studies suggest that circadian rhythms do confer some *intrinsic* fitness advantage to organisms even under constant conditions, even though only some processes continue to be retained under circadian control of endogenous pacemakers while others may have become arrhythmic or continue to oscillate with non-circadian periods. An alternative explanation could be that these traits are maintained even in the absence of selection favouring them due to phylogenetic inertia, but this is unlikely as laboratory studies of evolution using *Drosophila* show that traits not conferring any fitness advantage to the organism under the given culture conditions get affected by random genetic drift and mutation accumulation fairly rapidly (within 100-200 generations) (Mueller, 1987; Service et al., 1988).

1.5 Studies that link circadian rhythms with fitness

Pittendrigh and Minis (1972) assayed the longevity of tumourous strain (tu^g) and wild type *D. melanogaster*, that had been routinely maintained in LD 12:12 h regime, under five different assay light regimes: LD cycles of 12:12 h, 10.5:10.5 h, 13.5:13.5 h, constant light (LL), and DD. They reported that the longevity of these flies was greatest in LD 12:12 h as compared to other light regimes This led to the notion of circadian resonance: the enhanced performance of organisms in an LD regime whose periodicity is closest to their own intrinsic periodicity (Pittendrigh and Minis, 1972). Similar results were obtained in blow flies *Phormia terraenovae* that were routinely maintained under LD 12:12 h. The longevity of the flies when assayed in LD 10:10 h regime decreased compared to their longevity in LD 12:12 h and LD 14:14 h regimes, whereas there was no difference in longevity between the flies assayed in LD 12:12 h and 14:14

h (von Saint-Paul and Aschoff, 1978). However, in a more recent study Klarsfeld and Rouyer (1998) found that in case of male *D. melanogaster*, *per* mutant flies had lower longevity compared to wild type flies in all light regimes, including those whose periodicities closely matched their own free running period, whereas female longevity was not affected by the periodicity of the environment.

Although LL has often been believed to be deleterious (Pittendrigh, 1960), the effect of LL on fitness is not consistent across organisms. While some reports suggest that LL is harmful for plants (Highkin and Hanson, 1954), others claim that the growth rates increased in LL (Highkin, 1960). It was speculated that in LL a loss of mutual entrainment between the oscillatory subsystems that constitute the circadian organisation of an organism might be responsible for the deleterious effects (Pittendrigh, 1960). However, arrhythmic mutant hamsters (Menaker and Vogelbaum, 1993) and mice (Takahashi 1995; King et al., 1997) did not differ in longevity from wild type individuals, when assayed in the laboratory. Similarly, lesioning of the suprachiasmatic nucleus (SCN), considered the site of the circadian pacemaker in mammals, did not cause a reduction in longevity under laboratory conditions in golden-mantled ground squirrels *Spermophilus lateralis* (Zucker et al., 1983; Dark et al., 1985; Ruby et al., 1996), or in Siberian chipmunks *Eutamias sibiricus* (Sato and Kawamura, 1984). Yet, a study of mutant (tau^{+}/tau^{s}, tau^{s}/tau^{s}) and wild type (tau^{+}/tau^{+}) golden hamsters *Mesocricetus aureus* revealed that heterozygotes showed fragmented locomotor activity rhythms and reduced longevity in LD 14:10 h as compared to the wild type which entrained to the LD cycle, and homozygous recessive mutant which free ran. In LL of low intensity (20 - 50 lux) however, the longevity of the three genotypes did not differ, and all three genotypes showed free running locomotor activity rhythms (Hurd and Ralph, 1998). In the same study, it was also demonstrated that the

locomotor activity rhythms could be restored, and post transplant life expectancy enhanced, by grafting ageing individuals with foetal SCN.

One drawback of most of these studies has been the use of longevity as the principal indicator of fitness. A trade-off between fecundity (a very important component of fitness) and longevity has been documented in many species, including *Drosophila* (Rose et al., 1996; Sheeba et al., 2000). Therefore, using longevity as the sole indicator of fitness and drawing conclusions about adaptation to an environment based on knowledge of longevity alone could be erroneous.

In a recent experiment, the issue of adaptive significance of circadian rhythms was rigorously examined, using the asexual cyanobacterium *Synecchococcus spp.* strain PCC7942, for the first time taking into consideration an overall measure of fitness (Ouyang et al., 1998). Three different mutant strains with distinct free-running periods of luminescence rhythm were competed against one another by subjecting pair-wise combinations of the strains to competitive conditions under different light regimes. Pairs of strains were mixed together and an aliquot was plated to form single colonies to determine the initial composition of the culture. The strains were grown together for about 27 days in batch culture under different light regimes: LD 12:12 h; LD 11:11 h and LD 15:15 h. Subsequently another aliquot was plated from each of these mixed cultures. The results showed that under competitive conditions the strain whose periodicity most closely matched with the environmental light regime outcompeted the other and, thus, for the first time demonstrated a direct link between the periodicity of the circadian oscillations and fitness in particular periodic environments. However, as the authors have noted, the actual mechanism by which the reproductive advantage is gained is not yet clear (Ouyang et al., 1998).

1.6 Studies of circadian rhythms affecting fitness of organisms in the wild

Although it is generally believed that circadian rhythms confer some adaptive value to the organisms in the natural environment, where both biotic and abiotic factors can vary with a periodicity of 24 h, there are only a few studies that have empirically tested this hypothesis. Daan and Tinbergen (1980) showed in a study on guillemot *Uria lomvia* fledglings that those individuals which jumped off their nests on cliffs during a certain time in the evening suffered a lower mortality due to predation by gulls than at other times of the day. In another set of studies, DeCoursey and co-workers found that lesioning of the SCN in antelope ground squirrels *Ammospermophilus leucurus* and eastern chipmunks *Tamias striatus* caused increase in mortality compared to the SCN intact controls (DeCoursey et al., 1997; DeCoursey and Krulas, 1998). Such decrease in the life expectancy due to absence of functional time keeping mechanism was partly attributed to increase in predation. The SCN lesioned ground squirrels were active and above the ground surface at night as opposed to the intact controls which were strictly diurnal. Consequently, a higher proportion of the SCN lesioned squirrels were killed by predators as opposed to their controls (DeCoursey et al., 1997). The SCN lesioned chipmunks, too, showed significantly shorter life span as compared to their controls (DeCoursey and Krulas, 1998).

1.7 Studies of clinal variation in traits that exhibit circadian rhythms

Several studies on *Drosophila* species have examined the geographical distribution of traits that exhibit circadian rhythms. Weak latitudinal clines were reported for both free running period and phase of the eclosion and adult diapause rhythms of about 57 European populations

of *D. littoralis* collected from localities ranging from 30° to 70°N latitude (Lankinen, 1986). The northern populations showed shorter free running period and early phase as compared to the southern populations. Under LD regime, the phase of peak eclosion showed a latitudinal cline, with the northern populations eclosing earlier than the southern ones (Lankinen, 1986). The amplitude of both free-running and entrained eclosion rhythms was also reduced in the northern relative to the southern populations. The critical day length for diapause induction was also strongly correlated with latitude, being longer in the northern populations. Similar relationships between latitude and the phase, free running period, and amplitude of the eclosion rhythm were reported in a study of 12 populations of *D. pseudoobscura*, a non-photoperiodic species, from Scandinavia and the Canary Islands (Lankinen, 1993). Latitudinal clines for oviposition rhythm in 15 European and African strains of *D. melanogaster* (latitudes 0° to above 60° N) have also been observed (Allemand and David, 1976). Two traits, the proportion of eggs laid during the light phase of the LD 12:12 h cycle, and an index characterising peak size, were found to decrease with latitude. Flies from equatorial regions laid more than 80% of their eggs in the dark phase, while the flies from the northern latitudes laid a little less than 50% of their eggs in the dark phase. Moreover, the egg laying peaks in the equatorial strains were narrower and taller than those from the northern populations. The observed regular latitudinal clines were taken as evidence for the adaptive value of these two traits, and as an indication that perhaps factors such as light intensity and temperature play a role in determining the phase and the manner in which these behavioural traits evolved.

The PERIOD protein coded by the *per* gene, which forms an integral part of the circadian organisation in *D. melanogaster*, includes a repetitive threonine-glycine (Thr - Gly) region. This repeat region is polymorphic in length and two of the major variants $(Thr\text{-}Gly)_{17}$ and $(Thr\text{-}Gly)_{20}$

are distributed in a highly significant latitudinal cline. The number of (Thr-Gly) repeats in 18 populations of *D. melanogaster* from Europe and North Africa is greater in the northern than in the southern populations (Costa et al., 1992). Subsequently, other studies suggested that these clines may be related to temperature compensation, as locomotor activity rhythm of flies with $thr\text{-}gly_{20}$ allele of the *per* gene showed more efficient temperature compensation than the $thr\text{-}gly_{17}$ allele at both 18 °C and 29 °C (Sawyer et al., 1997). Similar repeats of amino acid sequences were also found in the protein products of *frq* and *wc1* and *wc2* genes of *Neurospora*, as well as in the mammalian *per* homologues.

However, the existence of clinal variation for traits can only be taken as indirect evidence for adaptation as there may be many factors that are uncontrolled in these studies such as the ancestry and other geographical factors that may be different in the different regions. Recent studies using *D. melanogaster* illustrates the hazards of relying on clinal studies alone (without supporting data using other approaches) as evidence for adaptive evolution (Hoffmann and Harshman, 1999; Hoffmann et al., 2001 a). Moreover, the fact that these studies have been mostly done on inbred cultures that have been raised in the laboratory from isofemale lines further complicates the issue due to the possibility of fortuitous fixation of alleles at various loci affecting the rhythms being studied (Sharma and Joshi, 2002).

More recently, the (*thr-gly*) repeats in the *per* gene in *Drosophila spp.* have been found to vary widely among species, leading to the speculation that the repeats coevolve with their immediately flanking regions. This hypothesis was tested by Peixoto et al (1998) by juxtaposing (*thr-gly*) repeats of one species with the flanking region of another. The results of their experiments showed that transgene constructs which consisted of DNA from two different species resulted in arrhythmic or poorly temperature compensated phenotypes, whereas

constructs made of repeats and flanking regions from conspecifics rescued the wild type circadian rhythm phenotype. The results of Peixoto et al (1998) support the coevolutionary interpretation that the repeats and their flanking regions in the PER molecule have evolved in tandem and serve some function related to temperature compensation, and is a very good example of a study that has demonstrated that clinal variation trait is also associated with fitness differences.

1.8 The significance of laboratory selection studies of circadian parameters:

Laboratory selection studies to address evolutionarily pertinent questions have been conducted time and again by biologists, but the approach and methodology used in addressing them have often been inadequate (Rose et al., 1996). This has been especially true of most studies that attempted to understand the evolutionary significance of circadian rhythms. Most previous selection experiments have used one selected population and its control to arrive at evolutionary conclusions about the adaptive significance of circadian rhythms. In a population, allele frequencies can change from one generation to the next purely by chance. Therefore in selection studies using only one selected population and one control population, any differences observed between them cannot unambiguously be assigned to selection alone, as drift cannot be ruled out. Indeed, if evolutionary conclusions are to be drawn from a study, the unit of replication needs to be the population. For example, *D. pseudoobscura* populations selected for early and late eclosion for 50 generations under LD 12:12 h gave rise to a divergence of 4 h in the peak of eclosion among the divergent stocks (Pittendrigh, 1967). A similar selection protocol on the pink bollworm *Pectinophora gossepiella* for 9 generations resulted in a divergence of 5 h

between the peaks of eclosion in the divergently selected stocks (Pittendrigh and Minis, 1971). In both species the difference among the stocks was maintained when tested under different photoperiods. The free running periods of the early eclosing strains were shorter than the late eclosing strains in both species. Correlated responses to selection for phase of eclosion was seen in the case of *P. gossipiella* in the phase of egg hatching rhythm, while activity and oviposition rhythms did not show a correlated response. Since there was no replication at the level of the population within selection regime in both these studies, it is not clear whether the differences seen can be ascribed to selection. When replicate populations and their matched controls are used, one can unambiguously assign selection as being the cause for differences between the stocks and their controls as it is unlikely that random genetic drift will drive all the replicate populations to undergo the same sequence of genetic changes.

Another serious problem with many laboratory studies is related to the size of the population under investigation. The effective population size (an index which indicates the degree to which non ideal populations are affected by random genetic drift, and is a number which is usually smaller than the actual number of individuals) plays a very crucial role in selection studies. That without variation evolution cannot take place is a well-established premise (Kirkpatrick, 1996). Therefore, if the population on which selection is imposed is relatively small, the actual subset of potential genotypes that is realised at each generation is small. On the contrary, for a naturally out breeding population, the potential number of different multi-locus genotypes that can be produced is extremely large, due to the possibility of reshuffling of genes during meiotic recombination and fertilisation. Moreover, inbreeding and drift in small populations can fortuitously generate artefactual genetic correlations among traits.

Therefore laboratory studies using organisms maintained at small population sizes are susceptible to erroneous interpretation of results.

Mutant lines, chromosome extraction lines and isofemale lines, which are popular systems for laboratory experiments on *Drosophila spp.* are typically inbred by the virtue of how they are generated. Even if crossing of several wild caught isofemale lines is employed (as is quite often done) to overcome the shortage in genetic variation in such studies, the potentially confounding large linkage disequilibrium effects persist unless they are maintained for more than 25 generations in the laboratory after initiation (Weir and Cockerham, 1979). Owing to the relatively small sizes of most laboratory populations the likelihood of encountering genetic artefacts of random genetic drift and inbreeding on the selected populations is very high. Such results therefore cannot be used to reliably infer the course of evolution in species that typically occur as large and out-breeding populations in nature.

Similarly, when wild caught populations are brought into laboratory environments, or even when populations from one laboratory are taken to another wherein there are small and inadvertent differences in the maintenance regime, loci previously not exposed to selection may be expressed in these novel environments. Those loci with alleles with positive or negative effects on several components of fitness segregate in the population for several generations before reaching genetic equilibrium. As the population adapts to the novel environment, fitness trade-offs may not be apparent in the beginning but slowly reappear as the population adapts to the new environment. Hence the use of wild caught populations moved to the laboratory to study fitness associated traits may result in unpredictable appearance/disappearance of fitness trade-offs between traits over several generations (Service and Rose, 1985; Joshi and Thompson, 1995).

Over the past two and half decades rigorous criteria have been developed to test the adaptive significance of traits empirically. The demonstration that specific environmental regimes relevant to the trait being studied have differential effects on components of fitness and that the trait does evolve differently under these different environmental regimes have become important criteria that need to be established to show conclusively that adaptation of an organism to a specific environment has taken place. Moreover, it has now become increasingly apparent that careful and systematic long-term studies on laboratory populations of organisms is a highly rewarding approach to understanding adaptive evolution. This approach has yielded high dividends in the study of evolutionary genetics of several traits in *Drosophila.* The study of evolution of life histories under age-specific (reviewed by Rose et al., 1987, 1990, 1996) and density-dependent selection (reviewed by Joshi, 1997 b; Mueller, 1995, 1997), and the evolution of resistance to environmental stresses such as of starvation and desiccation (Chippindale et al., 1996, 1998; Hoffmann and Harshman, 1999; Hoffmann et al., 2001 b), and temperature stresses like cold and heat stresses (Gilchrist et al., 1997) are good examples of highly successful studies. It has also become apparent that the evolutionary process is exceedingly subtle, and evolutionary responses to even seemingly trivial changes in the environment can be quite large and often, counterintuitive. Laboratory selection studies to understand adaptive value of traits are no doubt time consuming and labour intensive, but provide relatively clean answers to well posed questions, as one may assess additive genetic correlations between traits by examining correlated responses of many different fitness traits to selection. Moreover, the use of replicate populations and their matched controls allows for statistically more powerful tests for unambiguously detecting selection.

In this thesis, I describe results from a series of experiments that are part of a long-term study to examine the evolutionary genetics of circadian rhythms. This study was initiated with four populations of *Drosophila melanogaster* that had been reared for more than 600 generations in the laboratory as large, outbred populations under constant light, temperature and humidity. Moreover, these four populations have been maintained as separate entities without any gene flow between them and, therefore, served as independent replicates for our studies. These populations were studied to examine whether populations kept under constant light for more than 600 generations retain the capacity to exhibit circadian rhythms. We also initiated three separate sets of populations from these four baseline populations and subjected them to three different selection regimes: two were aperiodic environments LL, and DD, and one periodic environment with a LD 12:12 h. In order to look for evidence for adaptive evolution, we assayed all the twelve populations for components of fitness and circadian parameters such as free running period and phase angle difference in all the three selection regimes at intervals of about ten generations of selection. We also examined the developmental plasticity of circadian rhythms and the possible multi-oscillatory nature of circadian organisation in these populations.

Chapter 2

Studies On The Circadian Parameters Of Baseline Populations

2.1 Introduction to the baseline populations

In this section the ancestry and maintenance schedule of the four baseline populations of *Drosophila melanogaster* used in some of our experiments are described (Fig. 1). These populations also served as the set of ancestral populations from which three sets of populations were derived and subjected to selection for adaptation to different light regimes. The baseline populations were derived from populations which were initiated from a wild caught population (IV) from South Amherst, Massachusetts, U.S.A, about 30 years ago (Ives, 1970). They were maintained in the laboratory for about 110 generations under constant light (LL), constant temperature (25 ± 1 °C) and constant humidity on a 14-day discrete generation cycle. Five populations (B1..B5) were derived from the IV population, and then reared under laboratory conditions similar to the ones in which the IV population was maintained (Rose and Charlesworth, 1981; Service and Rose, 1985). After about 360 generations, a set of 5 populations were derived from the five B (B1..B5) populations (UU1..UU5; Joshi and Mueller, 1996). The UU populations were maintained under constant light, temperature and humidity on a 21-day discrete generation cycle. and reared in the laboratory for about 170 generations after which four populations (JB1..JB4; our baseline populations, described in Sheeba et al., 1998) were derived from four UU populations (UU1, 2, 3, 5).

All the populations starting from the IV up to the UU were reared on banana-molasses food. The four populations (JB1..4) are maintained in our laboratory at 25 °C on a 21 day discrete generation cycle, under constant light (2.5 W/m^2 or 300 lux), constant temperature and constant humidity (80-90%) at moderate densities of about 60-80 larvae per vial (9.0 cm h x 2.4 cm dia), consisting of approximately 6 ml banana-jaggery food (henceforth, banana food). At every generation, adults of each population are allowed to lay eggs for about 18 hours on petri

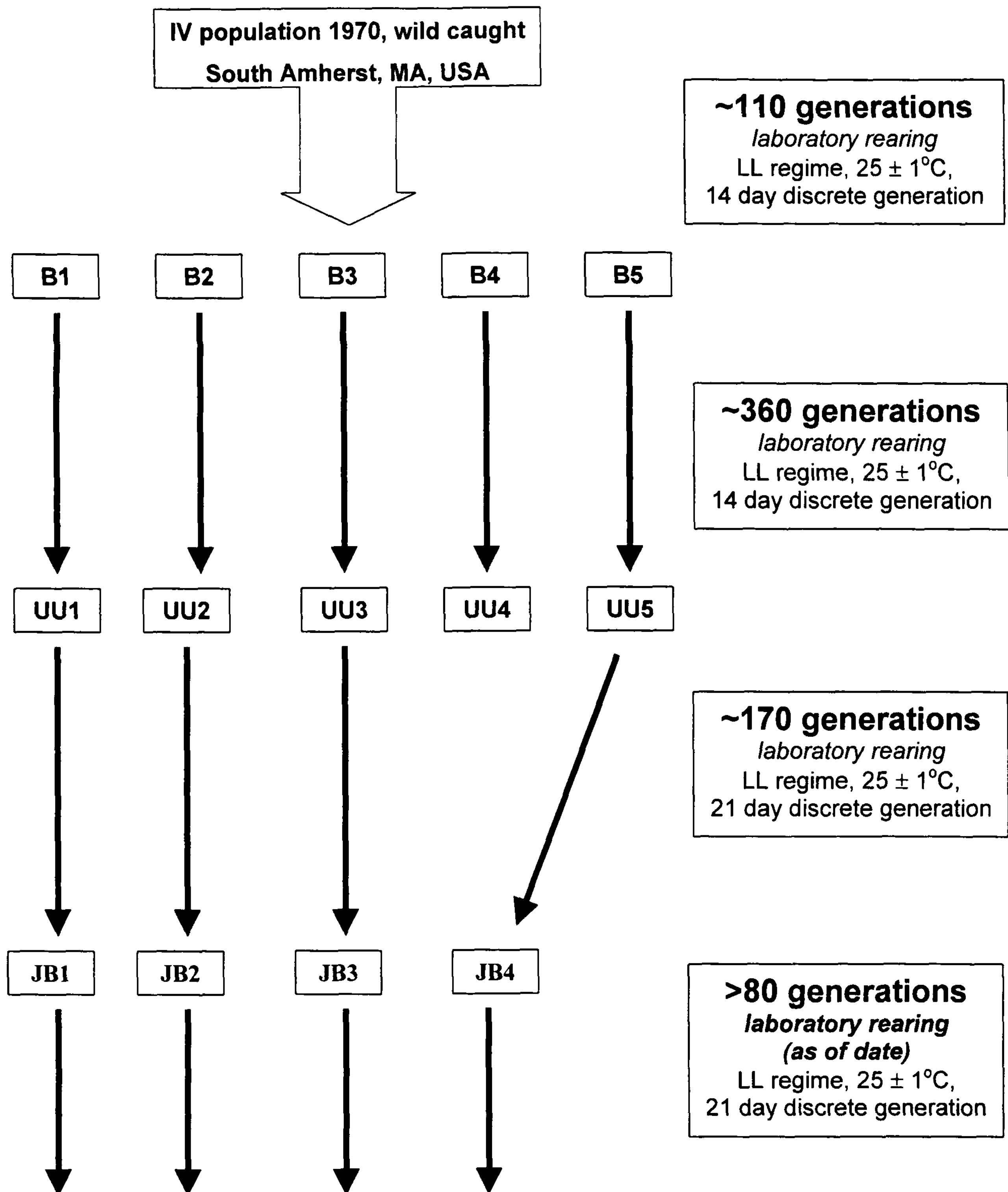

Fig. 1 Schematic representation of the ancestry and maintenance of the baseline populations

plates of fresh banana food placed in a plexiglas cage (25 x 20 x 15 cm^3). From these petri plates, 60-80 eggs are collected into each of 40 vials in which larvae then develop into adults. Adults eclosing from these vials are transferred to fresh food vials on day 12, 14 and 16, after egg lay. On the 18th day after egg lay, adult flies are transferred into plexiglas cages and supplied with banana food supplemented with live yeast paste for two days, after which eggs are collected to initiate the next generation and the adults discarded. The population typically consists of about 1500 flies at this stage and at the time of writing these populations are in their 80th generation. These four populations of *D. melanogaster* (JB1..4) are unique because of the fact that they have not been exposed to any circadian time cues for over 600 generations. Therefore, our first question was to examine whether these flies still retain the ability to exhibit circadian rhythmicity. In subsequent sections of this chapter, experiments carried out in order to answer this question by assaying eclosion rhythm (a populational level rhythm), locomotor activity and oviposition rhythm (individual level rhythms) in these flies are described.

2.2 Persistence of eclosion rhythm

The persistence of circadian rhythmicity over a very large number of generations in populations inhabiting environments where biotic and abiotic factors do not vary regularly with time may be taken to suggest an intrinsic adaptive value of possessing circadian periodicity, deriving, perhaps, from the need to synchronise various processes within the organism (Pittendrigh and Minis, 1972). To examine this possibility, we assayed the circadian rhythm of eclosion in the four JB populations (JB1..4), which have not been exposed to any time cues for several hundred generations.

2.2 a Materials and methods

We first conducted a pilot assay of the eclosion rhythm in LL, light/dark cycle 12:12 hours (LD 12:12 h) and constant darkness (DD) regimes using one of the four baseline populations (JB1) (henceforth referred to as experiment 1), after which a larger experiment was performed using all the four baseline populations (JB1..4) (henceforth referred to as experiment 2). The light phase in these treatments was achieved by means of fluorescent white light sources (approximate intensity 2.5 W/m^2 or 300 lux), whereas the dark phase was actually dim red light ($\lambda > 640$ nm), which facilitated observation and manipulation of flies without interrupting the dark phase. The continuous darkness (DD) in our experiments was also continuous dim red light ($\lambda > 640$ nm). In both experiments, free-running period (τ, the periodicity of a rhythm in the absence of any external time cues) measured in aperiodic regimes LL and DD, and the phase angle difference (ψ, measured as the time interval between the peak of eclosion and 'lights-on' of the LD cycle) in LD 12:12 h regime, were estimated.

The populations were maintained at controlled moderate density in an incubator under constant light (LL; of intensity 2.5 W/m^2 or 300 lux), temperature ($25^{o} \pm 1$ ^{o}C) and humidity (~90%), with food and mates being available *ad libitum.* In experiment 1, developmentally asynchronous eggs were collected from the adult flies in the population and introduced into LL (in the incubator), LD 12:12 h, and DD (in temperature controlled walk-in chambers with light intensity, temperature and humidity maintained at the same levels as in the incubators). In each light regime, 128 vials, containing 30 eggs per vial were assayed. The number of eclosing flies was recorded every 2 h, yielding a time series comprising of 5 consecutive 24-h-days for each environmental treatment (Fig. 2). The time series thus obtained for each light regime was subjected to Fourier Spectral Analysis using STATISTICA ^{TM} (Statsoft Inc, 1995). Statistical

significance of observed peaks in the periodogram obtained was tested using the techniques described in Siegel (1980).

In experiment 2, eggs were collected from adult flies in each of the four populations and placed at high densities of ~300 eggs per vial into vials containing 6 ml of food medium. One hundred and eighty such vials were collected per population. Sixty vials in five batches of twelve vials each were assayed in LL, LD 12:12 h and DD regimes. Each batch was separated by an egg collection window of six hours. Unlike in experiment 1, vials that were assayed in LL regime were first subjected to a dark stimulus of 12 hours before they were reintroduced to LL regime. The vials were monitored for the darkening of pupae and the number of eclosing flies were counted and sexed at 2 h intervals. This was continued for 10 consecutive days, or until most of the pupae had eclosed. The time series data thus obtained for males and females from each vial was subjected to Fourier Spectral Analysis using STATISTICA ™ (Statsoft Inc, 1995). Statistical significance of observed peaks in the periodogram was tested using the technique of Siegel (1980). The single value of τ for males and females obtained from each of the replicate vials kept in LL (τ_{LL}) and DD (τ_{DD}) regimes were used as data in a mixed model analysis of variance (ANOVA), where the populations were treated as random blocks, light regime was a fixed factor crossed with block, batch was nested within the interaction between block and light regime, and sex was a fixed factor crossed with all other factors. Kolmogorov-Smirnov two-sample tests were also performed in order to test for differences in the distribution of τ pooled over populations in each of LL and DD regimes. The ψ of eclosion rhythm in LD 12:12 h was estimated as the average time interval between peak eclosion and 'lights-on' over 10 consecutive days. The estimation of ψ was done separately for males and females eclosing from each vial,

and the values thus obtained were subjected to an ANOVA where populations were treated as random blocks, batch was nested within block, and sex was crossed with all other factors.

2.2 b Results

The results of experiment 1 showed that eclosion was rhythmic in all three light regimes. The flies entrained to an external LD cycle of 12:12 h, and under constant conditions of LL and DD regimes, the eclosion rhythm free-ran with τ_{LL} of 28.8 h and τ_{DD} of 24 h, respectively (Fig. 2). In experiment 2, as well, all four populations of flies exhibited rhythm in eclosion in all the three light regimes. The flies entrained to an external LD cycle of 12:12 h, and under constant conditions of LL and DD regimes the rhythm free-ran with τ_{LL} of 21.80 ± 2.72 h (mean ± 95% C.I.) and τ_{DD} 22.72 ± 4.7 h, (mean ± 95% C.I.) respectively. It should be noted that in experiment 2, rhythms in LL regime were assayed after the flies were exposed to 12 h of darkness during the larval stage. The ANOVA on τ_{LL} and τ_{DD} regimes in experiment 2 did not reveal any significant main effect of light regime, although a marginally significant main effect of sex was seen (Table 1), with males having a shorter mean τ (± 95% C.I.) (21.73 ± 3.85 h) compared to females (22.79 ± 3.59 h). The ANOVA also revealed a significant main effect of the random factor, block ($p < 0.001$) (Table 1). The block × light regime × sex interaction was also significant, probably because in one of the populations (JB1), the males were found to have a higher mean τ compared to the females, under LL regime. Moreover, the distribution of τ_{LL} was not significantly different from τ_{DD} ($p > 0.05$) (Fig. 3 a, b). The ANOVA on the ψ of eclosion rhythm did not reveal any significant effect of batch or sex, although a significant main effect of block (random factor) was seen ($p < 0.001$). The ψ of eclosion rhythm in LD 12:12 h was distributed unimodally with a range from + 0.67 to + 7.0 h with a mean of + 3.19 h (Fig. 4).

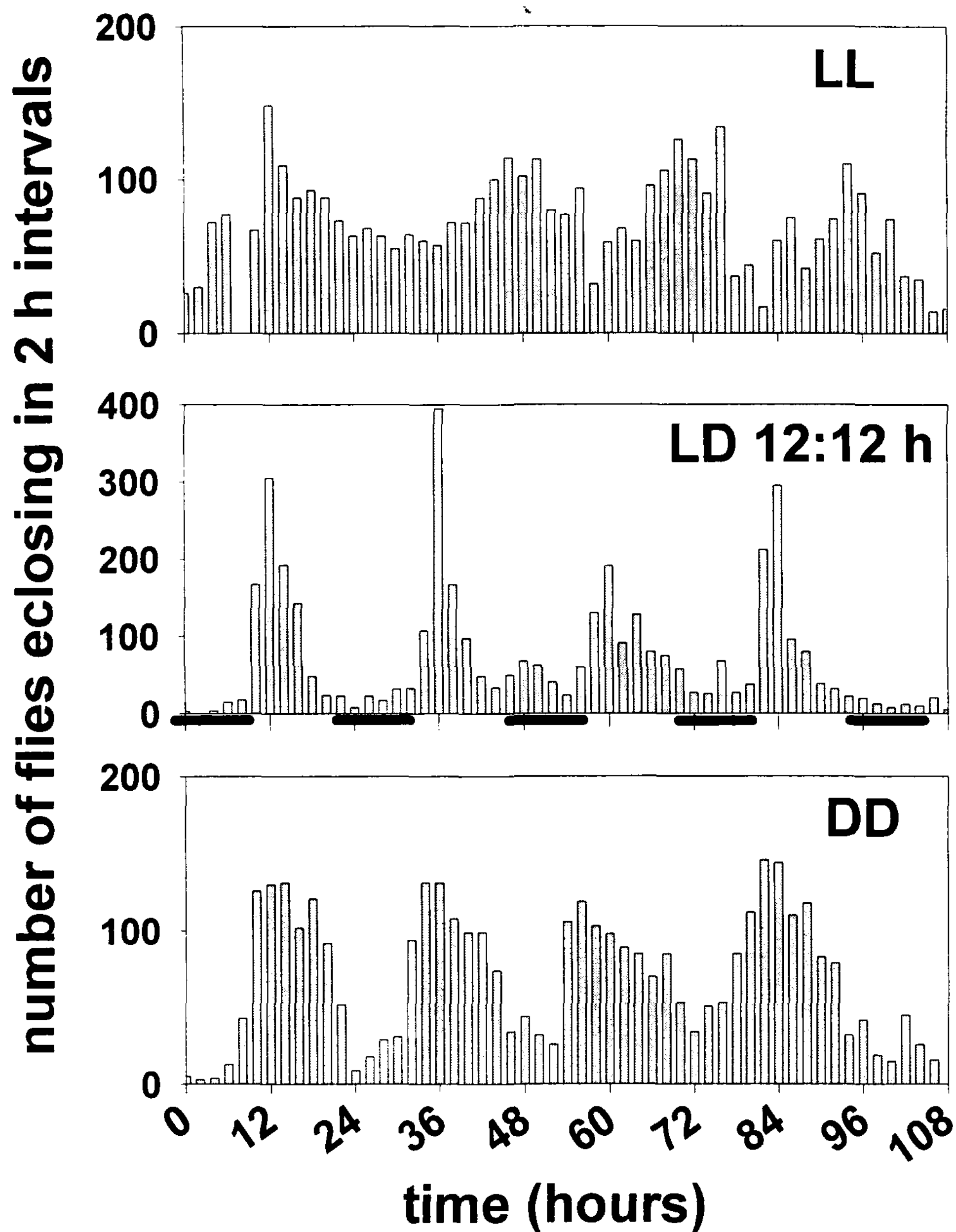

Fig. 2 Time course of eclosion of flies from population JB 1 under constant light (LL), light/dark cycles (LD 12:12 h), and constant darkness (DD). In LD regime lights were switched on at 08.00 h and switched off at 20.00 h (dark bands on the x axis for the LD regime plot indicate the 12 h dark phase).

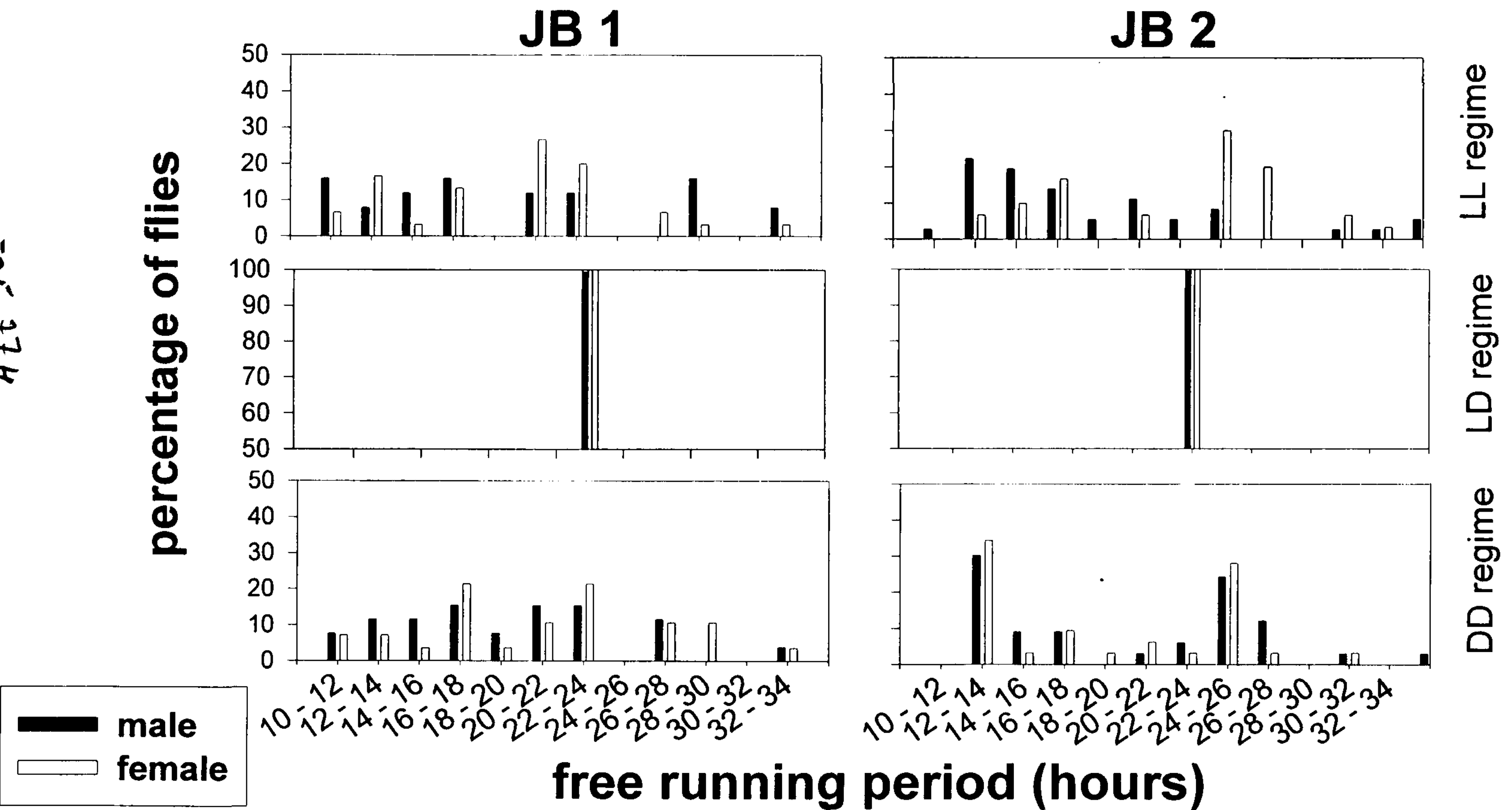

Fig. 3 a Frequency distribution of periodicity of eclosion rhythm of males and females from populations JB1 and JB2 in LL (top panels), LD (middle panels) and DD (bottom panels) regimes.

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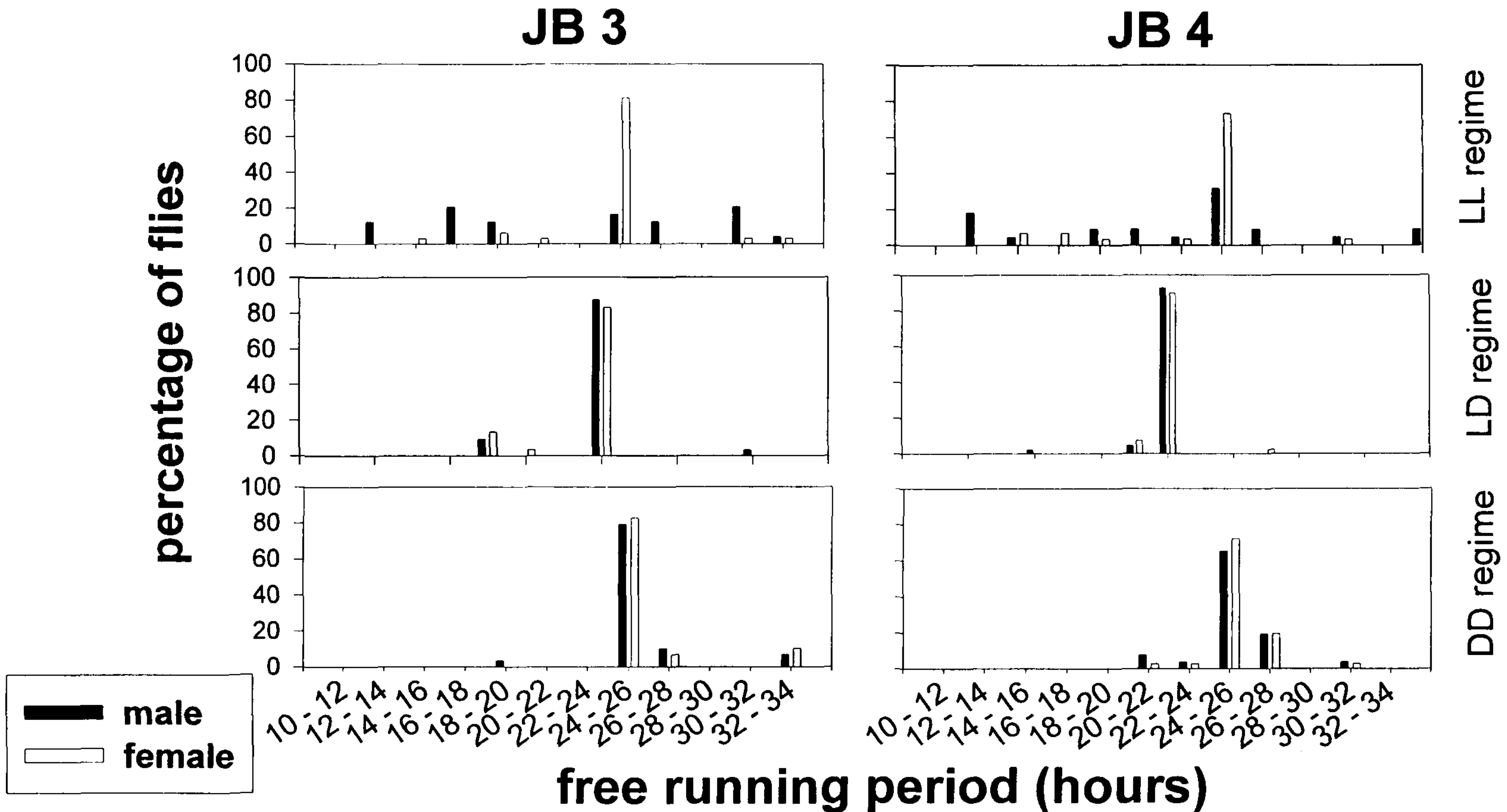

Fig. 3 b Frequency distribution of periodicity of eclosion rhythm of males and females in populations JB 3 and JB 4 in LL (top panels), LD (middle panels) and DD (bottom panels) regimes.

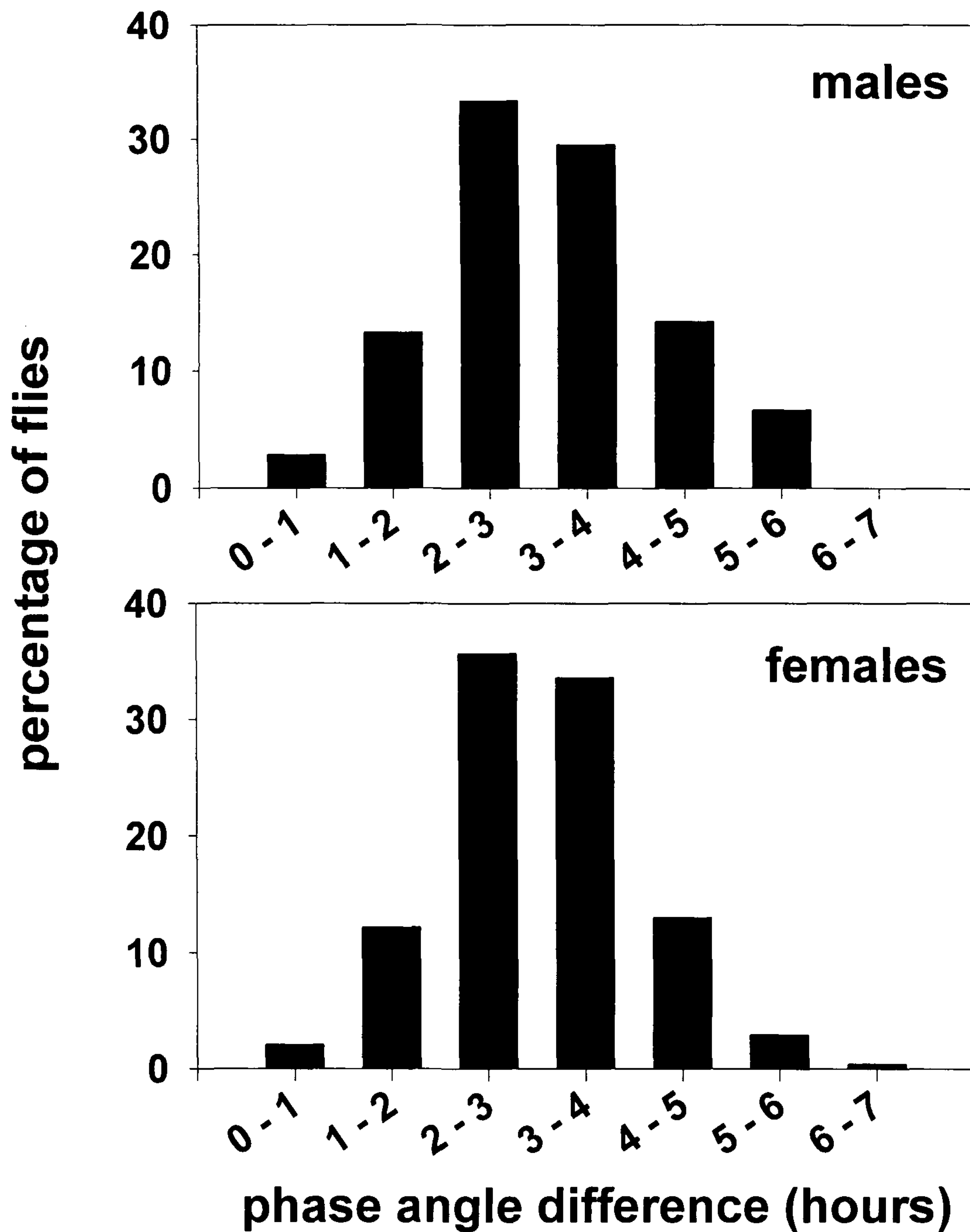

Fig. 4 Frequency distribution of phase angle difference of eclosion estimated separately for males and females as the time interval between peak eclosion and 'lights on' over 10 consecutive days.

Table. 1 Results of analysis of variance (ANOVA) on the free running period of eclosion rhythm assayed under LL (τ_{LL}) and DD (τ_{DD}) regimes of flies from the four JB populations.

Effect	***df***	**MS**	***F***	***p*-level**
Block (B)	3	570.636	21.688	**p <0.0001**
Light regime (L)	1	89.194	1.760	0.277
Batch (C)	32	30.753	1.169	0.246
Sex (S)	1	121.563	10.660	**0.047**
B × L	3	50.692	1.927	0.125
B × S	3	11.404	0.433	0.729
L × S	1	16.321	0.233	0.662
C × S	32	28.504	1.083	0.350
B × L × S	3	70.094	2.664	**0.048**

2.2 c Discussion

The differences in τ between the two experiments could be attributed to the differences in the experimental protocols. In the first experiment only one value of τ was estimated for data pooled across 128 vials. In the second, τ was estimated separately for males and females for each of 60 vials. In the first experiment the vials contained eggs at very low densities of 30 eggs each while in the second experiment each vial contained ~300 eggs. Moreover, the length of the time series in the first experiment was only half that of the second experiment. The first experiment served as a pilot study, whereas the second experiment was used to study eclosion rhythm in detail. Since the results of the second experiment are based on data from larger samples, multiple samples, and over a longer time series, they will be taken as representative of the eclosion rhythm parameters in these populations. The fact that the τ of eclosion in the two aperiodic regimes LL and DD are not significantly different from each other suggests that the τ of eclosion rhythm in these populations is not altered by the presence of light. The variation in τ of the eclosion rhythm in LL and DD regimes in our populations was quite large with values of for both males and females ranging from 12 to 34 h. Typically, flies sampled from the wild, or from populations that have been reared under light/dark regimes in the laboratory for many generations, exhibit much less variation among samples (Pittendrigh, 1981). The rearing of our populations in an aperiodic environment for several hundred generations may have caused a relaxation in the selection pressure to maintain an endogenous periodicity close to 24 h. Nevertheless, the persistence of circadian rhythm of eclosion in LL and in DD regimes, albeit with fairly variable τ, suggests that these flies have retained a functional circadian regulatory mechanism that controls their eclosion rhythm even after 600 generations in an aperiodic environment where there was no apparent need to synchronise to external environmental cycles.

In laboratory studies of evolution using *Drosophila*, it is typically seen that traits that do not confer any fitness advantage to the organism under the given culture conditions are affected by mutation accumulation and random genetic drift within 100-200 generations (Mueller, 1987; Service et al., 1988). If the trait in question happens to have some cost to it, the decline can be even faster, with mean trait values often reverting to those similar to control populations within 10-20 generations (Rose et al., 1996; Joshi, 1997 a). The fact that the capacity of exhibiting a circadian rhythm of eclosion is preserved in these populations even after 600 generations of rearing in an aperiodic environment strongly suggests that there may be an intrinsic adaptive significance to possessing a functional circadian clock.

Eclosion rhythm occurs at the level of the population, and some studies suggest that this rhythm may be under the control of a circadian oscillator different from that which regulates individual level rhythms like locomotor activity and oviposition (Engelmann and Mack, 1978; Helfrich-Förster, 1996; Sheeba et al., 2001 a). Therefore our next step was to investigate individual level rhythms in these populations of *D. melanogaster*. To examine whether individual level rhythms persisted in LL, LD 12:12 h and DD regimes, we assayed the locomotor activity of individual males and females, and oviposition in individual females from the same four populations in all three regimes (LL, LD 12:12 h and DD).

2.3 Persistence of locomotor activity rhythm

Locomotor activity of *D. melanogaster* is among the best studied circadian rhythms and almost all previous reports have shown that the rhythm is very robust in DD and LD regimes but damps out in LL regime (Konopka et al., 1989; Emery et al., 2000). Recent molecular genetic studies have demonstrated that the circadian rhythm of locomotor activity in

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D. melanogaster is influenced by the interaction of several genes and their protein products which form several feedback loops (Allada et al., 1998; Rutila et al., 1998). The roles of *period* (*per*) and *timeless* (*tim*) genes are by far the best studied, and their protein products PER and TIM are now known to inhibit the transcription of their own genes through a negative feedback loop (reviewed in Hall, 1998; Dunlap, 1999; Edery, 1999). Under LD cycles, the rhythmicity is known to be generated in the following manner by two proteins CLOCK (CLK) and CYCLE (CYC) which bind to E-box DNA elements and activate a slow transcriptional induction of PER and TIM proteins at around noon. Although *per* and *tim* mRNA levels begin to rise in the cytoplasm, phosphorylation of PER by the protein DOUBLE TIME (DBT) prevents the build up of PER levels in the cell cytoplasm (Edery, 2000; Geibultowicz, 2000). After nightfall, the level of light labile protein TIM increases in the cytoplasm, which rescues PER from being sequestered by DBT (Price et al., 1998). The TIM protein forms heterodimers with PER, following which PER-TIM heterodimers begin to enter the nucleus by midnight (Edery, 2000; Young, 2000). PER and TIM then prevent the heterodimer CLK:CYC from binding to the E-box promoter of *per* and *tim* genes (Scully and Kay, 2000). This causes the cessation of transcription of *per* and *tim* genes. The half lives of *per* and *tim* mRNA determine the rate of fall in their protein concentrations. With daybreak, another protein CRY that acts as a photoreceptor sequesters the protein TIM and renders it incapable of functioning as a transcriptional regulator. TIM ultimately becomes ubiquinated, phosphorylated and degraded via the proteosomal pathway. By noon, PER and TIM levels becomes so low that it may not be able to inhibit CLK:CYC activity, and then a new round of synthesis of *per* and *tim* mRNA is initiated. The CLK and CYC cycle is thus interlocked with the PER and TIM cycle and works in antiphase with the PER:TIM regulation, thus allowing for the very finely orchestrated functioning of the

circadian organisation in the flies (Glossop et al., 1999). Thus, current understanding of the circadian organisation in *D. melanogaster* implies that the lengthening or shortening of the overt rhythm assayed under different light regimes may be a reflection of changes in the molecular mechanisms in the circadian organisation underlying overt circadian rhythms. In DD, in the absence of light induced degradation of TIM, the PER-TIM heterodimer formation and its subsequent nuclear entry occurs at shorter intervals than that in LD or LL regimes, which suggests that the τ of the overt rhythm in DD regime should be shorter than τ in LL regime.

Since most previous studies report damping of overt circadian rhythms under LL regime, we studied the populations of *D. melanogaster* that have been maintained for several hundred generations under LL regime to examine what fraction of individuals exhibit circadian rhythmicity in locomotor activity in LL, LD 12:12 h and in DD regimes. We also examined whether the type of assay light regime, or the light regime experienced during pre-adult development, affects the fraction of flies exhibiting circadian rhythmicity in locomotor activity using four experimental protocols (henceforth referred to as experiments 1..4) (Table 2).

2.3 a Materials and methods

In all four experiments, the pre-adult development took place in LL regime. In experiment 1, the locomotor activity of the adults was monitored in LL regime up to 30 days. In experiment 2, the eggs were subjected to 12 h of darkness immediately after egg lay, after which the same protocol as in experiment 1 was followed. In experiment 3, the locomotor activity of adults was first monitored under LD 12:12 h for 15 days, after which they were monitored in DD regime for the next 15 days, while in experiment 4 the adult locomotor activity was directly

Table. 2 The four experimental protocols to assay locomotor activity of adult flies reared as pre-adults under LL regime.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Pre-adult light regime	LL	LL, with dark stimulus of 12 h immediately after egg collection	LL	LL
Adult light regime day 1-15	LL	LL	LD 12:12 h	DD
Adult light regime day 16-30	LL	LL	DD	--

Table. 3 The fraction of males and females which exhibited a circadian rhythmicity in locomotor activity when subjected to different experimental protocols.

	Fraction of rhythmic males	Fraction of rhythmic females
Experiment 1	0.30[a]	0.23[a]
Experiment 2	0.48[b]	--
Experiment 3	0.87[c]	0.70[b]
Experiment 4	0.85[c]	0.67[b]

monitored in DD (Table 2). In experiments 1, 3 and 4 both males and females were assayed, while in experiment 2 only male flies were assayed.

From the running cultures of the four populations, eggs were collected at densities of approximately 50 eggs per vial into vials containing ~6ml of food medium. Eight such vials were collected per population. The adults that emerged were collected and separated by sex within 6 hours of emergence, and the virgin flies were used to assay the locomotor activity. The flies were transferred to the locomotor activity-monitoring set-up within 24 to 48 h after emergence. Individual flies were monitored using a set-up that uses infra red beams to detect the vertical movement of the fly in narrow glass tubes (80 mm h, 6 mm dia) with sugar crystals at the bottom and cotton wicks moistened with water at the upper end. The up and down movement of the flies was monitored by two pairs of infra red (IR) emitters and sensors which were placed perpendicular to one another in such a manner that when a fly cut the IR beams, the event was recorded in 5 min bins by a computerised recording and display system. The flies were classified as being rhythmic, arrhythmic or having ultradian rhythmicity based on visual observation of the actograms. The fraction of flies in each population that were rhythmic was estimated, for each of the four experimental protocols, and the arcsine square root transformed values (Freeman and Tukey, 1950) were used as data in two separate mixed model analyses of variance (ANOVA). In both analyses, replicate populations were treated as random blocks. In one analysis, only data from experiments 1, 3 and 4 were used, and the experimental protocol and sex were considered as fixed factors crossed with blocks. In the other analysis, data from only the males in all four experiments was used, and the experimental protocol was considered as a fixed factor crossed with block.

2.3 b Results

The regime in which the locomotor activity was assayed is seen to clearly influence the fraction of flies that exhibited a circadian rhythm in locomotor activity. While only 26.4% of flies exhibited a circadian rhythm in locomotor activity when assayed in LL, more than 77% flies were rhythmic when assayed in DD regime (Table 3, Fig. 5). The ANOVA using both male and female data from three experiments (1, 3 and 4) revealed a significant main effect of experiment (Table 4 a). Multiple comparisons revealed that the fraction of rhythmic flies was significantly smaller in flies that were assayed in the LL regime as compared to those assayed in the other regimes (Tukey's test, Table 3). A significant effect of sex was also seen, with higher fraction of males exhibiting a circadian rhythm compared to females ($p < 0.01$). The ANOVA using data from males in all the four experiments revealed a significant main effect of the experiment (Table 4 b). Multiple comparisons revealed that the fraction of rhythmic flies when assayed in LL without dark pulse (experiment 1) was significantly less than the other three protocols, while those assayed in LL with dark pulse (experiment 2) was significantly less than those assayed in DD (experiments 3 and 4) (Tukey's test) (Fig. 5, 6, Table 3). There was no significant difference between the fraction of rhythmic flies assayed in DD after 15 days in LD 12: 12 h and those directly assayed in DD (experiments 3 and 4) (Fig. 5).

2.3 c Discussion

The results of our experiments suggest that a large fraction of individual flies have retained their ability to exhibit locomotor activity rhythm with circadian periodicity, even after 600 generations of rearing in an aperiodic environment (Fig. 6 a). It is also clear that LL has a direct suppressive effect on the expression of the circadian rhythm of locomotor activity in

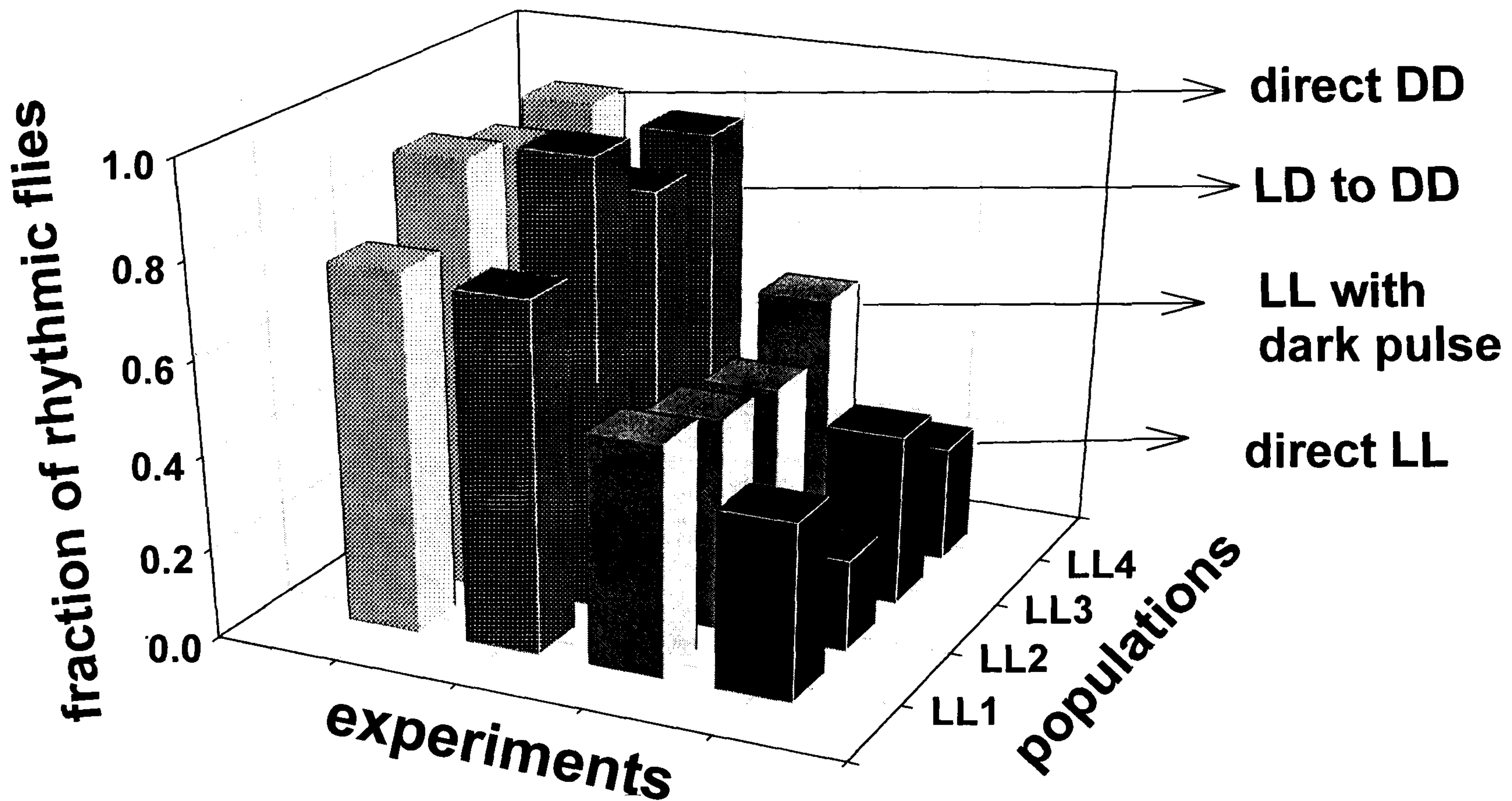

Fig. 5 Fraction of flies that exhibited a circadian rhythm in locomotor activity when assayed under four different experimental protocols.

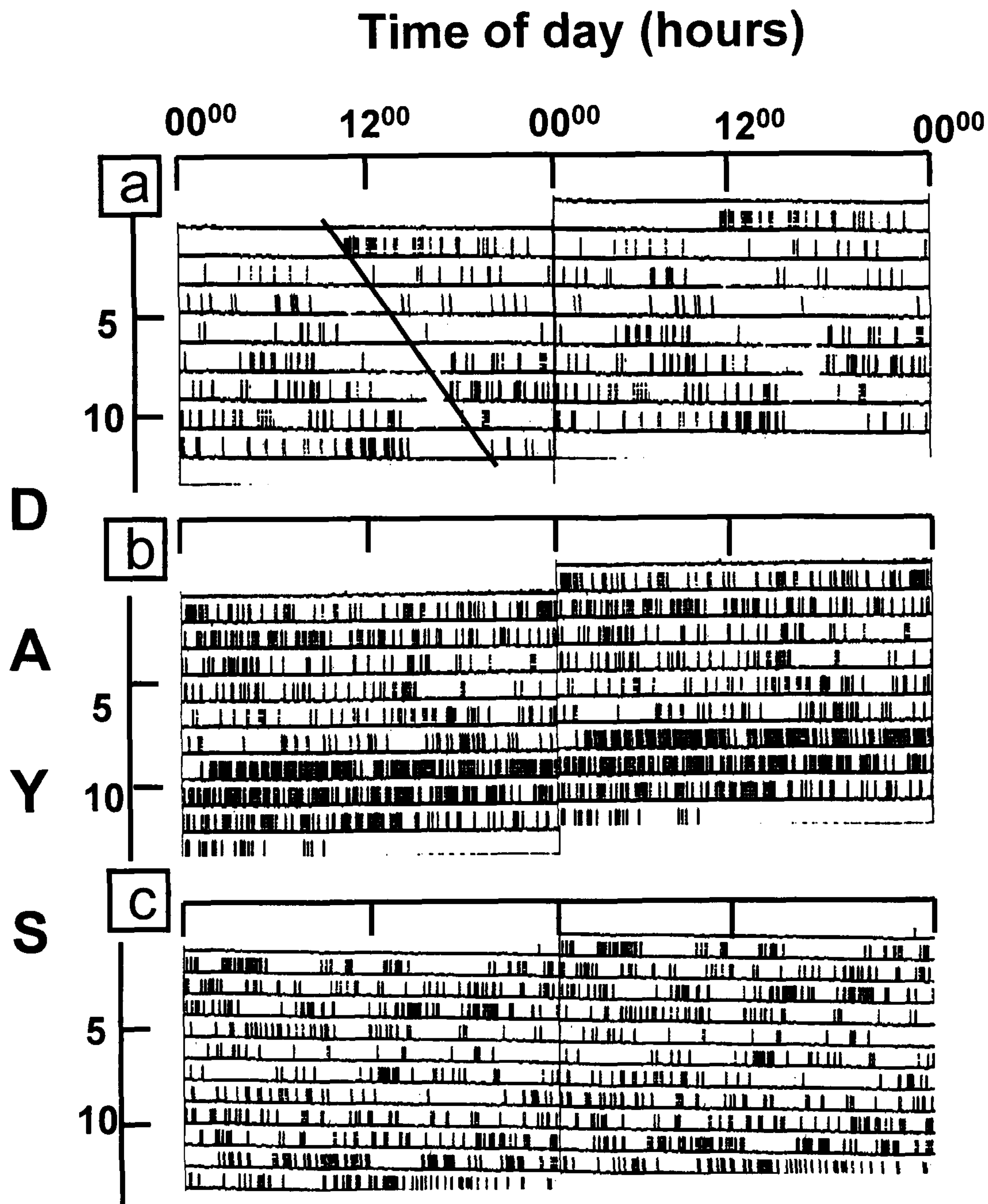

Fig. 6 Representative locomotor activity records of flies assayed in LL regime exhibiting (a) circadian rhythm (b) arrhythmicity and (c) ultradian rhythm. Abscissa shows time of day while ordinate shows number of days. Thick bars indicate activity, while horizontal lines indicate rest.

Table. 4 a Results of analysis of variance (ANOVA) on the fraction of males and females from each population exhibiting circadian rhythm in locomotor activity under three experimental protocols (1, 3 and 4). Block and interactions involving block cannot be tested for significance in this design.

Effect	*df*	**MS**	***F***	***p*-level**
Sex (S)	1	0.256	38.422	**0.008**
Experiment (E)	2	1.106	21.096	**0.002**
Block (B)	3	0.025	--	--
S × E	2	0.028	1.822	0.241
S × B	3	0.007	--	--
E × B	6	0.052	--	--
S × E × B	6	0.015	--	--

Table. 4 b Results of analysis of variance (ANOVA) on the fraction of males from each population exhibiting circadian rhythm in locomotor activity under four experimental protocols. Block and the interaction of block with light regime cannot be tested for significance in this design.

Effect	*df*	**MS**	***F***	***p-level***
Experiment (E)	3	0.574	33.78	$\boldsymbol{p < 0.0001}$
Block (B)	3	0.011	--	--
E × B	9	0.017	--	--

adults, as a significantly smaller fraction of adults exhibited a circadian rhythm when assayed in LL as compared to the other two regimes (LD 12:12 h and DD). Although in the case of experiments 1 and 2 the activity of most flies appeared to be arrhythmic (Fig. 6 b), a small fraction of flies (7-25%) showed very distinct ultradian rhythmicity (Fig. 6 c). Among the two experimental protocols in which flies were assayed in LL after being raised as pre-adults in LL, significantly greater fraction of flies were rhythmic when they were subjected to a dark stimulus of 12 h during larval development. This suggests that the dark stimulus given during the egg and early larval stages can initiate a circadian locomotor activity rhythm in the adults even when assayed in LL regime. The fact that these results are consistent across four independent populations of flies (Fig. 5) further strengthens our results.

2.4 Persistence of oviposition rhythm in individual flies

Oviposition rhythm in insects has been the subject of several studies, but almost all of them have used groups of females to study the rhythm. In one such study, the yellow fever mosquito *Aedes aegypti* showed well-defined peaks in oviposition both in the field and in the laboratory under LD cycles of 12:12 h (Haddow and Gillett, 1957). The oviposition peaks in this study were seen to coincide with the end of the light period of the LD cycle. In a separate study, *A. aegypti* raised in DD were found to exhibit a weak rhythm of oviposition when assayed in DD (Gillett et al., 1959). However, after exposure to a single brief light pulse, a well-defined periodicity in oviposition appeared, whereas the rhythm disappeared in LL. The results of these and other similar experiments (Gillett et al., 1961) suggested that the oviposition rhythm in mosquitoes depends on external time cues, and that in DD a trigger is required to set the circadian pacemaker in motion. Similarly, the oviposition rhythm in the pink boll worm

Pectinophora gossypiella was found to be suppressed by light, although a transfer from LL to DD initiated the rhythm with a periodicity of 22.66 h (Pittendrigh and Minis, 1964; Minis, 1965). The same pattern of effects was observed in the European corn borer *Ostrinia nubilalis* in which the oviposition rhythm disappeared in LL and was reinitiated with a free running period of 22.8 h in DD when individuals were transferred from LL to DD (Skopik and Takeda, 1980).

Rhythmic oviposition has also been reported in fruit flies (*Drosophilidae*) including *Drosophila* species (Rensing and Hardeland, 1967; Gruwez et al., 1972; David and Fouillet, 1973; Allemand, 1974, 1976 a, b, 1977) and in *Zaprionus* (Allemand, 1976 c). However, most of these studies were also carried out on groups of flies in LD cycle of 12:12 h, and hence do not shed any light on the possible endogenous nature of the rhythm in individual females. The studies on *Drosophila* oviposition rhythm (Rensing and Hardeland, 1967; Gruwez et al., 1972; David and Fouillet, 1973; Allemand, 1974; Allemand, 1976 a, b, c, 1977) have demonstrated that egg laying was rhythmic in a periodic environment (LD 12:12 h) and that the peak of egg laying occurred shortly after 'lights off' of the LD cycle. The rhythm was unimodal when the intensity of light in the light phase of LD cycle was greater than 60 lux, whereas at lower intensities of light the rhythm was found to be bimodal (Allemand, 1976 a, 1977). It was also found that in LD cycle of 12:12 h, oocyte development was periodic and there was a maximum retention of chorionated eggs just before peak oviposition (Allemand, 1976 b). This suggests that the oviposition rhythm in these flies may be an outcome of the rhythmicity in oocyte development and egg retention. In one of the first attempts to probe the possible endogenous control of the oviposition rhythm, groups of flies that were reared in DD were found to be arrhythmic when assayed in DD (Allemand, 1977). Moreover, a transfer from LD cycle of 12:12 h to DD did not elicit rhythmicity in oviposition, although a rhythm in vitellogenesis was found to persist

(Allemand, 1977). However, it is possible that inter-individual variation in the periodicity of the oviposition rhythm could eliminate the overall (group level) rhythm in LL and DD. The lack of rhythmicity in oviposition under LL and DD in the studies of Allemand (1977), could be due to the fact that oviposition was assayed on groups of females. A study of rhythmicity using groups might not unequivocally reveal the nature of the rhythm of the individuals that constitute the group, especially if there is high variation in the periodicity exhibited by the individuals. The only report that describes studies on the oviposition rhythm in individual flies demonstrated that the rhythm in egg laying occurred in LD cycles, whereas in continuous dim light the rhythm disappeared (Fleugel, 1978). While the conclusion from the early studies on the oviposition rhythm in *Drosophila* was that this rhythm is likely to be light driven and probably of exogenous origin (Fleugel, 1978; Saunders, 1982; Ashburner, 1989), more recent experiments have shown that this rhythm phenotype is affected by the *per* locus (McCabe and Birley, 1998). The study reported here is the first to clearly demonstrate that endogenous time keeping mechanisms control circadian oviposition rhythm at the level of individual *D. melanogaster* females, and that these individual level rhythms can persist even in populations subjected to an aperiodic environment (LL) for more than 600 generations.

2.4 a Materials and methods

From the running culture of one of the baseline populations JB1, arbitrarily chosen 1-2 day old male - female pairs were introduced into vials containing ~ 5 ml of food medium; 24 such vials were set up in each of the three light regimes, LL, LD 12:12 h and DD. At 2 h intervals, the flies were transferred to fresh food vials and the number of eggs laid within that interval was counted, yielding a time series of two hourly egg production for each individual fly.

In LL regime this procedure was carried out for 25 days, in LD 12:12 h regime for 10 days and in DD regime for 13 days. In case of death or escape of flies, they were replaced by flies from a cohort of matching age that were maintained in the respective light regime as backups. Care was taken to ensure that the experimental set up, including vials, the type of food medium, and food transfer protocols, was similar to that of the maintenance regime, thus ensuring minimal influence of extraneous factors in the assaying of the rhythm.

The periodicity of the egg laying rhythm was estimated by subjecting the primary data to Fourier spectral analysis using STATISTICA ™ (Statsoft Inc, 1995). Statistical significance of observed circadian periods in the periodogram was tested using the technique of Siegel (1980). The flies in each light regime were then classified as having one significant period (single), two significant periods (double), or no significant period (none). A 3×3 contingency table was used to test whether the distribution of flies exhibiting single, double and no period differed among the three light regimes. The distributions of periodicities of the individuals exhibiting a single significant period were then subjected to Kruskal-Wallis Analysis of Variance (ANOVA) by ranks, to determine whether the distribution varied among the three light regimes. Pair-wise comparisons among light regimes were done by Kolmogorov-Smirnov two-sample tests. Finally, data from all the females within each light regime were pooled and the average number of eggs laid (per 2 h interval per fly) was subjected to Fourier spectral analysis in the same manner as for individual flies.

2.4 b Results

In all the three light regimes (LL, LD 12:12 h, and DD), at least half of the individuals showed circadian rhythm in oviposition (Table 5). There was no significant difference in the

Table. 5 The number of flies which exhibited no significant periodicity, two significant periods and a single significant period in oviposition rhythm in the three light regimes.

	LL REGIME $n = 41$	**LD REGIME** $n = 23$	**DD REGIME** $n = 30$
No significant period	20	8	14
Two significant periods	2	2	1
One significant period	19	13	15

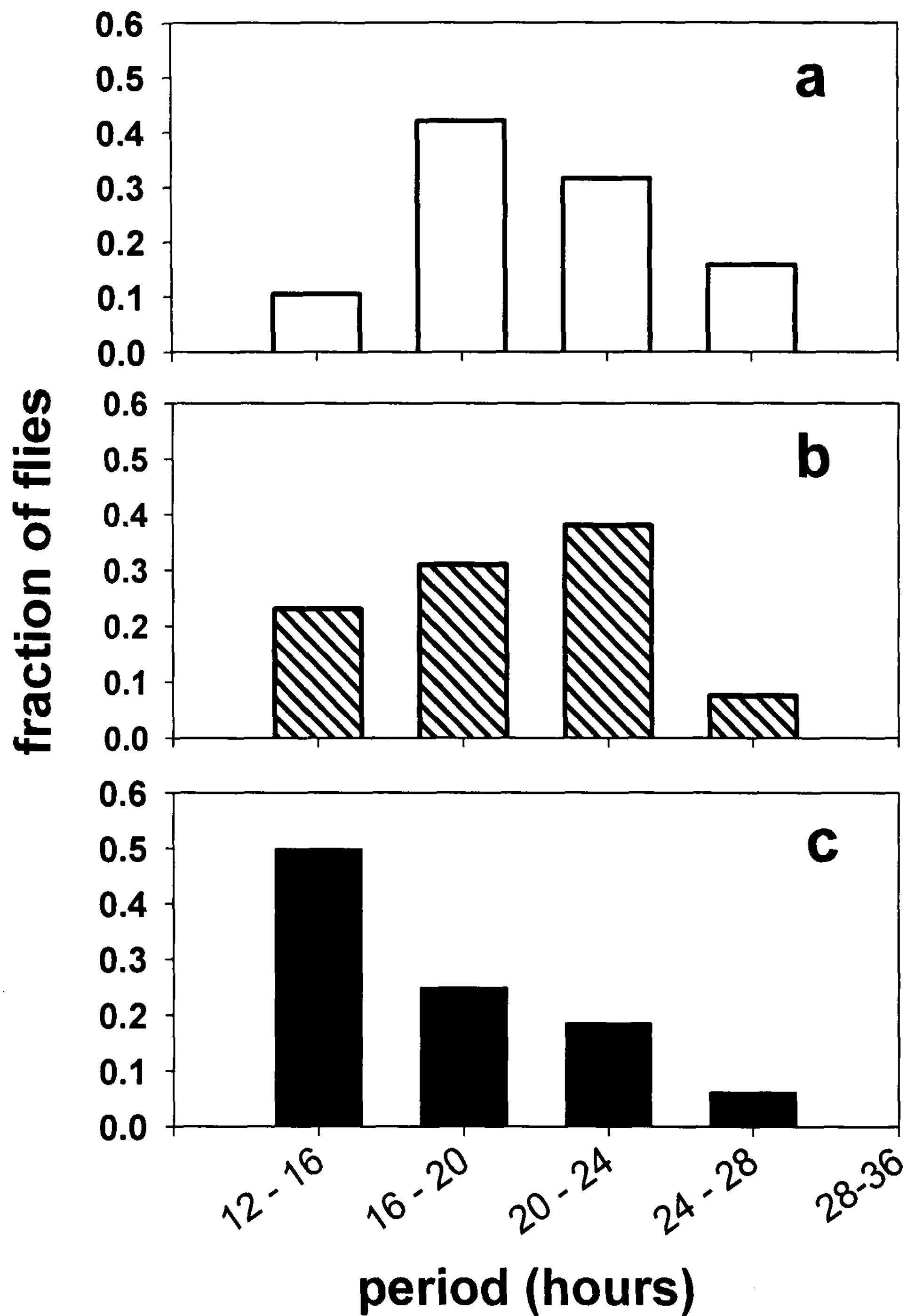

Fig. 7 Frequency distribution of periodicity in individual flies that exhibit a single significant periodicity of oviposition under (a) LL, (b) LD and (c) DD regimes. Individuals are grouped into 4 h class intervals.

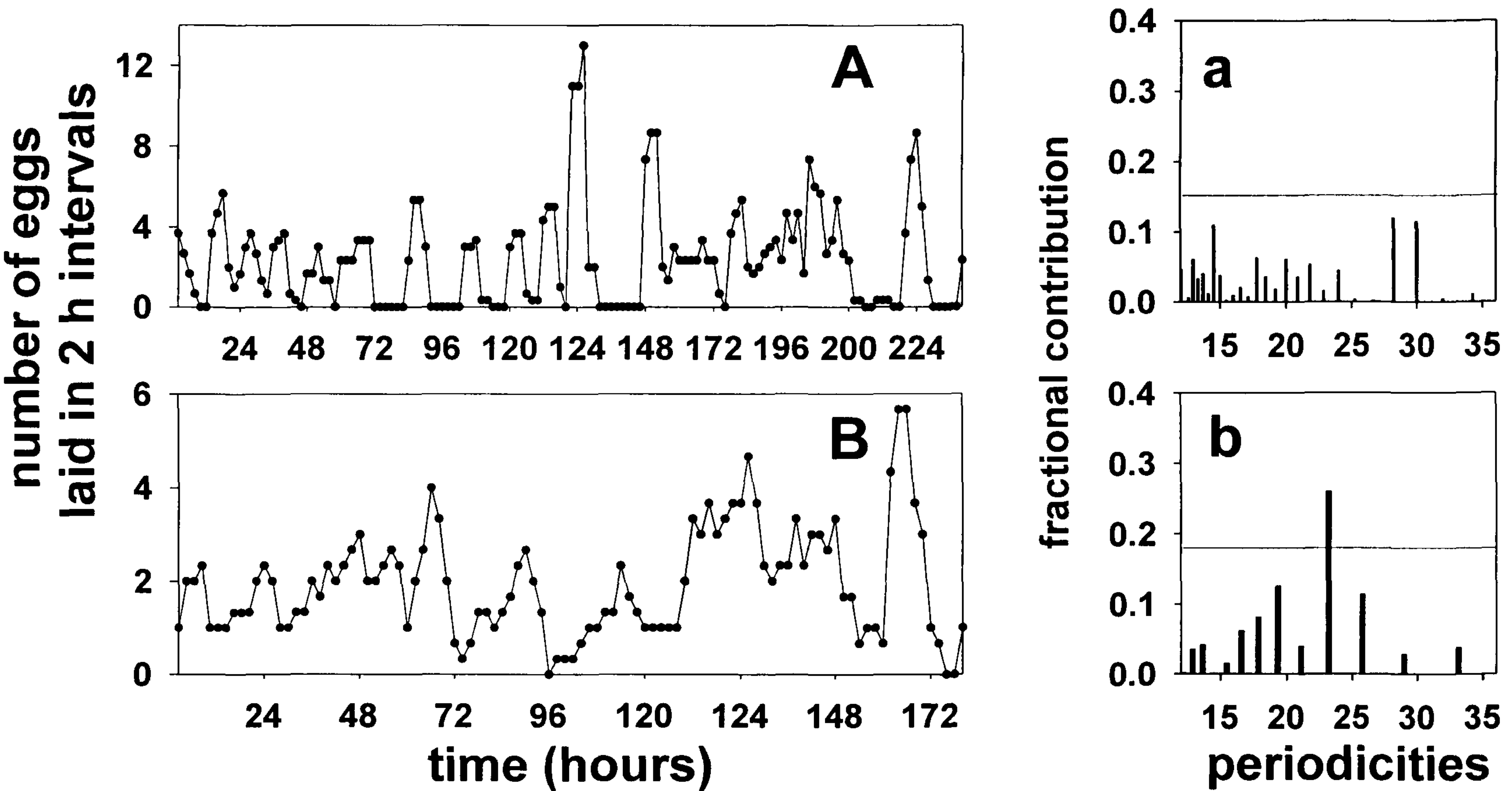

Fig. 8 Time series data of oviposition rhythm of individual flies in LL regime (A, B), and corresponding periodograms (a, b). The top panel (A) shows oviposition with no significant circadian periodicity (a), bottom panel (B) shows oviposition with periodicity of 23.2 h (b).

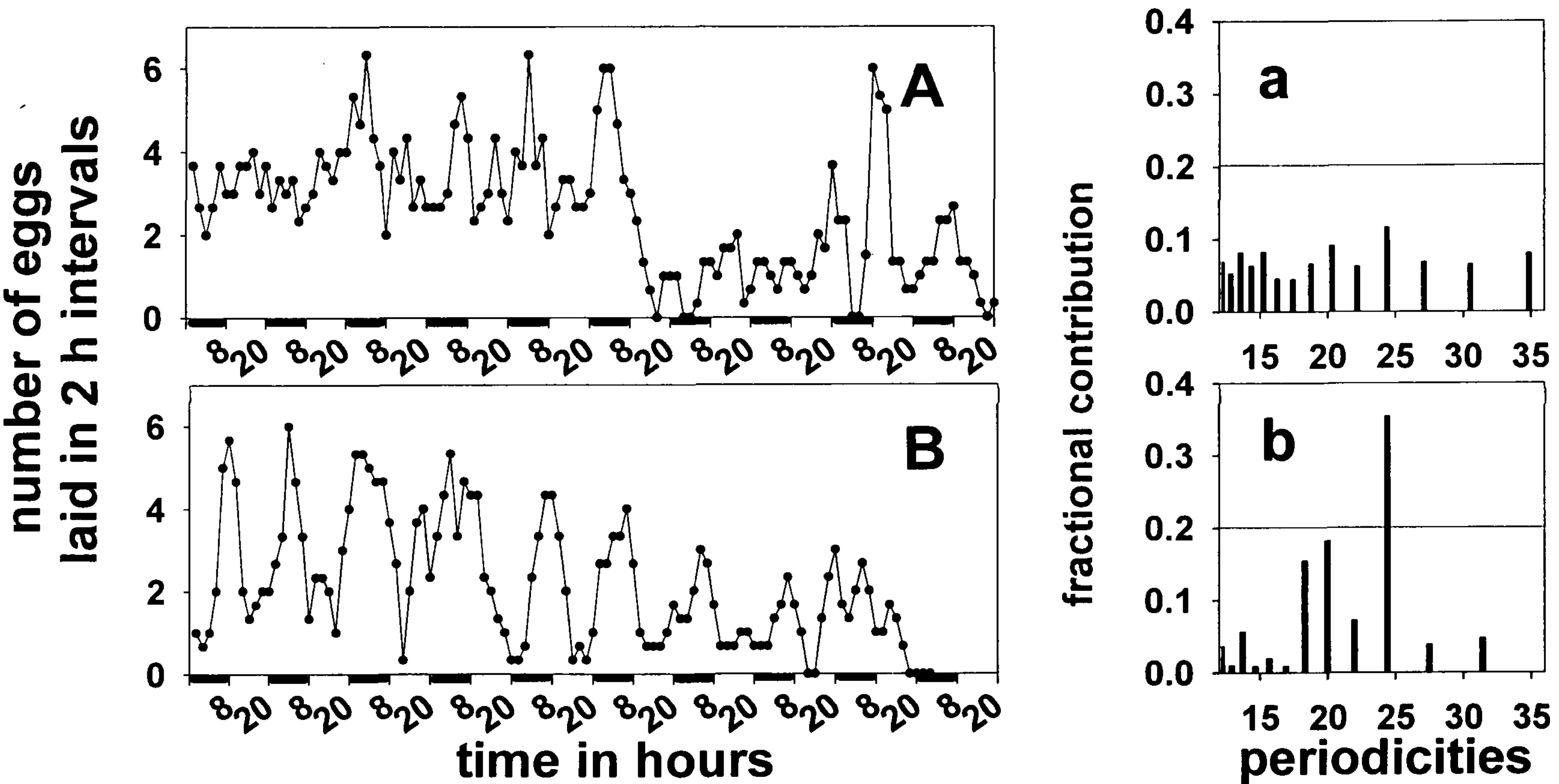

Fig. 9 Time series data of oviposition rhythm of individual flies in LD regime (A, B), and corresponding periodograms (a, b). The top panel (A) shows oviposition with no significant circadian periodicity (a), bottom panel (B) shows oviposition with periodicity of 24.4 h (b). The lights were switched on at 08.00 h and switched off at 20.00 h (dark phase is indicated by black bars on the x axis).

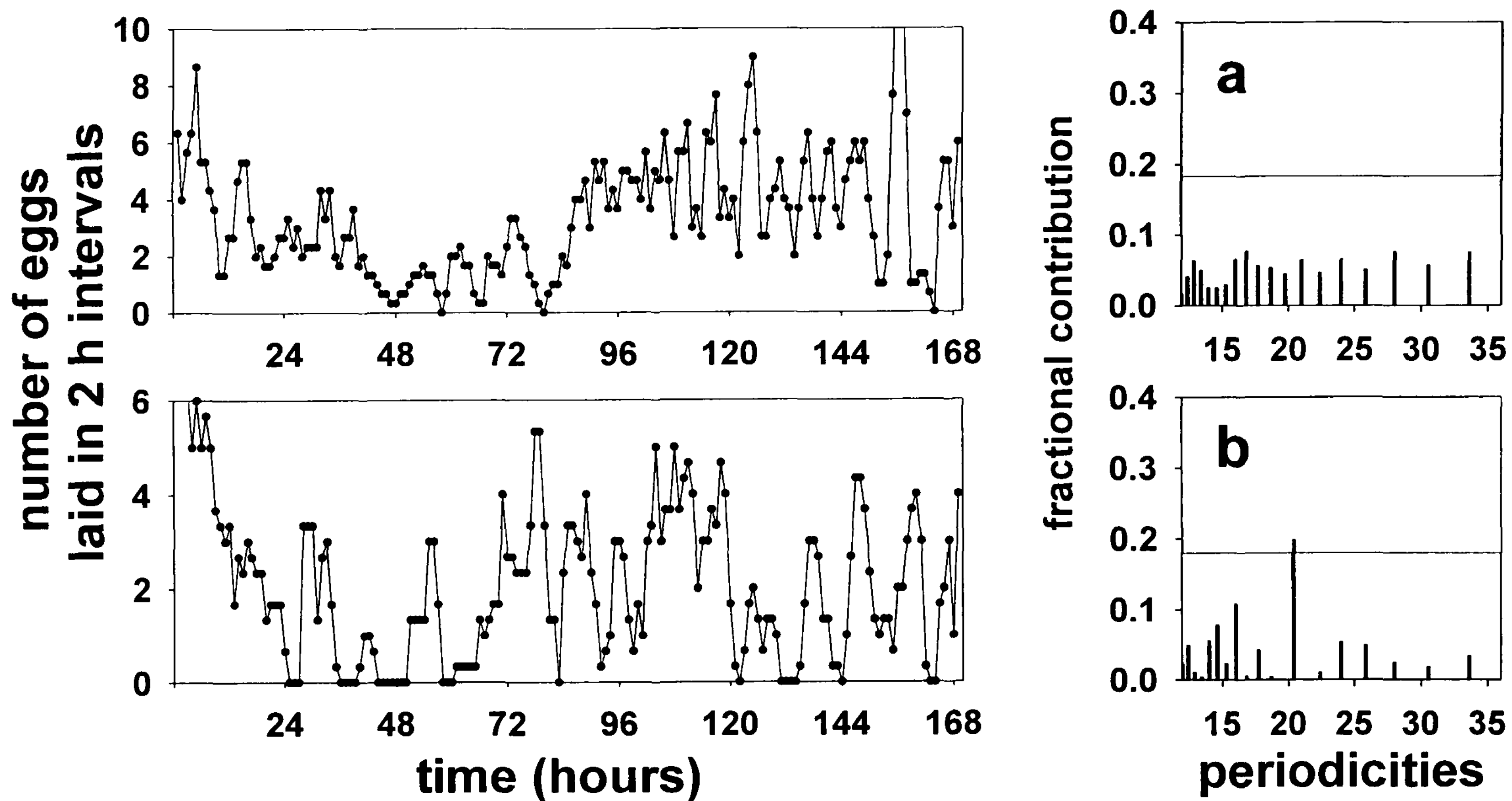

Fig. 10 Time series data of oviposition rhythm of individual flies in DD regime (A, B), and corresponding periodograms (a, b). The top panel (A) shows oviposition with no significant circadian periodicity (a), bottom panel (B) shows oviposition with periodicity of 20.2 h (b).

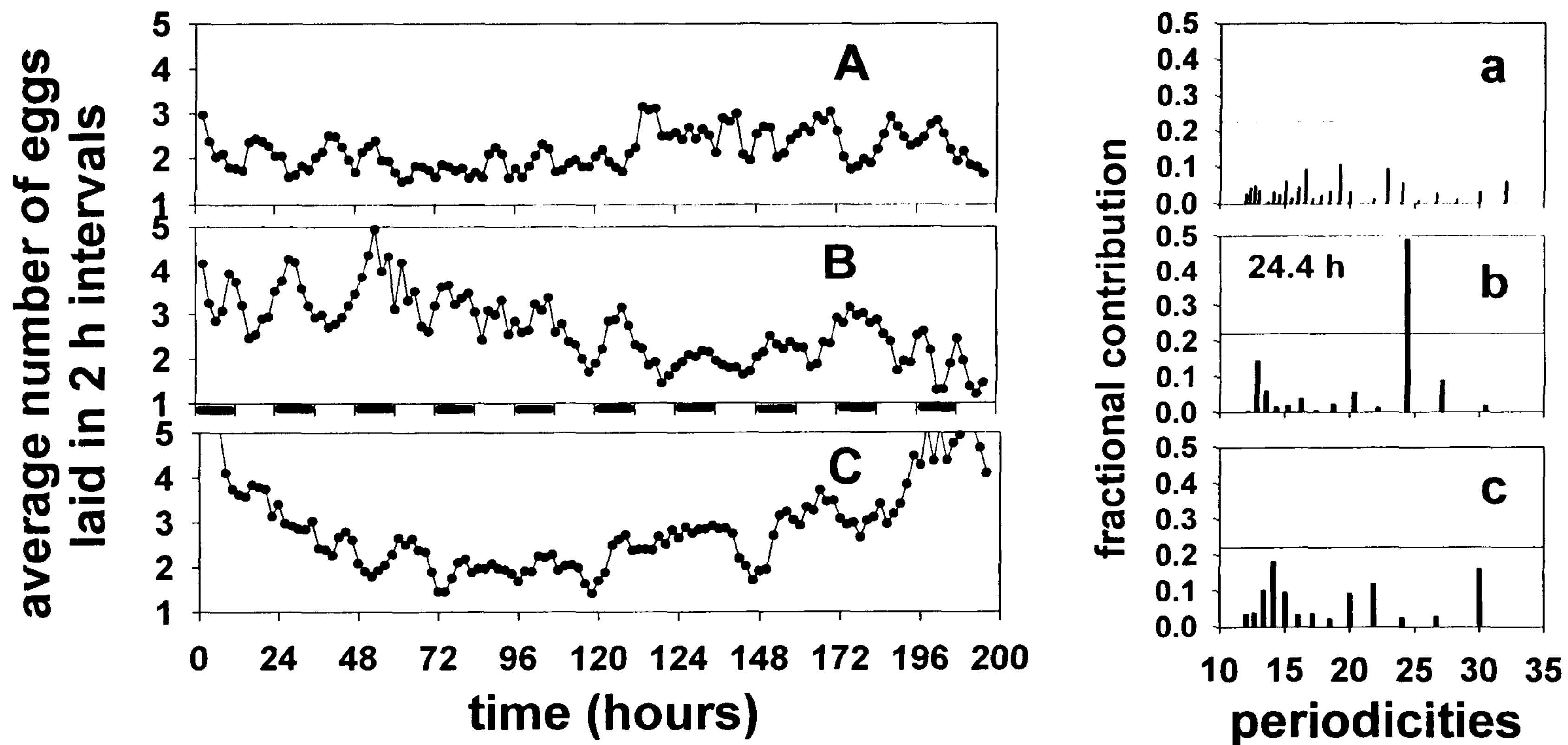

Fig. 11 Time series data averaged across groups of females in (A) LL, (B) LD and (C) DD regimes. Corresponding periodograms show that oviposition in the group occurs with no circadian periodicity in LL (a) and DD (c) regimes, while in LD, oviposition of the group is rhythmic with a periodicity of 24.4 h. Lights were switched on at 08.00 h and switched off at 20.00 h in the LD regime (dark phase is indicated by the black bars on the x-axis).

distribution of the number of flies exhibiting single, double and no period among the three light regimes ($\chi^2 = 1.4247$, $p > 0.05$). In the LL regime, 19 out of 41 individuals assayed showed rhythm in oviposition, and of these more than half exhibited a rhythm with a distinct single periodicity in the range of 16.67 - 27.2 h (Fig. 7). In the DD regime, 15 out of 30 flies assayed showed a significant single periodicity in egg laying rhythm in the range 12.4 – 25.84 h, whereas in the LD regime, 13 out of 23 flies assayed showed a significant single peak in egg laying rhythm with periodicity ranging between 12.2 – 25.5 h (Fig. 7). Some representative time series of individual flies in each regime are shown in Figures 8, 9, 10. The mean (± S.D.) periodicity of the flies which exhibited oviposition rhythm with a single period was 20.23 ± 3.8 h ($n = 18$), 19.42 ± 4.82 h ($n = 13$) and 17.68 ± 3.9 h ($n = 15$) in LL, LD and DD regimes respectively. The Kruskal–Wallis test did not reveal any significant difference in the distribution of periodicity among the flies that exhibited a single period in the three light regimes ($H = 3.6867$, $p = 0.1583$) (Fig. 7). However, pair-wise Kolmogorov-Smirnov two-sample tests revealed that the distribution of periodicity in LL was significantly different from that in DD ($p < 0.05$), while the distribution in LD was not different from that in the LL and DD regimes. The analysis of data averaged across all the flies in each of light regimes yielded no significant periodicity in LL and DD regimes, whereas oviposition was periodic in LD regime with τ of 24.4 h (Fig. 11).

Although results of our experiments when analysed using Kruskal-Wallis ANOVA by ranks did not reveal a statistically significant difference in the distribution of periodicities in the three different light regimes, pair-wise comparisons suggest that the distribution of τ in LL is different from that in DD. The lack of statistical significance overall may be due to the sample sizes of flies showing a single period being low (n ranging from 13-19), and the Kruskal – Wallis ANOVA by ranks being a non-parametric and less powerful test, not picking up differences

among the three light regimes. Moreover, since the flies used in this assay have been reared in LL for more than 600 generations, this may have resulted in periodicity of individual flies to be very deviant from each other. The large variation among individual flies probably contributes to the failure of the statistical test to pick up any differences among the three light regimes.

2.4 c Discussion

The fact that in our experiments the oviposition rhythm persists in individual flies of *D. melanogaster* in LL and in DD regimes clearly supports the view that this rhythm is endogenously controlled. Several studies in *D. melanogaster* have demonstrated that TIM protein which is considered as one of the molecular oscillators that control several overt rhythms, is degraded in presence of light (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996; Dunlap, 1999) as a result, several rhythms regulated by these light sensitive proteins should damp out in LL. On the contrary, a number of experiments including ours demonstrate that circadian rhythms do persist in the presence of light. Rhythmicity in oviposition and locomotor activity under LL regime has been reported in *per* mutants of *D. melanogaster*; per^{+}, per^{0}, per^{s}, and per^{l} (McCabe and Birley, 1998). In LL, the locomotor activity of a small fraction of individual males and females is found to be rhythmic in populations of *D. melanogaster*, which have been reared for more than 600 generations in LL (Section 2.3). The eclosion of these flies is also found to be rhythmic in LL when subjected to a dark pulse during the first 12 h after egg collection (Section 2.2, Sheeba et al., 1999 a). These results suggest the possible involvement of some unknown genes and their protein products, which are probably light refractory, in the regulation of circadian rhythms of oviposition, locomotor activity and eclosion in LL, even though oscillation of TIM is suppressed. The apparent lack of rhythmicity

of oviposition in groups of flies in LL and DD, observed in earlier studies is most likely an artefact of assaying groups of flies, because inter-individual differences in periodicity in LL and DD could obliterate the group periodicity. This notion is supported by our observation that analysis of data pooled over all flies in each light regime yields results very different from those seen when data from individual flies is analysed separately (Fig. 11). In both aperiodic regimes LL and DD, pooled data does not reveal any significant circadian periodicity probably because the individual females rhythms of oviposition are not synchronised with each other in aperiodic regimes, while in LD the pooled data showed periodicity which was 24.4 h. The distributions of period of oviposition rhythm in the three regimes (Fig. 7) are such that the mode in LD regime is close to 24 h, indicating that most of the individuals exhibit some degree of synchronization to the LD cycle (Fig. 1). Our results, thus, clearly indicate that the study of groups of individuals in order to understand the nature of rhythms that occur at the level of the individual is very likely to lead to misleading results and interpretations.

Unlike previous experiments in which brief light or dark pulses or temperature pulses were used to trigger the rhythm under constant conditions (Saunders, 1982), the rhythmicity in egg laying under LL or DD regime in our experiment was not assayed after administering pulses of light, darkness or temperature. This suggests that endogenous time keeping mechanisms that control the oviposition rhythm in *D. melanogaster* females function independently of external time cues and do not require any trigger to set the oscillator in motion.

2.5 Conclusions

The results of studies on our baseline populations have demonstrated that they have retained the ability to exhibit circadian rhythmicity in eclosion, locomotor activity and

oviposition even after 600 generations of maintenance in an aperiodic environment. These results suggest that the ability to be rhythmic offers some advantage to organisms other than what would be gained by synchronising to a periodic external environment. Perhaps this advantage accrues from a need to maintain synchrony among various periodic processes within the individual. Our assays showed that the baseline populations also exhibit unusually higher inter-individual variation in τ, compared to flies that have been recently caught from the wild or flies that have been raised under laboratory LD cycles for many generations. This suggests that the ability to possess circadian rhythmicity *per se* may have conferred an adaptive value to these flies independent of the need to possess a circadian rhythm with a value very close to 24 h. We speculate that the absence of external environmental cycles of the order of 24 h, for over 600 generations has perhaps caused a relaxation in the selective pressure to retain an endogenous period very close to 24 h. Thus these populations have perhaps evolved under a situation where although there was no selection for possessing a periodicity very close to 24 h, there was still a need to possess circadian rhythmicity *per se*.

Chapter 3

Studies Of Components Of Fitness In The Baseline Populations

3.1 Introduction

For any trait to have an adaptive value it should necessarily confer some fitness advantage to the organism. Therefore, our next step in the studies of the baseline populations was to examine the effects of different light regimes on various components of fitness both at the pre-adult and adult stages. Rigorous empirical validation of the idea that circadian rhythms have an intrinsic adaptive value would require a demonstration of differential fitness effects of different light regimes, and the further demonstration of adaptive evolution in response to different light regimes in experimental populations. However, relatively few studies thus far have attempted to systematically address the possible intrinsic adaptive significance of circadian rhythms. A few studies have shown that organisms normally reared in LD conditions tend to perform better in LD cycles of periodicity similar to their rearing conditions. *Drosophila melanogaster* flies, normally reared on a LD cycle 12:12 h, were found to have the greatest longevity under LD 12:12 h, as compared to LD 13.5:13.5 h, LD 10.5:10.5 h, or LL (Pittendrigh and Minis, 1972). Similar observations have been made on the effect of LD cycles of varying periods (*T*) on growth rates in several plant species (Went, 1959). Moreover, deleterious effects of LL on tomato (*Lycopersicon esculentum*) have also been observed (Highkin and Hanson, 1954; Hillman, 1956), leading to a general assumption that LL conditions exert harmful effects on organisms habituated to LD cycles (Highkin and Hanson, 1954; Daan and Aschoff, 1982). It is not, however, clear that LL conditions are harmful *per se*, especially in animals, and it is entirely possible that populations routinely maintained in LL may evolve so as to better tolerate any adverse physiological effects of constant light. Indeed, in a more recent study, the growth rate in cultures of the cyanobacterium *Synechococcus* was seen to be greater in LL than in various LD cycles (Ouyang et al., 1998).

3.2 Effect of different light regimes on pre-adult fitness components

We examined two components of pre-adult fitness in the four baseline populations of *D. melanogaster* (JB 1..4). Egg to eclosion development time and survivorship in these populations was assayed under three different light regimes: LL, LD 12:12 h cycles, and DD, in order to study the effect of different light regimes on these two major components of pre-adult fitness.

3.2 a Materials and methods

From the running culture of each population in cages, eggs were collected by allowing females to lay eggs on a non-nutritive agar medium for exactly one hour. Exactly 30 eggs were collected into vials (9.0 cm h × 2.4 cm d) containing ~ 6 ml of banana-jaggery food. In this manner, 24 such vials were set up per population, of which 8 vials were kept under LL, 8 vials under light dark LD cycles of 12:12 h, and the remaining 8 vials in DD. The light phase in these treatments was achieved by means of fluorescent white light sources, whereas the dark phase was actually a period when the flies were maintained in red light ($\lambda > 640$ nm), to facilitate observation and manipulation without interrupting the dark phase. After pupation, the vials were checked every 6 h for eclosing adults. At each six-hourly check, the number of male and female flies that eclosed in each vial was recorded, and the flies discarded. This process was continued until no more flies eclosed in any of the vials for three consecutive checks. The entire assay was repeated thrice to ascertain the repeatability of results. The procedure used here for assaying development time is a standard one in fitness component studies in *D. melanogaster* (Chippindale et al., 1997 a; Santos et al., 1997; Prasad et al., 2000), and ensures a moderate and controlled density to minimise environmental variation in development time due to either overcrowding, or hardening of medium due to a very low larval density (Mueller, 1985). It

should be noted that the protocol for assaying development time is very different from those used for studying eclosion rhythms in *Drosophila* (Chandrashekaran, 1998); the latter typically involve high larval densities in order to spread out the eclosions through crowding effects, thereby ensuring that several cycles of data can be obtained. In studies such as the present one, such high densities, being very different from the density at which the populations are normally maintained, would increase the likelihood of observing genotype × environment interactions that could make the data very difficult to interpret (Leroi et al., 1994).

From the primary data from each assay, egg to eclosion development time (in hours) and survivorship (the fraction of adults that emerged per vial) were computed. The population mean development times (ln transformed) in each light treatment, for each sex from each experiment were used as data in a mixed model analysis of variance (ANOVA) in which replicate populations were treated as random blocks and the various light dark regimes and sex as fixed factors crossed with blocks. Similarly, the population mean survivorship values (arcsin square-root transformed) in each light regime were used as data in a mixed model ANOVA, with replicate populations being treated as random blocks and the various light dark regimes as a fixed factor crossed with block. For both development time and survivorship, the mean values for each combination of block × light regime × sex (for development time), or block × light regime (for survivorship), from each of the three experiments served as replicate observations in the analysis. We used population means in the analyses because we wish to draw evolutionarily relevant inferences about the differential effects, if any, of light treatment on the development time and survivorship. For such inferences to be drawn, the crucial unit of analysis and interpretation is the population and not the individual (Rose et al., 1996).

3.2 b Results

Survivorship from egg to eclosion did not differ significantly among the various light treatments, indicating that light regime did not differentially affect survivorship in these populations ($F_{2,3} = 1.06, p = 0.4$) (Table 1). On the other hand, light regime did have a significant effect on development time, with the fastest development being in LL (Fig. 1). The ANOVA on development time data revealed a significant treatment effect ($p < 0.0001$) (Table 2). Across all the three assays, development time in LL, LD and DD were all significantly different from each other (paired t-test: $p < 0.0001$ for all pair-wise comparisons) (Fig. 1). As expected in *D. melanogaster*, there was a significant effect of sex on development time ($p = 0.00049$), with females developing faster than males (Fig. 2). The ANOVA did not reveal any significant difference in development time among the four replicate populations (blocks) ($p > 0.9$), nor were any of the interactions involving block significant (Table 2).

3.2 c Discussion

In contrast to previous reports that, in general, LL has deleterious effects on organisms (reviewed in Pittendrigh, 1960; Daan and Aschoff, 1982), we found that two major components of pre-adult fitness (survivorship and development time) were not adversely affected by LL in populations of *D. melanogaster* maintained in LL for over 600 generations. Indeed, development time in these populations was shortest in LL, suggesting that maintenance in LL for many generations may have resulted in the evolution of specific adaptations to constant light, thereby allowing flies from these populations to complete development faster in LL, as compared to LD and DD regimes. Thus, our results, while strengthening the idea that organisms perform best in

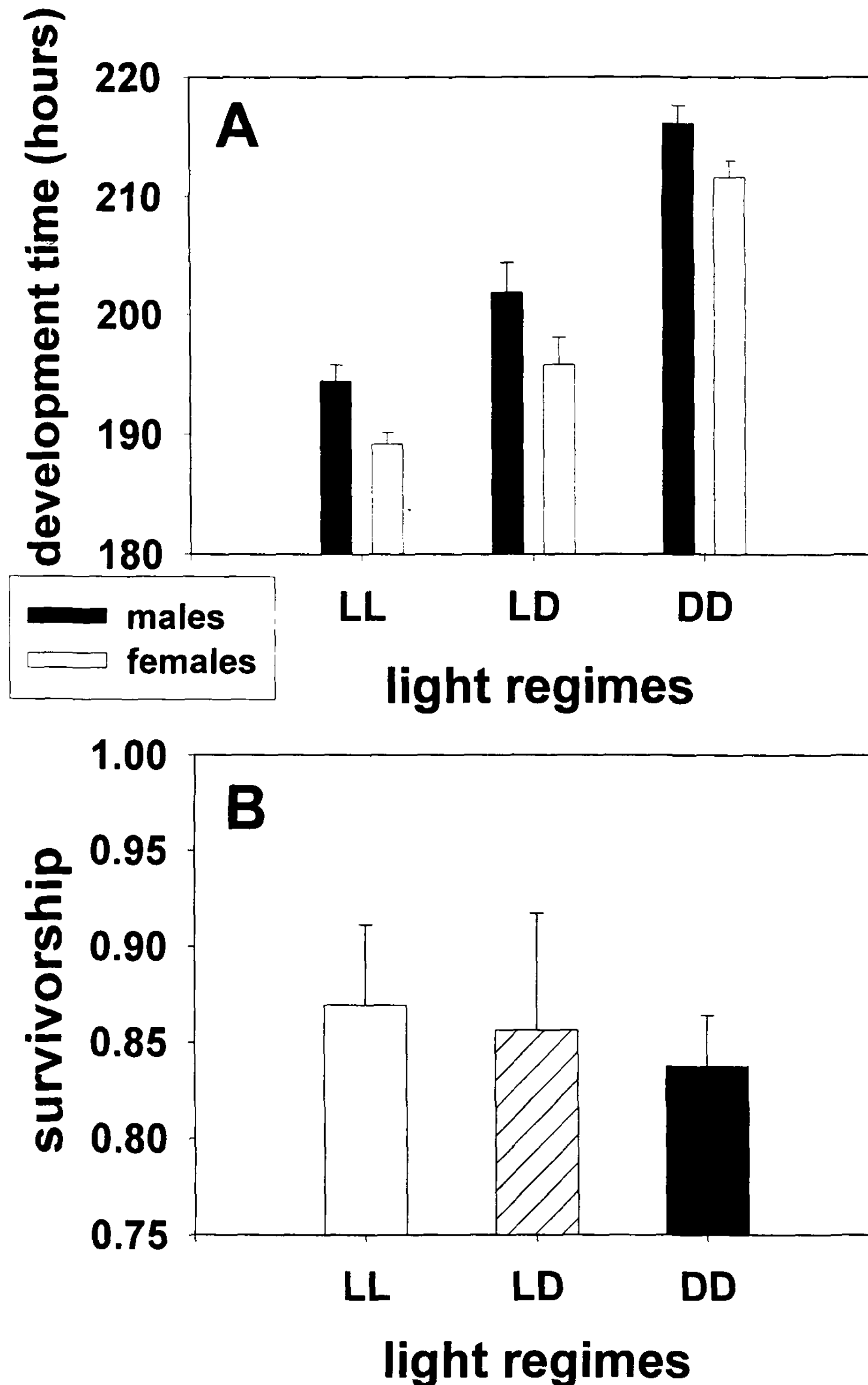

Fig. 1 Mean egg to eclosion (A) development time and (B) survivorship under LL, LD and DD (averged across 4 replicate populations and 3 experiments). Error bars represent 95% confidence intervals about the mean, constructed using variation among 4 replicate populations, after averaging across experiments for each population.

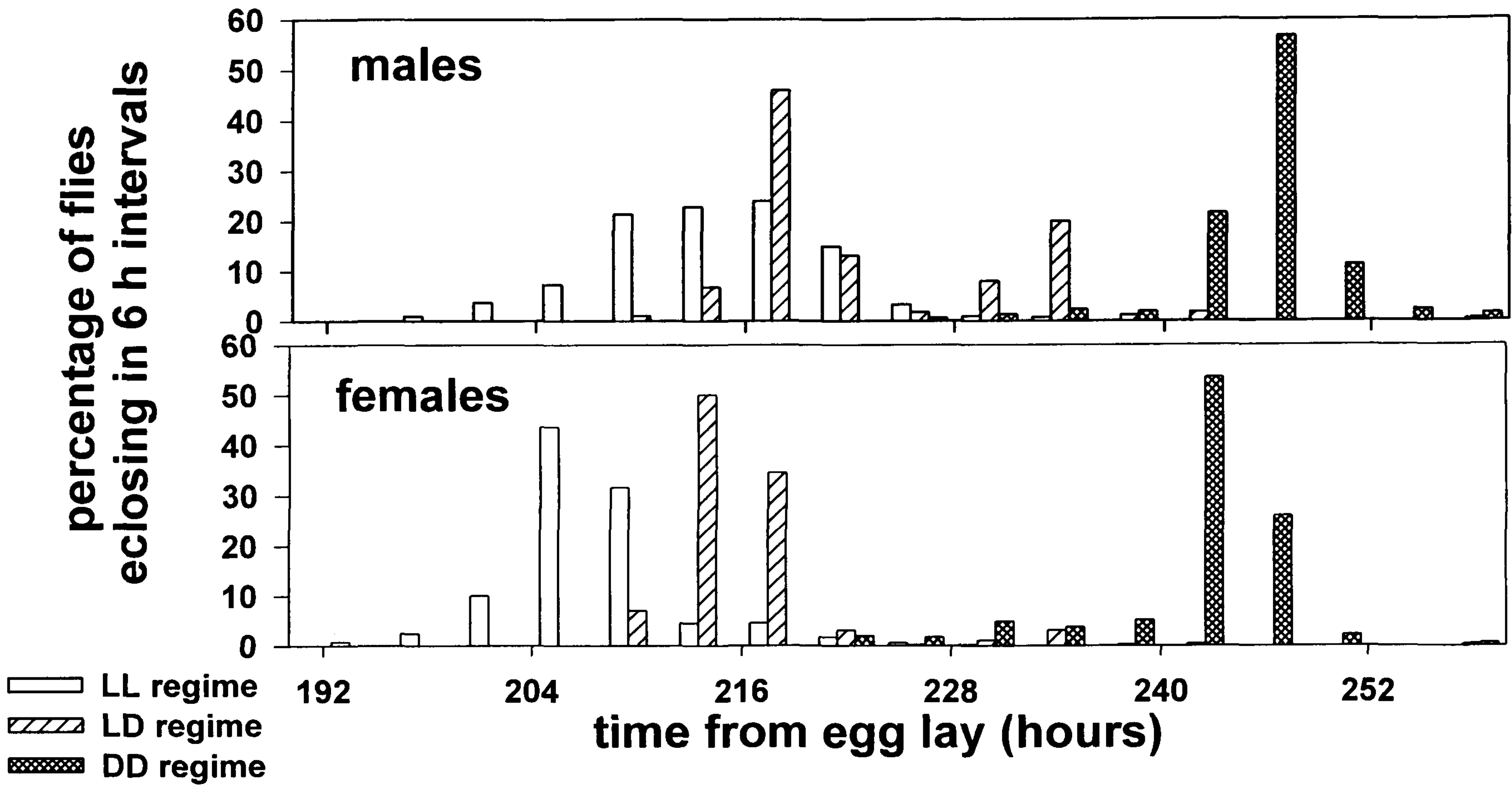

Fig. 2 Frequency distribution of egg to eclosion development time (pooled data from 4 replicate populations from one of the experiments) showing the degree of separation of the distribution of development time under LL, LD and DD regimes for both sexes.

Table. 1 Mean survivorship from egg to eclosion in the four JB populations under LL, LD and DD in three separate experiments. The 95 % confidence intervals are based on variation among the four replicate populations in each experiment × light regime combination.

	Experiment 1			**Experiment 2**			**Experiment 3**		
	LL	**LD**	**DD**	**LL**	**LD**	**DD**	**LL**	**LD**	**DD**
JB 1	0.82	0.65	0.78	0.93	0.88	0.87	0.90	0.91	0.85
JB 2	0.79	0.85	0.80	0.85	0.95	0.85	0.84	0.89	0.82
JB 3	0.92	0.79	0.87	0.89	0.90	0.86	0.83	0.82	0.85
JB 4	0.91	0.91	0.77	0.86	0.87	0.85	0.90	0.83	0.88
mean	**0.86**	**0.80**	**0.81**	**0.88**	**0.90**	**0.86**	**0.87**	**0.86**	**0.85**
95% C.I.	**0.09**	**0.18**	**0.07**	**0.06**	**0.06**	**0.02**	**0.06**	**0.07**	**0.04**

Table. 2 Results of mixed model ANOVA on ln-transformed development time. The population mean development times from each light regime × sex × experiment combination were used as the units of analysis. Replicate populations were treated as random blocks and light regime and sex as fixed factors crossed with block. The three experiments, thus, act as within-cell replicate observations for the analysis.

Effect	***df***	**MS**	***F***	***p*-level**
Block (B)	3	0.0003	0.032	0.9922
Light regime (L)	2	0.0681	1403.741	**<0.0001**
Sex (S)	1	0.0128	271.509	**0.0004**
B × L	6	<0.0001	0.004	1.0000
B × S	3	<0.0001	0.004	0.9996
L × S	2	0.0001	8.723	**0.0167**
B × L × S	6	<0.0001	0.001	1
Error	48	522.4183	--	--

the light regime that they are routinely reared in, extends this logic to organisms that have been maintained in LL, and suggest that there are no intrinsic, deleterious physiological effects of LL on pre-adult fitness in *D. melanogaster* that adaptive evolution cannot overcome.

Development time is known to be a highly labile trait, and the fact that all four populations studied exhibited a similar trend in development time in the three different light regimes, suggests that light conditions exert their influence on some highly conserved aspect of the developmental pathway. This idea is supported by the absence of any significant interactions involving block, which is unusual in studies on fitness components in *Drosophila* (A Joshi, *pers. comm.*). The fact that the effects of light regime were consistent across independent replicate populations is important because it allows us to rule out fortuitous effects due to either chance or the unique genetic composition of a particular population. Such populational level replication is crucial if one wishes to draw evolutionary conclusions from data on fitness effects of different environmental regimes (Mueller, 1995; Rose et al., 1996; Joshi, 1997 a) and has been a crucial aspect of experiments aimed at studying adaptive significance that has been neglected in most previous studies on the possible adaptive significance of circadian rhythms.

There is some evidence that subjective time estimation in organisms may feed back into determining the duration of different life-stages. *D. melanogaster per* mutants with relatively short τ have been seen to have relatively short development time and *vice versa* (Kyriacou et al., 1990), suggesting that development time may be a function of τ. Similarly, life-span in the prosimian primate *Microcebus murinus* has been seen to be shortened by experimentally subjecting the animals to an 8 month year (Perret, 1997). If the duration of pre-adult development is in fact correlated with subjective rather than chronological time, then the faster

development in LL could be a consequence of a direct effect of constant light speeding up the organism's biological clock (Pittendrigh, 1960).

If this is indeed the case, then our results suggest that the period of eclosion rhythm in LL may be expected to be shorter than that in LD, and the period in LD shorter than that in DD. Eclosion in LL for these flies is arrhythmic, but rhythmicity can be induced by exposing the eggs to 12 hours of darkness starting from the time of egg collection, and then transferring to LL regime (This experiment is described in detail in chapter 2, Section 2.2). We found no significant difference between the free running period of eclosion rhythm in LL and DD regimes. On the other hand, the free running rhythm of oviposition in LL was found to be greater than in DD regime (chapter 2, Section 2.4). In contrast, cultures of the cyanobacterium *Synechococcus* were found to exhibit shorter doubling time in LL, as compared to LD (Ouyang et al., 1998).

An alternative explanation for our results could be that the faster development of these populations in LL is a consequence of their having adapted to LL conditions through some specific adaptations that are entirely independent of the biological clock. Whatever the underlying cause of faster development in LL as compared to LD 12:12 h and DD, our results demonstrate that LL conditions are not necessarily deleterious to the adult fitness, at least in organisms that have experienced LL for many generations. Although pre-adult fitness components were not adversely affected by LL in this study, it is possible that LL conditions may, nevertheless, have deleterious effects on components of adult fitness, even in populations maintained under LL for many generations. For example, if longevity is positively correlated with development time, mean life span may be expected to be lower in LL than in LD or DD. Moreover, fast development in *Drosophila* is known to trade-off with traits related to adult fitness, such as body size (Zwaan et al., 1995; Chippindale et al., 1997 a; Prasad et al., 2000) and

fecundity (Hiraizumi, 1961), and such trade-offs could result in lowered adult fitness in flies raised under an LL regime.

3.3 Effect of different light regimes on adult fitness components

Previous studies of light regime effects on adult life span in insects suffer from the lack of detailed information provided by the authors about the maintenance regimes, and an exclusive focus on mean or median life span alone as a global index of physiological well being (Pittendrigh and Minis, 1972; Allemand et al., 1973; von Saint-Paul and Aschoff, 1978; Klarsfeld and Rouyer 1998). It is well documented from numerous studies on both laboratory and wild populations, that life span in many vertebrate and invertebrate species is directly and profoundly influenced by reproduction (Bell, 1984; Partridge and Harvey, 1985; Reznick, 1992; Roff, 1992; Stearns, 1992; Zwaan, 1999). Especially in *Drosophila,* the costs of reproduction, such as those involving tradeoffs between adult life span and egg production, courtship and mating, are well studied (Partridge and Harvey, 1985; Partridge et al., 1987; Chapman et al., 1995; Chippindale et al., 1993, 1997 b). It has also been shown that energetic metabolism determines at least part of the phenotypic trade-off between survival and reproduction in *Drosophila*, because nutrition invested in gametic function is not available for somatic maintenance, thereby reducing survival (Kirkwood and Rose, 1991; Chippindale et al., 1993, 1997 b). Moreover, seemingly small differences in maintenance regimes can yield surprisingly large effects on *Drosophila* life-history traits, such as female fecundity and adult life span, and on the correlations among them (Leroi et al., 1994; Rose et al., 1996; Joshi, 1997 b).

In most previous studies of light regime effects on fitness in *Drosophila*, adult life span alone was examined in different light regimes, without taking into account any possible changes

in reproductive output of individuals in the various treatments. Given the close inverse relationship between life span and reproductive output, and the fact that reproductive output contributes to fitness far more directly than adult life span, conclusions about fitness effects of different light regimes that are based on life span data alone can, at best, be considered speculative.

Greater insight can be gained about how exactly various treatments affect life span if the effects of treatments on age-independent mortality, and on the rate at which mortality rates increase with age, are considered separately. This is often done by fitting the Gompertz equation (Gompertz, 1825) to survivorship data (Finch, 1990; Johnson, 1990; Tatar et al., 1993; Masoro, 1995; Joshi et al., 1996; Joshi and Mueller, 1997). The Gompertz equation, $\mu(X) = A\, e^{\alpha X}$, describes the mortality rate $\mu(X)$ as a function of age, X, where A and α represent the age-independent mortality rate and the age-dependent acceleration of mortality rate, respectively. The parameter α is often considered a measure of the 'rate of ageing' (Finch, 1990; Rose, 1991), and although there is some disagreement about the ability of the Gompertz equation to accurately represent the mortality rates at extremely advanced ages (Carey et al., 1992; Mueller and Rose, 1996; Pletcher and Curtsinger, 1998), it is a very good tool for separating age-independent and age-dependent effects of different treatments on mortality and, therefore, life span (Joshi et al., 1996; Nusbaum et al., 1996). For example it has been shown that selection for extended life span in *Drosophila* involves a reduction in α (Nusbaum et al., 1996), while many environmental manipulations alter life span through effects on A (Joshi et al., 1996). In the beetle *Callosobruchus maculatus*, mating appears to affect α, whereas egg production affects age-independent mortality A (Tatar et al., 1993).

In contrast to the resolution attained in studies of life span in the context of life-history evolution, the evidence for the importance of biological clocks or light regimes in determining the life span of animals is sketchy and often inconsistent, as has been noted by several workers (DeCoursey and Krulas, 1998; Hurd and Ralph, 1998; Ouyang et al., 1998; Klarsfeld and Rouyer, 1998). Indeed, there have been no systematic studies of the effects of different light regimes on the various components of fitness taken together. This is a serious shortcoming given the complex network of correlations that typically exists among fitness components (Rose et al., 1987, 1990, 1996; Joshi, 1997 b; Mueller, 1997). We assayed two major adult fitness components, life time fecundity and adult life span of the baseline populations (JB1..4) under LL, LD 12:12 h and DD regimes, in an attempt to elucidate the possible role of differences in reproductive output in mediating light regime effects on adult life span in *Drosophila*. The lifetime fecundity profile of another set of four populations (LL1..4) which were derived from the baseline populations and continued to be retained in LL regime in separate cubicles where light intensity, temperature and humidity were kept constant and comparable. At the time of these experiments, the LL populations had been separated from the JB populations for 13-18 generations.

3.3 a Materials and methods

Adult life span assays on reproducing flies

Eggs were collected from the running culture of each of the four JB populations by allowing females to lay eggs on a fresh petridish with food medium for exactly one hour. For each population, approximately 50 eggs were collected into each of 24 vials which were retained in LL regime. Adults eclosing in these vials were used to set up the life span assays. Freshly

eclosed males and females were collected and kept in vials at a density of four males and four females per vial. Twenty four such vials were set up for each population, eight in LL, eight in LD 12:12 h and eight in DD. The vials were checked everyday for deaths, and, flies were transferred to fresh food vials every alternate day. The procedure used here for assaying life span is more rigorous than most previous studies of light regime effects on life span that used much smaller sample sizes, and had a longer time interval between checking for deaths. This assay was done on the four JB populations as well as the four LL populations. In the case of the JB populations, the larvae experienced LL during development until eclosion, whereas in the case of the LL populations, the larvae experienced LD 12:12 h during pre-adult development.

Adult life span assays on virgin flies

The method used to assay the life span of virgin flies was similar to that used for reproducing flies, except that each vial was set up with either eight males or eight females. Four vials of males and four vials of females were kept under each of the three light regimes (LL, LD 12:12 h, and DD).

Lifetime fecundity assay

Flies were obtained from the running cultures of the four LL populations in the same manner as described for the assays on life span. One male and one female each were introduced into vials containing approximately 3 ml of food medium. Forty such pairs were set up per light regime (LL, LD 12:12 h and DD). Everyday, at nearly the same time, the flies were transferred to fresh food vials and the number of eggs laid by each female over the preceding 24 h was recorded. This procedure was carried out until the last female died. Cohorts of males of all populations were maintained in all three light regimes as backups. In the event of the death of a

male in any of the assay vials, the dead fly was removed and replaced by another male of the same age.

From the primary data of each life span assay (JB reproducing, JB virgin, and LL reproducing), mean adult life span (in days) was computed. We also estimated the Gompertz parameters A and α for each assay × light regime × sex combination, using a maximum likelihood method that utilizes the untransformed survivorship data (Mueller et al., 1995). Estimates of population mean adult life span, A, and α for each assay × light regime × sex combination were then used as data in separate mixed model analyses of variance (ANOVA). In these analyses, replicate populations were treated as random blocks, whereas assay (JB reproducing, JB virgins, LL reproducing), light regime and sex were treated as fixed factors crossed with block.

From the primary data on daily egg production of females in the lifetime fecundity assay, the total number of eggs produced by an individual female throughout its life was computed, and the mean across the 40 females was estimated for each population in each light regime. These mean values were then used as data in a two way mixed model ANOVA with light regime as the fixed factor and block as the random factor. We used population means in the analyses because we wish to draw evolutionarily relevant inferences about the differential effects, if any, of light treatment on life span or fecundity and the crucial unit of replication, analysis and interpretation is, therefore, the population and not the individual (Rose et al., 1996; Mueller, 1997).

3.3 b Results

In all three life span assays, flies kept in LL tended to have a shorter life span than those kept in LD 12:12 h or DD (Figs. 3, 4, 5). The ANOVA on mean life span in the three assays

revealed a significant main effect of light regimes (Table 3), and multiple comparisons revealed this to be a consequence of flies having significantly shorter mean life span in LL ($p < 0.0001$) compared to either LD 12:12 h or DD (Fig. 6). Mean life span was also affected by the reproductive status and source of flies assayed (assay); reproducing females from JB populations had significantly lower mean life span than those from LL populations, and reproducing females had significantly lower mean life span than virgins (Fig. 6, Table 3). There was a significant interaction between assay and sex (Table 3), due to reproducing females of both JB and LL populations having lower mean life span compared to virgin females, whereas in the case of males, life span did not differ significantly among the assays (Fig. 6). There were some differences in the pattern of light regime effects on life span in males and females. In females, mean life span in LL was significantly less than that in LD 12:12 h and DD for the JB populations, regardless of reproductive status (Fig. 6). However, in case of the LL populations, life span in DD was significantly greater than that in LL or LD 12:12 h; life span did not significantly differ between the latter two regimes (Fig. 6). In the case of virgin females, the same trend was seen, with life span being least in LL. In reproducing males of JB and LL populations, mean life span was least in LL, whereas life span of virgin males did not differ significantly among the light regimes (Fig. 6). These differences were reflected in a significant assay × sex × light regime interaction in the ANOVA (Table 3).

The ANOVA on the Gompertz parameter A revealed significant main effects of assay, sex and light regime as well as a significant light regime × sex interaction (Table 4). In general, estimates of A in males did not differ among assays or light regimes, except for significantly greater A in reproducing JB males when assayed in LL, as compared to LD 12:12 h or DD (Fig. 7). Estimates of A for virgin females did not differ significantly among light regimes, whereas

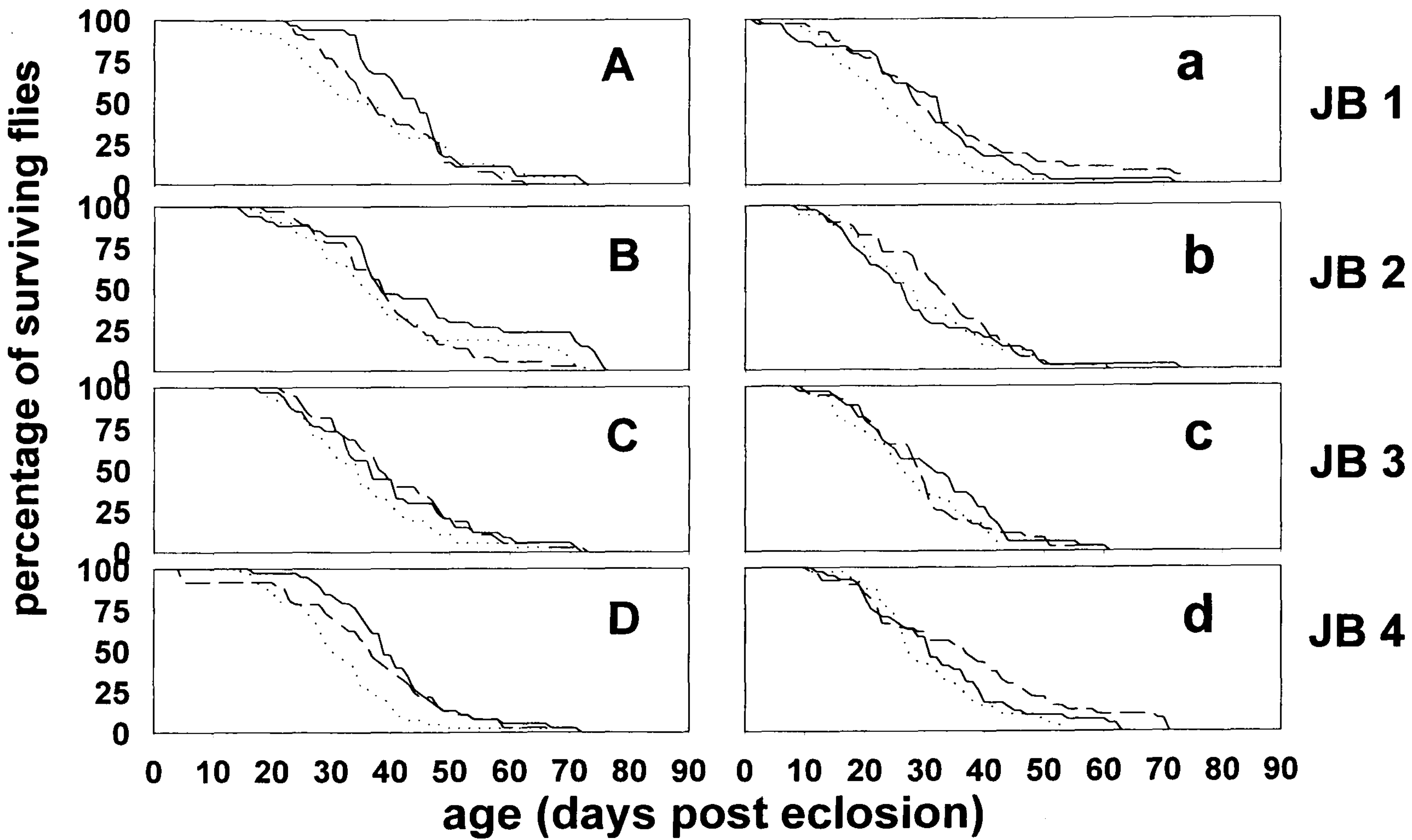

Fig. 3 Adult survivorship curves for reproducing males (A, B, C, D) and females (a, b, c, d) from 4 JB populations under LL (dotted line), LD (dashed line) and DD (solid line) regimes.

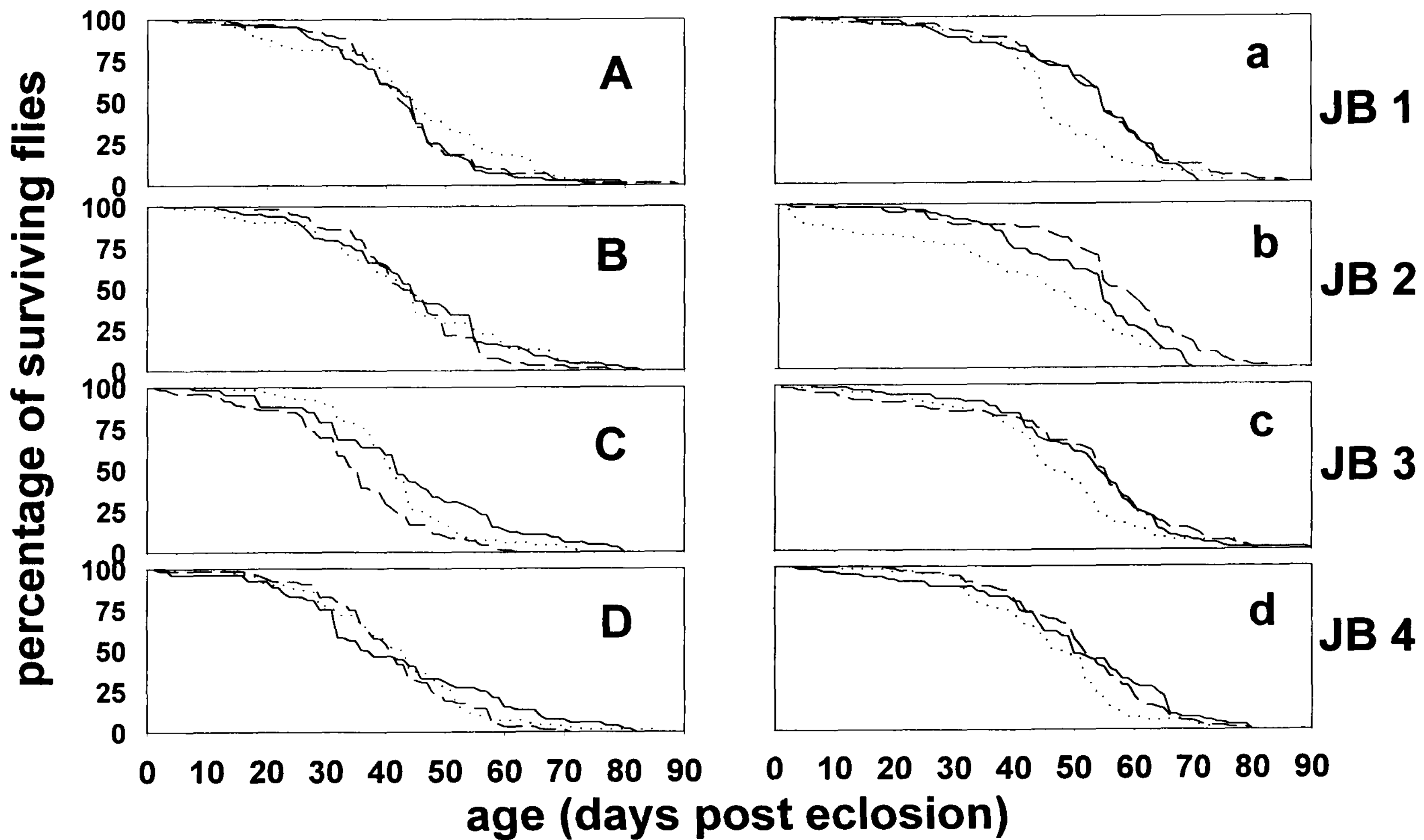

Fig. 4 Adult survivorship curves for virgin males (A, B, C, D) and females (a, b, c, d) from 4 JB populations under LL (dotted line), LD (dashed line) and DD (solid line) regimes.

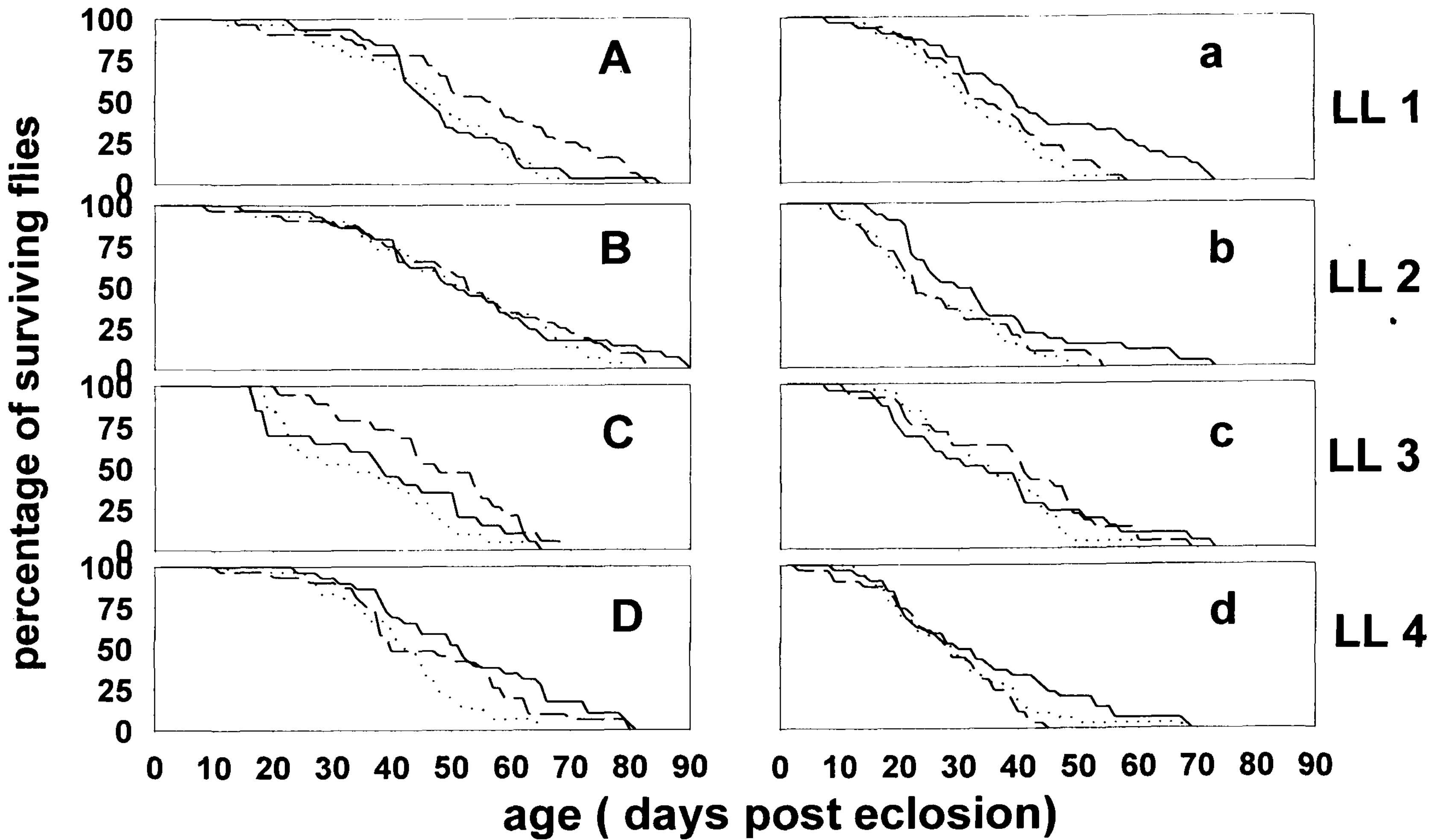

Fig. 5 Adult survivorship curves for reproducing males (A, B, C, D) and females (a, b, c, d) from 4 LL populations under LL (dotted line), LD (dashed line) and DD (solid line) regimes.

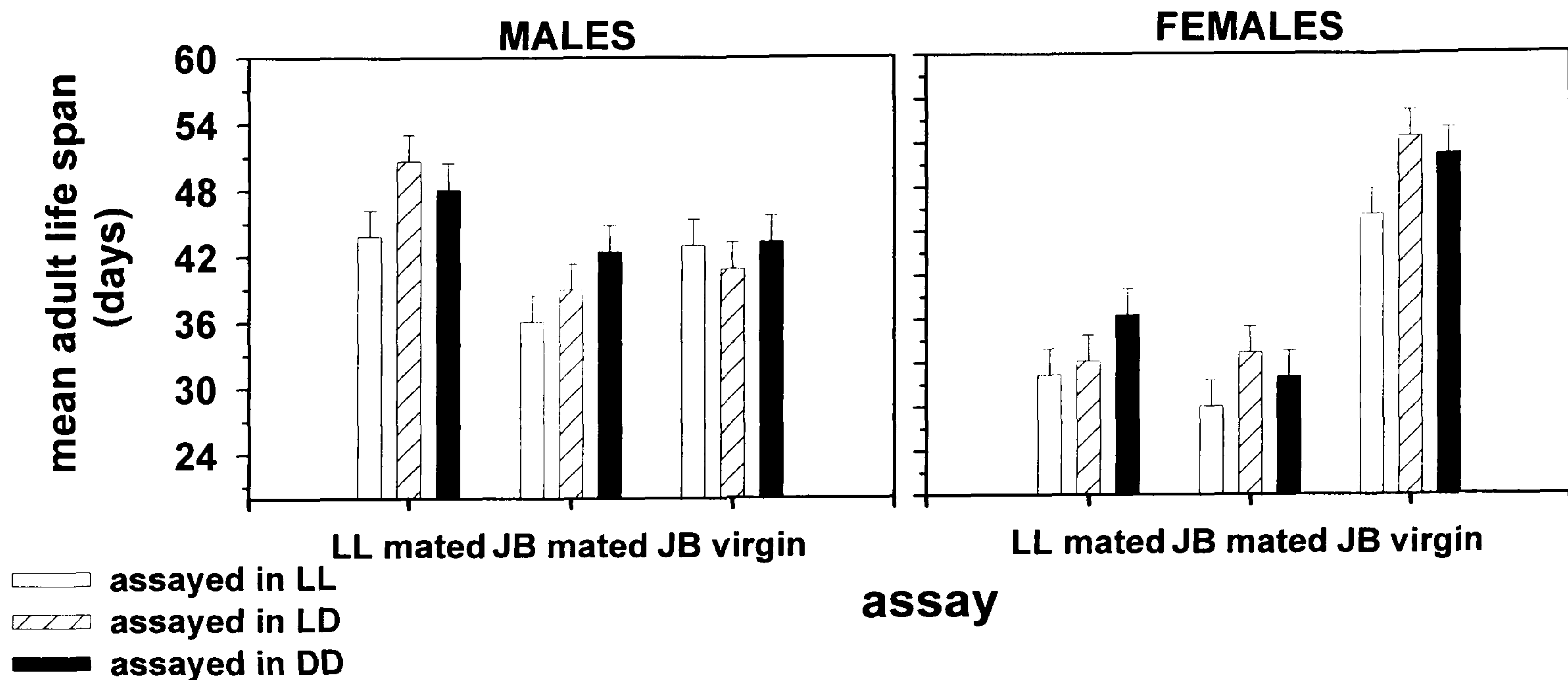

Fig. 6 Mean adult life span under LL, LD and DD, of males and females of JB and LL populations in the three lifespan assays. Error bars represent 95% C.I. about the mean of the 4 replicate populations in each assay, calculated using least squares estimates of the standard errors of the appropriate cell means in the randomised block ANOVA and can, therefore, be used for visual hypothesis testing.

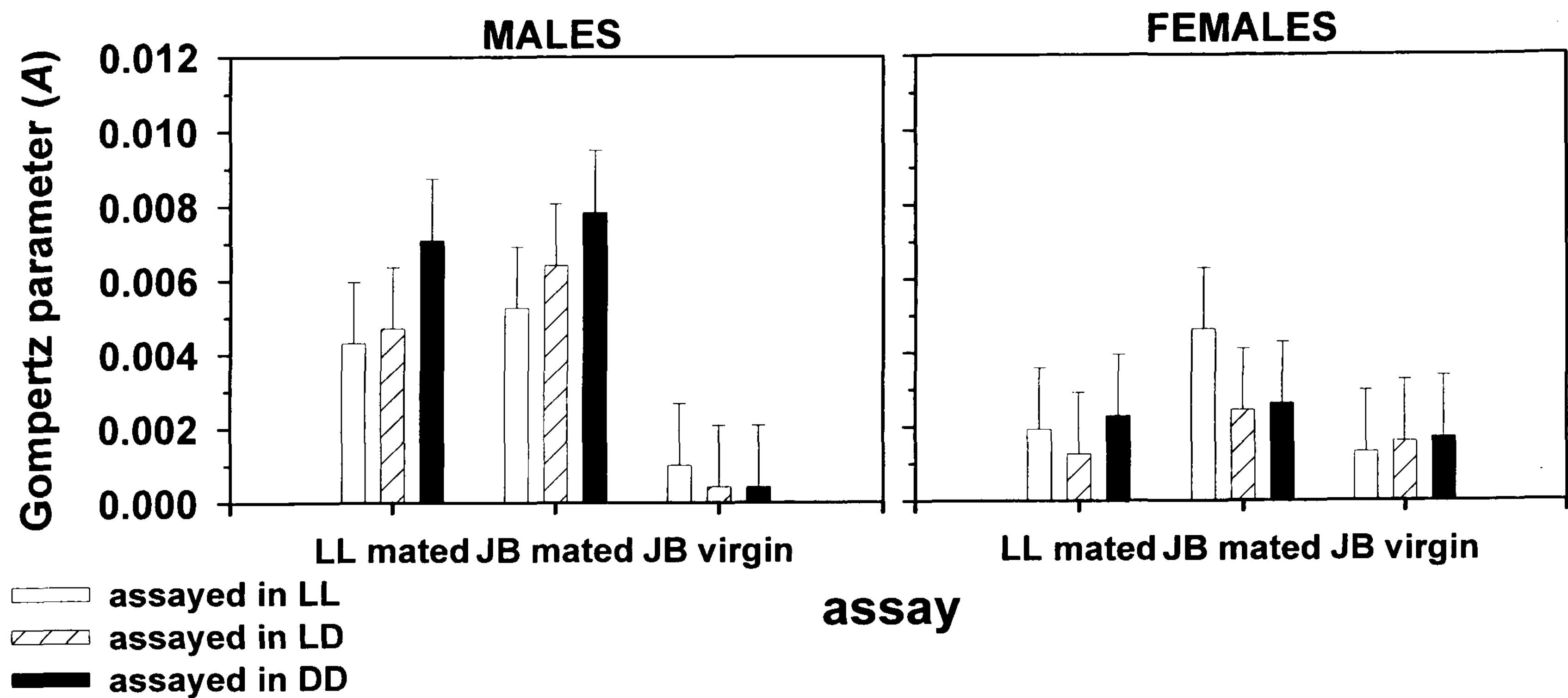

Fig. 7 Estimated values of age-independent mortality (Gompertz parameter *A*) under LL, LD and DD regimes, of males and females of JB and LL populations in the 3 lifespan assays. Error bars represent 95% C.I. about the mean of the 4 replicate populations in each assay, calculated using least squares estimates of standard errors of the appropriate cell means in the randomised block ANOVA and can, therefore, be used for visual hypothesis testing.

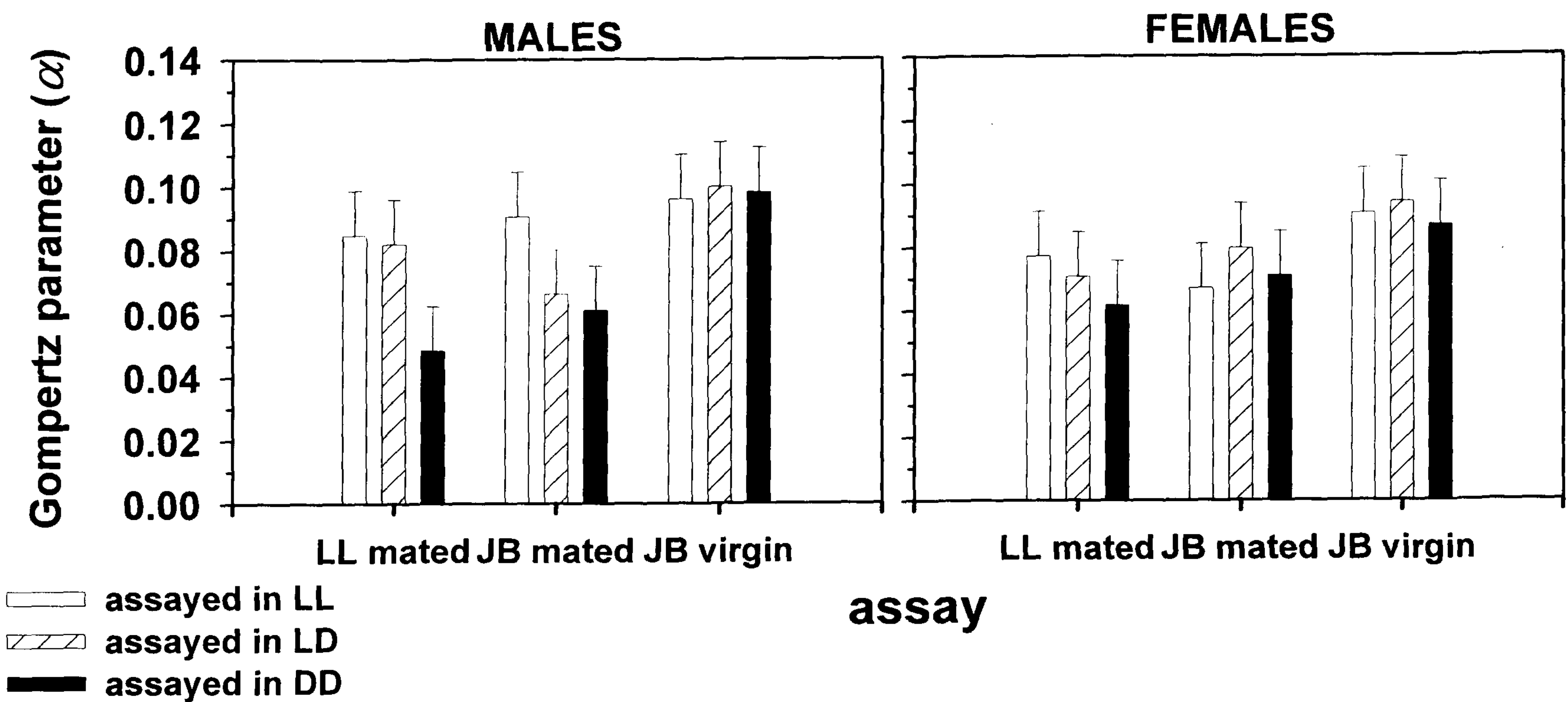

Fig. 8 Estimates of the Gompertz 'rate of aging' (α) under LL, LD and DD, of males and females of JB and LL populations in the 3 life span assays. Error bars represent 95% C.I. about the mean of the 4 replicate populations in each assay, calculated using least squares estimates of standard errors of the appropriate cell means in the randomised block ANOVA and can, therefore, be used for visual hypothesis testing.

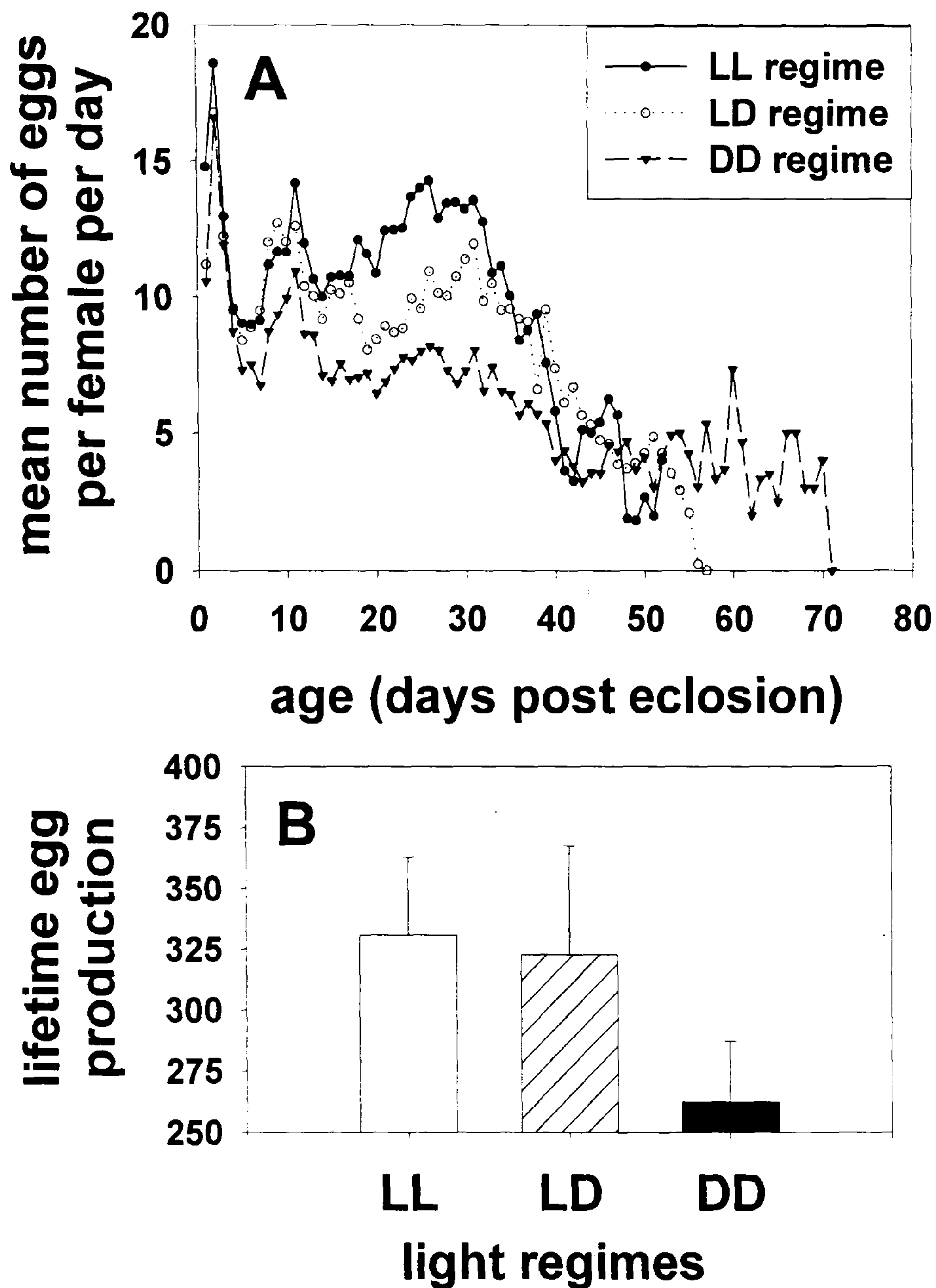

Fig. 9 (A) Mean daily fecundity over the lifetime of females from LL populations assayed in LL, LD and DD regimes. (B) Total lifetime egg production of flies assayed in LL, LD and DD averaged across 40 flies from four populations. Error bars represent standard error of the mean across the 4 replicate populations.

Table. 3 Results of analysis of variance (ANOVA) on mean adult lifespan of flies from LL and JB populations in the three lifespan assays. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance.

Effect	***df***	**MS**	***F***	***p*-level**
Assay (A)	2	748.0181	99.3838	**<0.0001**
Sex (S)	1	500.6736	8.6165	0.0607
Light regime (L)	2	116.611	57.0730	**0.0001**
Block (B)	3	32.3184	--	--
A × S	2	752.6984	20.7855	**0.0020**
A × L	4	2.5627	0.2449	0.9072
S × L	2	5.3070	0.9553	0.4363
A × B	6	7.5265	--	--
S × B	3	58.1059	--	--
L × B	6	2.0431	--	--
A × S × L	4	39.9435	8.3984	**0.0018**
A × S × B	6	36.2125	--	--
A × L × B	12	10.4611	--	--
S × L × B	6	5.5550	--	--
A × S × L × B	12	4.7560	--	--

Table. 4 Results of analysis of variance (ANOVA) on the age-independent mortality (Gompertz parameter A) of flies from LL and JB populations in the three lifespan assays. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance.

Effect	***df***	**MS**	***F***	***p*-level**
Assay (A)	2	8.93E-05	16.0484	**0.0039**
Sex (S)	1	6.96E-05	20.6301	**0.0199**
Light regime (L)	2	4.60E-06	6.2100	**0.0345**
Block (B)	3	3.54E-06	--	--
A × S	2	3.76E-05	5.8990	**0.0383**
A × L	4	2.00E-06	0.8391	0.5262
S × L	2	6.11E-06	7.4783	**0.0234**
A × B	6	5.56E-06	--	--
S × B	3	3.37E-06	--	--
L × B	6	7.41E-07	--	--
A × S × L	4	4.33E-06	1.8096	0.1918
A × S × B	6	6.37E-06	--	--
A × L × B	12	2.39E-06	--	--
S × L × B	6	8.17E-07	--	--
A × S × L × B	12	2.39E-06	--	--

Table. 5 Results of analysis of variance (ANOVA) on the Gompertz 'rate of ageing' (α) of flies from LL and JB populations in three lifespan assays. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance.

Effect	*df*	MS	*F*	*p*-level
Assay (A)	2	0.0041	21.8484	**0.0017**
Sex (S)	1	0.0002	2.5805	0.2065
Light regime (L)	2	0.0012	5.5010	**0.0439**
Block (B)	3	0.0005	--	--
A × S	2	9.32E-05	0.2719	0.7708
A × L	4	0.0003	1.5311	0.2550
S × L	2	0.0004	1.5850	0.2801
A × B	6	0.0002	--	--
S × B	3	6.94E-05	--	--
L × B	6	0.0002	--	--
A × S × L	4	0.0004	2.4129	0.1065
A × S × B	6	0.0003	--	--
A × L × B	12	0.0002	--	--
S × L × B	6	0.0002	--	--
A × S × L × B	12	0.0001	--	--

Table. 6 Results of analysis of variance (ANOVA) on the population mean lifetime fecundity (averaged across 40 females per population). Block and the interaction of block with light regime cannot be tested for significance in this design.

Effect	*df*	MS	*F*	*p*-level
Light regime	2	5561.63	7.3632	**0.0242**
Block	3	13092.00	-	-
Light regime × Block	6	755.32	-	-

for reproducing females of both LL and JB populations, estimates of A in DD were significantly greater than those in LL or LD 12:12 h (Fig. 7). In general, estimates of A for virgin flies were lower than those for reproducing flies, although the bulk of this effect was due to females rather than males (Fig. 7).

The only significant effects in the ANOVA on the Gompertz parameter α were main effects of assay and light regime (Table 5). The pattern of light regime effects on α was different from that on A in females, with α in DD being the smallest in reproducing females compared to LL or LD 12:12 h (Fig. 8). Estimates of α in virgin females did not differ significantly among light regimes. Estimates of α in virgin and mated males did not differ significantly among assays or light regimes (Fig. 8).

The course of lifetime fecundity in the three light regimes was similar for the first 5 days or so, after which daily fecundity in LL tended to be higher than in LD 12:12 h or DD for the next 30 days or so (Fig. 9). Thus, over the bulk of their reproductive life, females kept in LL laid substantially more eggs than those kept in LD 12:12 h or DD. The ANOVA revealed a significant effect of light regime on lifetime fecundity (Table 6), with the highest mean lifetime egg production in LL, followed by LD 12:12 h and then DD. Multiple comparisons revealed that mean lifetime egg production in DD was significantly less than that in LL or LD 12:12 h, whereas mean lifetime egg production in LL and LD 12:12 h did not differ significantly.

3.3 c Discussion

Overall, our results were consistent with earlier observations that LL tends to reduce the adult life span in *Drosophila*, relative to LD 12:12 h or DD (Figs. 3 - 6). This effect of LL on life span was seen in reproducing males and females of both LL and JB populations, as well as in

virgin JB females, but not in virgin JB males. Given the well known tradeoff between fecundity and life span in *Drosophila* (Hiraizumi, 1985; Luckinbill and Clare, 1985; Partridge et al., 1987; Chippindale et al., 1993, 1997 b; Leroi et al., 1994; Rose et al., 1996), decreased life span in reproducing females in LL may partly be a consequence of enhanced fecundity in LL, compared to LD 12:12 h or DD (Fig. 9). Indeed, despite having shorter life span, females in LL laid a greater total number of eggs in their lifetime than those in LD or DD.

In virgin females, the differences in mean adult life span caused by light regimes are probably due to physiological process being modified by light. Well-fed virgin *Drosophila* females are known to store eggs in their ovaries (rather than not make any eggs) until mates are available (Chippindale et al., 1997 b). Virgin females also lay unfertilized eggs, although in much lower numbers than mated females. There is also evidence that egg-retention behavior in virgin females is under the control of a myotropic hormone produced in the pars intercerebrallis (Boulétreau-Merle, 1986). Fecundity reduction in virgin females, thus, occurs through mechanisms very different from those mediating the effects of dietary restriction. When *Drosophila* females are fed a calorie restricted diet, fecundity is reduced as a result of females not making eggs and, consequently, more stored lipid is available for somatic maintenance (Chippindale et al., 1997 b). This effect of poor nutrition on vitellogenesis is mediated by the juvenile hormone produced by the corpora allata (Bownes and Reid, 1990). It is, therefore, possible that LL triggers some hormonal or other physiological signals that induce reproductive females to lay more eggs, and the same signals also cause virgin females to divert relatively more resources into egg production and storage, thereby causing a reduction in virgin life span by a depletion of somatic lipid reserves. Overall, of course, virgin females have a greater life span than reproducing females (Fig. 6). This is not surprising because even though virgin

females do allocate some resources to egg production and storage, these are less than in reproducing females. Moreover, virgin females are exempt from life span costs associated with mating *per se* (Chapman et al., 1995).

In case of virgin males, however, there was no effect of light regime on mean adult life span. This suggests that in males the effect of light regimes is contingent upon reproduction. The lower life span of reproducing males in LL is, therefore, likely to be partly a consequence of mating more frequently with females in LL who are laying eggs at a higher rate than their counterparts in LD or DD. It is known that the presence of mates, courtship, and mating, all exert a cost on male adult life span in *Drosophila melanogaster* (Partridge et al., 1987; Luckinbill et al., 1988; Service, 1989). Moreover remating frequency in *Drosophila* depends upon the rate of egg laying by females (Trevitt et al., 1988). It is also possible that the repertoire of male courtship behaviors is somehow modified in the different light regimes. Perhaps there is a diminished level of courtship activity in DD, as compared to LL, while courtship in LD 12:12 h is restricted to the light phase, resulting in an overall lowering of the level of courtship activity. A recent study demonstrating that mating activity shows a robust circadian rhythm and that the female mating rhythm is governed by clock genes (Sakai and Ishida, 2001) strengthens this view.

Another explanation for the reduced life span in LL is that flies kept in LL have reduced duration of rest, a phase that is known to share many similarities with mammalian sleep (Shaw et al., 2000). It is, therefore, possible that flies in LL are stressed due to rest deprivation and that this contributes to enhanced mortality rates. However, although rest deprivation may be contributing to increased mortality in LL (durations of activity in the locomotor activity rhythm in these populations are longer in LL than in LD 12:12 h or DD), it cannot be the sole cause of reduced life span because the effects of LL on life span were less pronounced in virgin females,

and altogether absent in virgin males. It may also be the case that longer durations of activity in LL simply results in greater egg laying by females who are active for longer durations of time. However, this is unlikely to be a major contributor to the observed effects of LL on life span because egg laying is rhythmic in over half the females in these populations even in LL and DD, with mean free-running period of the oviposition rhythm in LL being significantly greater than that in DD, but not different from that in LD 12:12 h (Sheeba et al., 2001 b; chapter 2, Section 2.4.). Although stress due to rest deprivation may play some role in reducing life span of flies kept in LL, a large part of the reduction in life span is probably due to increased egg production. How exactly LL results in increased egg production is not clear; it is quite possible that there are some rhythm-related effects of LL on the amplitude of the oviposition rhythm. It is, however, clear that if a substantial part of the reduction in life span in LL is due to increased fecundity, it is not correct to say that LL is generally deleterious. Indeed in LL there is an increase in fitness due to increase in lifetime fecundity in these flies.

On examination of the Gompertz parameters, α in the reproducing females was found to be higher in LL as compared to DD (Fig. 8), indicating that mortality rates accelerate with age more rapidly in LL. This is consistent with the notion of a "wave of mortality" proposed by Sgrò and Partridge (1999), who suggest that mortality rates respond to early reproduction after a time delay of several days. This is also consistent with our observation that daily fecundity of females in LL was greater than that in LD and DD only after the first 5-6 days of adult life. It is not clear at this time why age-independent mortality, A, was found to be higher in DD compared to LD 12:12 h and LL in the reproducing females (Fig. 7). Interestingly, higher A of females in DD was coupled with lower α (Fig. 8), and, overall, life span in DD tended to be higher than in LL, despite greater early life mortality. This result suggests that females that survive the initial

mortality in DD are then able to survive for much longer in DD. Clearly, some as yet unrecognized aspect of maintenance in DD is exerting a strong selection pressure early in adult life. Comparison of virgin and reproducing females shows major differences in *A*, with reproducing females having very high *A* (Fig. 7), which contributes largely to their low mean adult life span (Fig. 6). Reproduction, therefore, seems to increase the age-independent mortality rate of females across light regimes.

Overall, the gross pattern of light regime effects on adult life span did not differ between the LL populations and their JB ancestors, as evidenced by the lack of a significant assay × light regime interaction in the ANOVA (Table 3). The difference in absolute values of mean adult life span between the two sets of populations can be explained by the differences in absolute values of the female fecundity. In the course of ~ 15 generations of maintenance in cubicles, as opposed to incubators, the LL populations have evolved lower fecundity (chapter 4), and this is presumably the cause of the higher life span of reproducing flies from LL populations, as compared to their JB counterparts.

Our results clearly suggest that the effect of constant light in reducing the mean life span in our populations is mediated at least in part through an increase in reproductive output. Thus, at least in these populations of *Drosophila* that have been maintained in LL for over 600 generations, there is no clear evidence for unequivocally deleterious effects of LL on fitness. These populations have been shown to develop faster to adulthood under LL (Sheeba et al., 1999 b), and in doing so, do not pay a cost in adult dry weight. Moreover, the present study shows that lifetime reproductive output in LL is higher compared to the LD 12:12 h and DD. In terms of the weighting of fitness components, reproductive output is far more important than life span in these populations, as the peak egg production occurs well before the average onset of mortality

in reproducing flies. Moreover, these populations are maintained on a 21-day discrete generation cycle, which means that for more than 600 generations they have not been under any selection pressure to live beyond approximately 11 days post eclosion. Hence, longevity beyond approximately 11 days of adult life does not contribute to fitness in these populations.

Thus, fitness in these populations appears to be enhanced in LL, relative to LD or DD. Our results, therefore, call into question the general belief in the deleterious effects of LL, at least in populations of *Drosophila* maintained in LL for many generations. In our opinion, our results also highlight the fact that the fitness effects of different light regimes are far more subtle than previously thought, and further studies on the reproductive physiology and courtship behavior in different light regimes are needed to gain a better understanding of exactly how light regimes affect adult fitness in *Drosophila*.

Chapter 4

Studies Of Circadian Rhythm Parameters And Components Of Fitness On Selected Populations

4.1 Introduction

The results of our studies on the circadian rhythm parameters of the baseline populations provided evidence for an intrinsic adaptive significance to exhibiting circadian rhythms, and the assays on components of fitness under different light regimes revealed that different light regimes affect components of fitness differentially. We observed that the baseline populations that have been reared for more than 600 generations in constant light (LL) regime had highest overall fitness in LL, the regime of their origin, which suggested that perhaps these populations have adapted to LL regime. However, contrary to a widely held notion that any traits that confer a fitness advantage to organisms will evolve, it is now well known that several genetic factors can constrain adaptive evolution (as I have described in chapter 1). Most previous studies that tested the adaptive significance of circadian rhythms used organisms that had been maintained under light/dark (LD) 12:12 h cycles and assayed their longevity in different periodic and aperiodic regimes. Their results suggested that organisms were most long lived in the periodic LD 12:12 h environment which was their rearing environment, as compared to other regimes in which periodicity was different from that of the rearing environment, or aperiodic environments LL and constant darkness (DD) (Pittendrigh and Minis, 1972; von Saint-Paul and Aschoff, 1978). But these studies are not really evidence for adaptation to the periodic LD 12:12 h regime, as concrete evidence for adaptedness to an environment can be provided only when a population shows greater fitness in that environment, compared to controls. The observation that a population has higher fitness in a certain environment compared to other environments does not constitute evidence for adaptation to that environment. In our studies, we adopted a rigorous and systematic approach based on principles and tools of evolutionary genetics to address this issue. We looked for evidence of increased fitness in populations reared in a periodic LD12:12 h

regime for many generations, after having been reared in LL for over 600 generations, by comparing them to populations that continued to be maintained in aperiodic regimes.

We derived three sets of selection lines from the four baseline populations (JB1..4) described in detail in chapter 2. One set of four populations (LL1..4, henceforth referred to as LL stocks) was introduced into a walk-in cubicle under LL (2.5 W/m^2 or 300 lux), a second set of four populations (LD1..4, henceforth referred to as LD stocks) was introduced into a cubicle within the same complex with LD cycle of 12:12 h, and a third set of four populations (DD1..4, henceforth referred to as DD stocks) was introduced into an adjacent cubicle under DD (Fig. 1). All three cubicles were maintained at constant temperature (25 ± 1 ^{0}C), and relative humidity ~ 80-90%. The entire selection experiment was in a block design, with populations LD-*i*, LL-*i*, DD-*i*, derived from JB-*i* (*i* = 1..4). In order to test whether circadian rhythmicity confers any adaptive advantage to flies in LD 12:12 h, we assayed the three sets of selected populations for (a) pre-adult and adult components of fitness, assayed in all the three light regimes, and, (b) circadian parameters τ and ψ of two rhythms, eclosion (populational level rhythm) and locomotor activity (individual level rhythm). To demonstrate unambiguously that organisms adapt to LD 12:12 h through evolutionary changes in circadian organisation, the LD stocks would have to exhibit greater mean fitness than LL and DD stocks in LD 12:12 h, and circadian rhythm parameters of the two rhythms should be seen to have diverged in expected ways among the selected populations. Given the large variation in τ in the ancestral baseline populations (chapter 2), we may expect to find that the mean τ of the two rhythms in the LD stocks has shifted closer to 24 h over generations.

In this chapter, I describe experiments that attempted to assess possible adaptive evolutionary changes over nearly 50 generations, in the populations under selection in the three

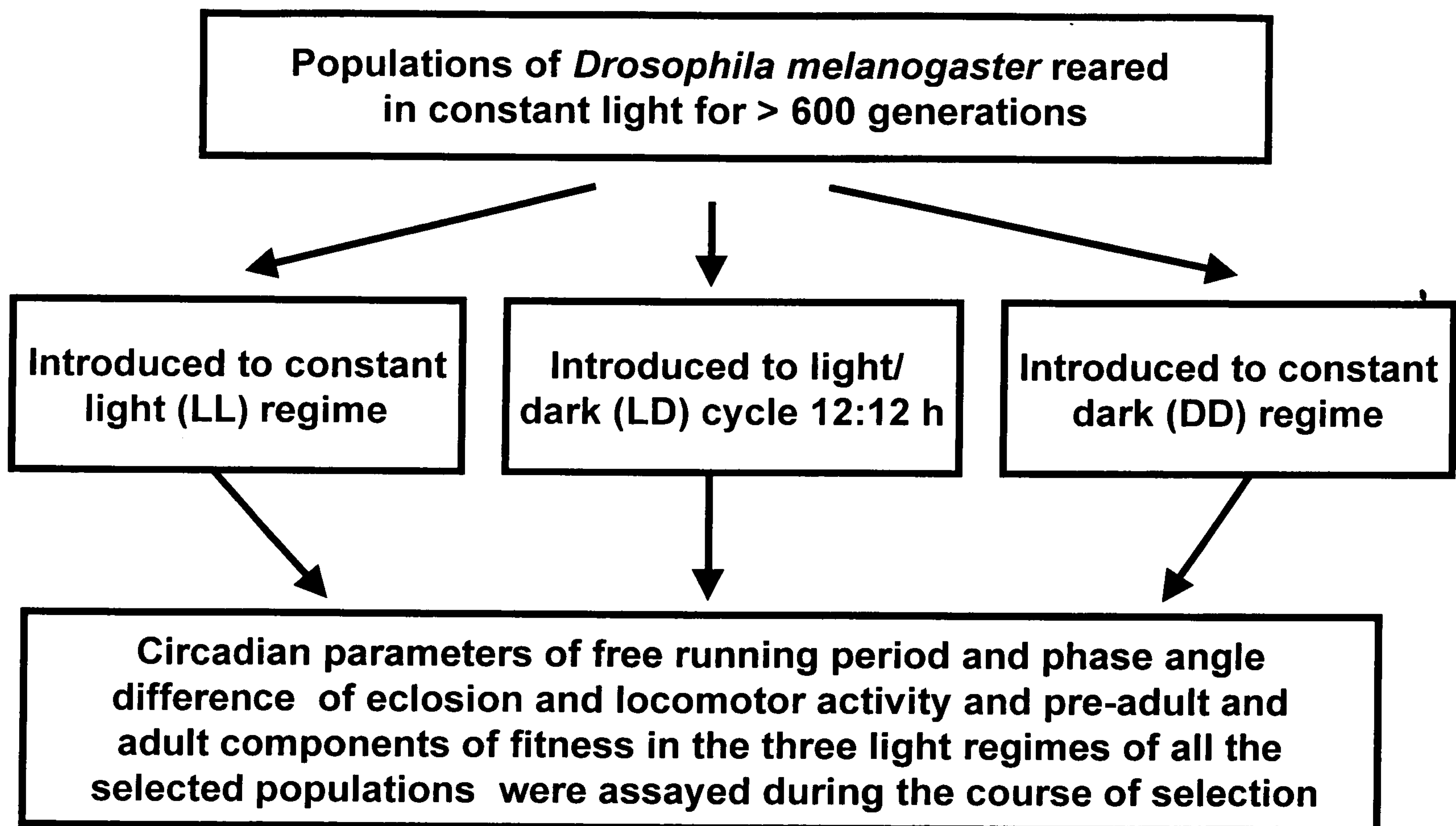

Fig. 1 Schematic representation of the manner in which the selected populations were derived from the baseline populations (the ancestry and other details of the baseline population are described in Chapter 2).

light regimes. In these studies, circadian parameters of eclosion and locomotor activity rhythms, as well as components of fitness in different light regimes were assayed. All assays were performed on all twelve populations (three selection light regimes × four replicate populations per regime), after they were subjected to a common rearing environment (LD 12:12 h regime) for one generation to obviate any maternal effects due to parental maintenance regime (Rose et al., 1996). The experiments carried out to assay the circadian rhythm parameters in the three sets of selected populations after ~30 generations of selection are discussed in Section 4.2 of this chapter, while the experiments on components of pre-adult and adult fitness are described in Section 4.3.

4.2 Assays on circadian parameters of selected populations

4.2 a Materials and methods

Standardisation of populations from the three selection regimes

From the running cultures of each of the three sets of selected populations (LL1-4; LD1-4; DD1-4), twenty four vials containing ~ 60 – 80 eggs each were introduced to LD 12:12 h regime. The adults that emerged from these vials (henceforth referred to as standardised flies) were collected into cages on the 12th day after egg collection. The progeny of these flies were used for experiments in which circadian rhythm parameters as well as components of fitness (Section 4.3) were assayed.

Eclosion rhythm

This rhythm was assayed after ~30 generations of selection. Eggs from each of the standardised populations were collected into vials containing 6 ml of food medium at densities of ~300 eggs per vial. Thirty six such vials were collected per population. Twelve vials each were

assayed in LL, LD 12:12 h and DD regimes. The vials that were assayed in LL regime were first subjected to a dark stimulus of 12 h before they were introduced to LL regime. Once pupae began to darken, the vials were monitored every 2 h and eclosing adults were collected and their number and sex was recorded. This was continued for 10 consecutive days, or until most of the pupae eclosed. The time series of eclosion events thus obtained from each vial was subjected to Fourier spectral analysis using STATISTICA™ for Windows Release 5.0 B (StatSoft Inc, 1995) in order to estimate the periodicity of the rhythm. Statistical significance of observed peaks in the periodogram was tested using the technique of Siegel (1980). The values of periodicity of eclosion in LL and DD regimes (τ_{LL} and τ_{DD}) for the replicate vials were subjected to a three way mixed model analysis of variance (ANOVA), wherein assay light regime and selection regime were fixed factors crossed among themselves and with block (replicate populations) which was considered as a random factor. The ψ of eclosion rhythm in LD 12:12 h was estimated as the average time interval between peak eclosion and 'lights-on' over a minimum of 10 consecutive days for flies eclosing from each vial. The values for ψ were used as data in a two-way mixed model ANOVA, with selection regime as the fixed factor and replicate populations as random blocks.

Locomotor Activity Rhythm

This assay was done after ~ 35 generations of selection. From each of the standardised populations, eggs were collected at densities of ~ 50 eggs per vial into 24 vials containing ~6 ml of food medium and retained in LD 12:12 h regime. The adults that emerged were collected and segregated by sex within 6 hours of eclosion. Locomotor activity of these virgin flies was assayed for fifteen days in LD 12:12 h, following which the flies were shifted to DD regime and assayed for another 15 days. The flies were transferred to the locomotor activity monitoring set-

up within 24 to 48 h after eclosion. The locomotor activity of individual male and female flies was assayed using a set-up that uses infra red beams to detect the vertical movement of the fly in a narrow glass tube (described in chapter 2). At least 26 flies of each sex were assayed from each of the four populations of the three stocks.

The τ_{DD} of locomotor activity was estimated for each fly separately by fitting a least squares linear regression line to the onset of activity for at least 6 consecutive days. The mean τ_{DD} for each population was then used as data in a mixed model analysis of variance (ANOVA) in which replicate populations were treated as random blocks, and selection regime and sex as fixed factors crossed with block; post hoc comparisons were done using Tukey's test. A separate ANOVA was done on the variance in τ_{DD} within each combination of sex, stock and block. The ψ of locomotor activity rhythm for "onset of activity" in LD 12:12 h, was estimated by averaging the time interval between onset of activity and 'lights-on' for about 10 consecutive days. The distribution of the values of ψ for onset of activity for the flies of both sexes, from the three selection regimes were tested for differences using Kolmogorov-Smirnov two-sample tests due to extreme non-normality of the distributions. All statistical analyses were implemented using STATISTICA™ for Windows Release 5.0 B (StatSoft Inc, 1995).

4.2 b Results

The mean (± 95% C.I.) τ_{LL} of eclosion rhythm of the LL, LD and DD stocks were 24.08 ± 1.02 h, 24.39 ± 1.63 h and 23.91 ± 1.93 h, respectively, and the ANOVA showed that they were not significantly different from each other ($F_{2,6} = 0.43, p > 0.05$). The mean (± 95% C.I.) τ_{DD} of eclosion rhythm of the LL, LD and DD stocks were 23.75 ± 1.82 h, 24.28 ± 1.39 h and 23.64 ± 2.29 h, respectively, and these values, too did not significantly differ from one another

($F_{2,6} = 0.43, p > 0.05$). The analysis of periodicity of populations assayed in LD 12:12 h regime showed that the eclosion rhythm entrained to the LD cycle in all three stocks. The ψ of eclosion rhythm in LD 12:12 h for individuals from the three stocks was distributed over a range of –1 h to –3.3 h. The ANOVA on ψ of eclosion rhythm of the stocks revealed a significant main effect of selection regime (Table 1), with ψ of the LD stocks being significantly greater than that of DD or LL stocks ($p < 0.05$ for both comparisons; Tukey's test). The ψ of LL and DD stocks were not significantly different from one another.

The ANOVA on mean τ_{DD} of the locomotor activity rhythms of the three stocks revealed a significant main effect of selection regime (Table 2). Mean τ_{DD} of the LD stocks was significantly shorter than that of LL and DD stocks (Tukey's test), and was closer to 24 h, as compared to LL and DD stocks (Fig. 2, 3 a). There was no significant difference between the mean τ_{DD} of locomotor activity rhythm of LL and DD stocks (Fig. 3 a). The ANOVA on the variance in τ_{DD} in the different populations revealed a significant main effect of selection regime (Table 3). The variance in τ_{DD} of DD stocks was significantly larger than that of LL and LD stocks (Tukey's test) (Fig. 3 b). The Kolmogorov-Smirnov two-sample tests revealed that the distribution of ψ of onset of locomotor activity of LD stocks was significantly different from that of LL stocks ($p < 0.01$), but not significantly different from DD stocks ($p > 0.05$) (Fig. 4). Moreover, there was no significant difference between the distribution of ψ of LL and DD stocks ($p > 0.05$) (Fig. 4).

4.2 c Discussion

Eclosion and locomotor activity rhythms of the three sets of stocks were found to be rhythmic in the aperiodic LL and DD regimes, while exhibiting stable entrainment to the LD

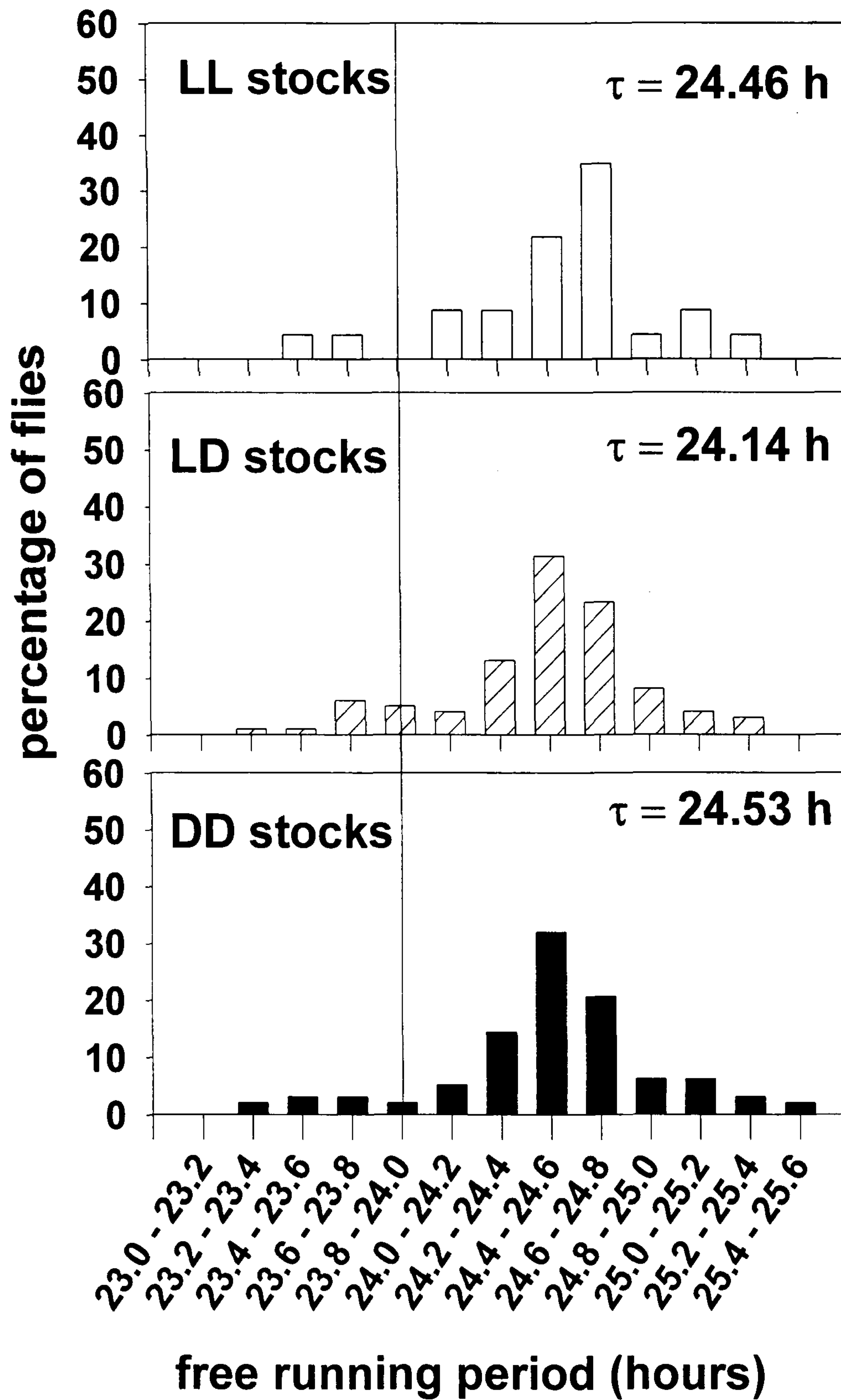

Fig. 2 Frequency distribution of free running period of locomotor activity rhythm in DD of flies from LL, LD and DD stocks.

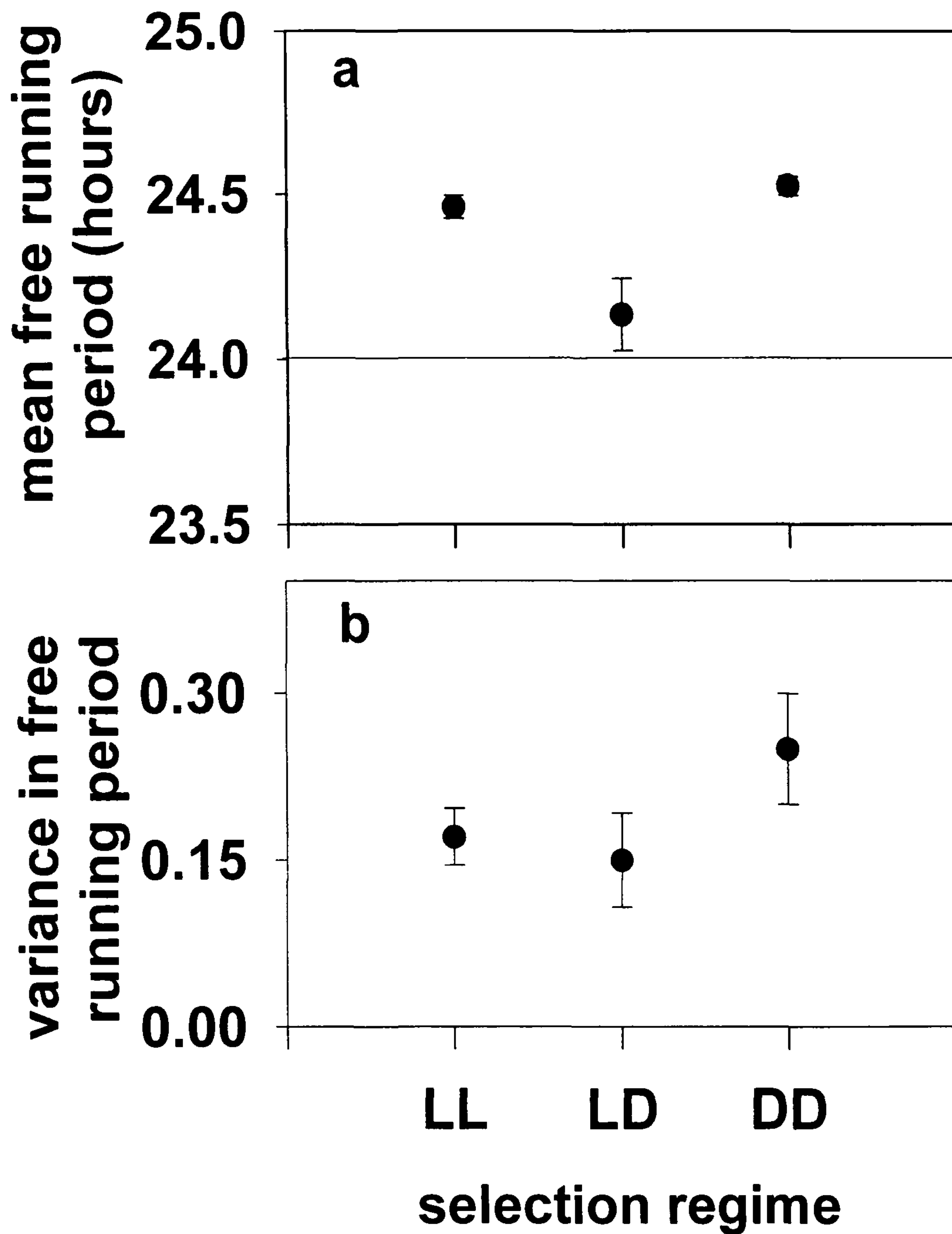

Fig. 3. (a) Mean and (b) variance in free running period of locomotor activity rhythm in DD of flies from LL , LD and DD stocks.

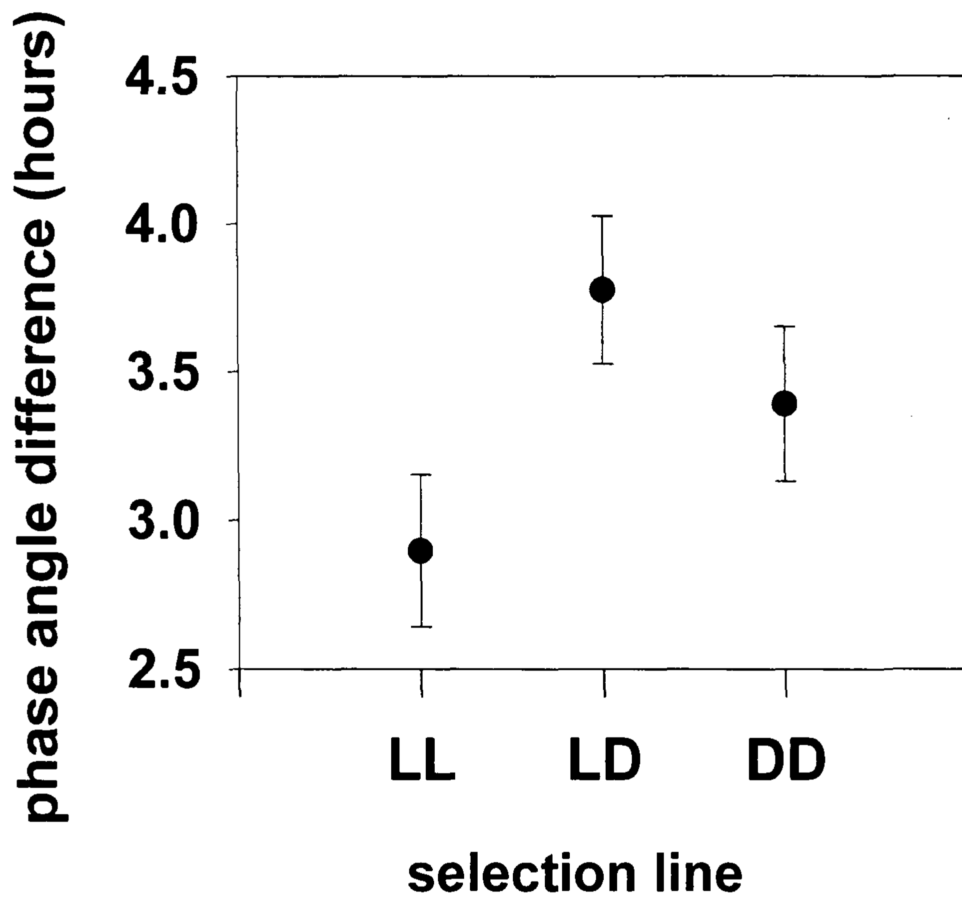

Fig. 4 Mean phase angle difference in LD 12:12 h regime of locomotor activity rhythm of flies from LL, LD and DD stocks.

Table. 1 Results of analysis of variance (ANOVA) on the phase angle difference (ψ) of eclosion rhythm of the LL, LD and DD stocks (selection regime) under LD 12:12 h regime. Since the analysis was performed on population means, block, and interactions involving block, cannot be tested for significance in this design.

Effect	***df***	**MS**	***F***	***p*-level**
Selection regime (R)	3	0.574	33.78	**<0.0001**
Block (B)	3	0.011	--	--
R × B	9	0.017	--	--

Table. 2 Results of analysis of variance (ANOVA) on the mean free running period (τ_{DD}) of locomotor activity rhythm of the LL, LD and DD stocks (selection regime). Since the analysis was performed on population means, block and interactions involving block cannot be tested for significance in this design.

Effect	***df***	**MS**	***F***	***p*-level**
Selection regime (R)	2	0.3497	12.94	**0.007**
Block (B)	3	0.0359	--	--
Sex (S)	1	0.0043	0.061	0.820
R × B	6	0.0270	--	--
R × S	2	0.0124	0.260	0.779
B × S	3	0.0694	--	--
R × B × S	6	0.0476	--	--

Table. 3 Results of analysis of variance (ANOVA) on the variance (within each combination of selection regime, block and sex) of free running period (τ_{DD}) of locomotor activity rhythm of the LL, LD and DD stocks (selection regime). Since the analysis was performed on population means, block, and interactions involving block, cannot be tested for significance in this design.

Effect	***df***	**MS**	***F***	***p*-level**
Selection regime (R)	2	0.0221	8.06	**0.0199**
Block (B)	3	0.0245	--	--
Sex (S)	1	0.0132	1.3899	0.3234
R × B	6	0.0028	--	--
R × S	2	0.0349	2.7869	0.1393
B × S	3	0.0095	--	--
R × B × S	6	0.0125	--	--

12:12 h regime, as was seen in the case of the ancestral baseline populations. We did not see any significant difference among the stocks maintained in different light regimes in the τ_{LL} or τ_{DD} of the eclosion rhythm. The analysis of the ψ of eclosion rhythm revealed a significant effect of selection regime, with the LD stocks exhibiting a significantly greater ψ than the LL and DD stocks. Although the importance of the phase of the eclosion rhythm has been the subject of several studies, it is not yet entirely clear as to what mechanisms drive the selection of the very distinct phase of eclosion in many insect species. From our results it appears that the stocks that have been entrained to a LD 12:12 h cycle for about 30 generations eclose in a window farther from 'lights-on', as compared to LL and DD stocks. The lack of significant difference in τ_{LL} and τ_{DD} of the eclosion rhythm of the three stocks may be due to the lower resolution of eclosion rhythm assay, compared to the locomotor activity assay. Data for eclosion rhythm were sampled only once every two hours, whereas locomotor activity data were sampled every 5 minutes.

The results of locomotor activity rhythm assays reveal that the τ_{DD} of the locomotor activity rhythm of the flies in the LD stocks has diverged from the ancestral value, with the LD stocks now exhibiting a mean τ_{DD} closer to 24 h as compared to that of LL stocks (which continued to be maintained in the ancestral LL regime) and DD stocks. Although τ_{DD} of DD stocks has not significantly diverged from the ancestral value, the variance in τ_{DD} in the DD stocks has become greater than the LL (ancestral) stocks. We also detected a difference in another circadian rhythm parameter, the ψ of onset of locomotor activity rhythm in LD cycle of 12:12 h among the three stocks. These results suggest that within ~ 30 - 40 generations of selection under an environment with a periodicity of 24 h, the LD stocks have diverged from the ancestral populations in terms of the circadian organisation that regulates locomotor activity and eclosion rhythms. These results are the first empirical demonstration of evolutionary change in

circadian rhythm parameters occurring in response to maintenance in a periodic regime. The LD selected populations appear to have been subjected to selection for possessing an endogenous periodicity closer to 24 h so as to synchronise with the external periodic LD cycles for over 30 generations. We may, therefore, expect fitness of LD stocks to be greater than that of LL or DD stocks, when assayed in LD regime.

4.3. Assays on components of fitness of selected populations

4.3 a Materials and methods

We studied components of pre-adult fitness such as egg to adult development time, survivorship and adult dry weights at eclosion in the three stocks (LL, LD and DD) at approximately every 10 generations (generation 10, 21, 33 and 45) during the course of selection in order to assess the effects of selection on these three traits.

Development time and survivorship assays

Egg to adult development time and survivorship assays were conducted following a procedure similar to the assay of baseline populations as previously described in chapter 3. Unlike the assays performed on the baseline populations, we obtained greater resolution of data in these experiments as the number of eclosing flies were monitored at intervals of two hours instead of six. We assayed the egg to adult survivorship in the three light regimes for all three stocks following the protocol described in chapter 3.

Dry weights at eclosion

Eggs were collected from the 12 populations of the three stocks at a larval density of 30 eggs per vial and introduced to three assay light regimes, LL, LD 12:12 h and DD, and the flies eclosing within a window of about 6 h corresponding to the peak of eclosion were used for the assay. The dry weight of freshly eclosed adults was measured by freezing adults within 2 h of eclosion to kill them and then subjecting them to dry heat at ~70 °C for 36 h. The adults of each sex were then weighed in batches of five. Six such measurements were taken for each sex per population per assay light regime.

For each of the three traits considered (development time, egg to adult survivorship and dry weights) the primary unit of analysis was the population mean trait value for a given

combination of block × generation of assay × selection regime × assay light regime × sex (except survivorship, where sex was not a factor). Analyses of variance (ANOVAs) were conducted according to a mixed model, completely randomised block design wherein generation, selection regime, assay light regime, and sex (except in the case of survivorship) were treated as fixed factors crossed among themselves and with random blocks based on ancestry. In such a design it is not possible to test for significance of block or any interactions involving block. ANOVAs were done on untransformed data for development time and dry weights since the units of analysis were population mean values, which can be assumed to follow a normal distribution to a reasonable degree.

Assays of adult components of fitness

Lifetime fecundity, age-specific body weight and adult lifespan were assayed for all the 12 populations of the three stocks under the three light regimes LL, LD 12:12 h and DD. The lifetime fecundity of forty individual females per population per light regime was assayed following the protocol described in chapter 3 after 35-40 generations of selection. The body weight profile of adult flies of all 12 populations was assayed after 33-36 generations of selection by sampling adult males and females from all three assay light regimes during a fixed time interval on each of the six days: day 2, 4, 6, 8, 10 and 12 post-eclosion. The dry weights were estimated following the same method as described in the previous section. The adult lifespan of mated male and female flies was assayed in the three light regimes LL, LD 12:12 h and DD following the method described in chapter 3 after 10-15 generations of selection.

From the primary data on daily egg production of females in the lifetime fecundity assay, the following were computed for each female: the average number of eggs laid per day, the total number of eggs produced throughout its life, the total number of eggs laid from day 1 to day 20,

the total number of eggs laid between day 20 to day 40, and the number of eggs laid on day 11 (which corresponds to the day on which eggs are collected during regular stock maintenance). For each of these estimates, the mean across the 40 females was calculated for each of the 12 populations in each assay light regime. These mean values were then used as data in separate three way mixed model ANOVAs with selection regime and assay light regime as fixed factors and block as the random factor.

The primary data on the adult dry weights were subjected to an ANOVA where the age at which the assay was performed, the selection regime, the assay light regime and sex were fixed factors crossed among themselves and with random blocks. From the primary data, we also computed the coefficient of variation of the dry weights assayed on different days for each combination of block × selection regime × assay light regime × sex. The arcsine square root (Freeman and Tukey, 1950) transformed values of coefficient of variation were then used as data in a mixed model ANOVA where selection regime, assay light regime and sex were fixed factors crossed among themselves and with random blocks.

From the primary data of each lifespan assay, mean adult life span (in days) and Gompertz parameters A and α (details in chapter 3) were estimated for each selection regime × assay light regime × sex combination, using a maximum likelihood method that utilizes the untransformed survivorship data (Mueller et al., 1995). Estimates of population mean adult life span, A, and α for each selection regime × assay light regime × sex combination were then used as data in separate mixed model ANOVAs in which replicate populations were treated as random blocks, whereas selection regime, assay light regime and sex were treated as fixed factors crossed with block.

4.3 b Results

Assays of pre-adult components of fitness

The pattern of assay light regime effects on egg to adult development time (Fig. 5) was consistent with that of assays on the baseline populations (chapter 3, Fig. 1). The ANOVA revealed significant main effects of light regime and sex on the egg to adult development time (Table 4). Multiple comparisons revealed that the egg to adult development time was significantly shorter in LL regime as compared to LD 12:12 h and DD regimes. The development time in LD regime was in turn significantly less than in DD regime (Fig. 5). The males developed slower than females, as is usual in *D. melanogaster*. The interaction of generation of assay × assay light regime was also significant ($p < 0.001$). Multiple comparisons revealed that this was due to there not being any significant difference in development time between LL and LD assay regimes in the 45th generation assay. The interaction between generation and selection regime was also significant ($p < 0.048$), and multiple comparisons revealed this to be due to the DD stocks exhibiting longer development time as compared to the other stocks only in case of the 45th generation assay. The interaction between generation of assay × assay light regime × sex was also significant ($p < 0.02$), and multiple comparisons revealed this to be due to development time being significantly different among the three light regimes in case of generation 10 and 33 for both males and females, while in generation 21, the development time of both males and females in LD and DD assay light regimes were not significantly different from each other, and in generation 45, there was no significant difference in development time of females in LD and DD assay light regimes (Tukey's test). The interaction between assay light regime, selection regime and generation was not significant ($p = 0.29$). The ANOVA on egg to adult survivorship revealed no significant main effect of assay light regime, but a significant main effect of

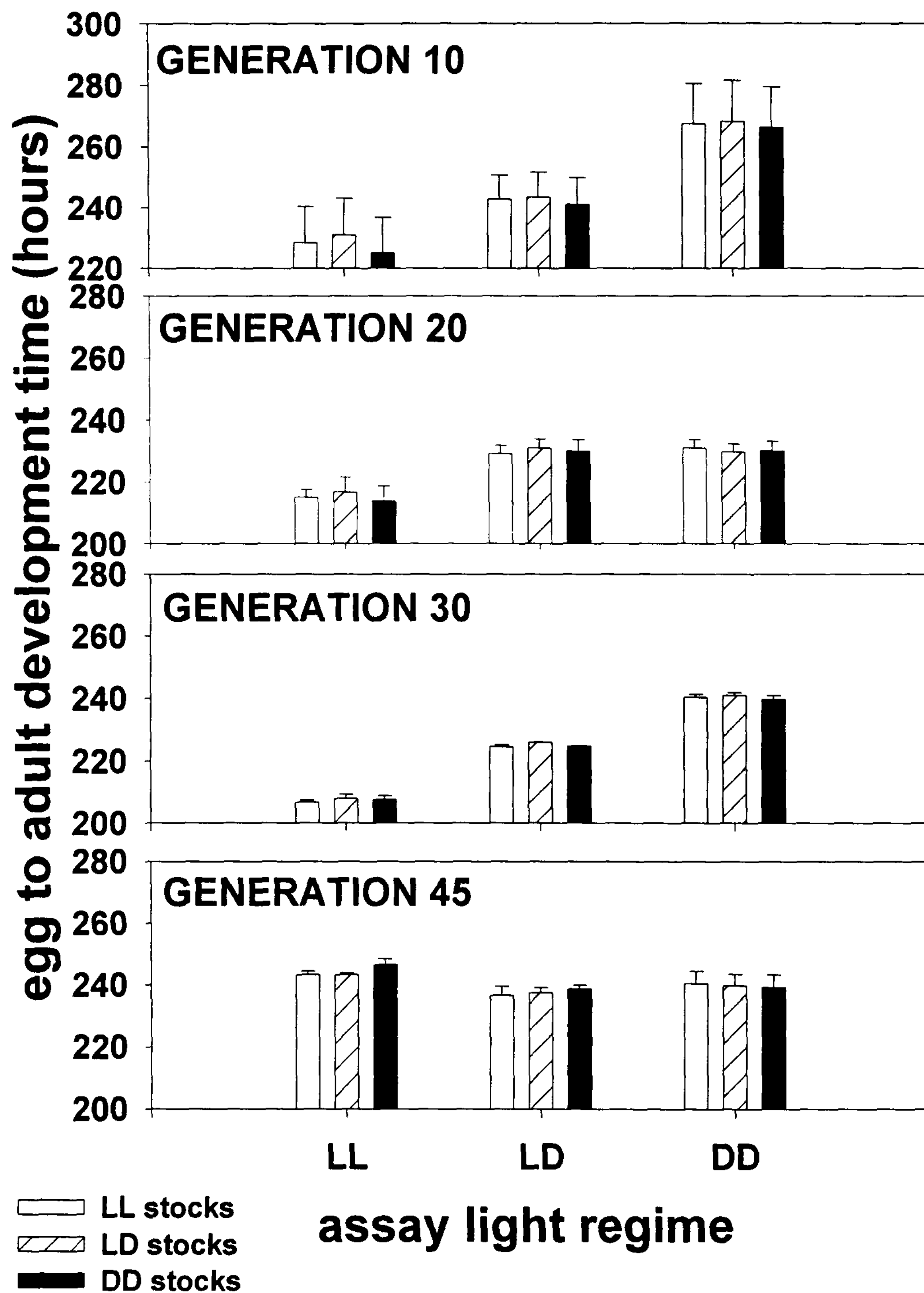

Fig. 5 Mean egg to adult development time of flies from LL, LD and DD stocks, assayed under LL, LD and DD regimes. Error bars are standard errors about the mean of the four replicate populations.

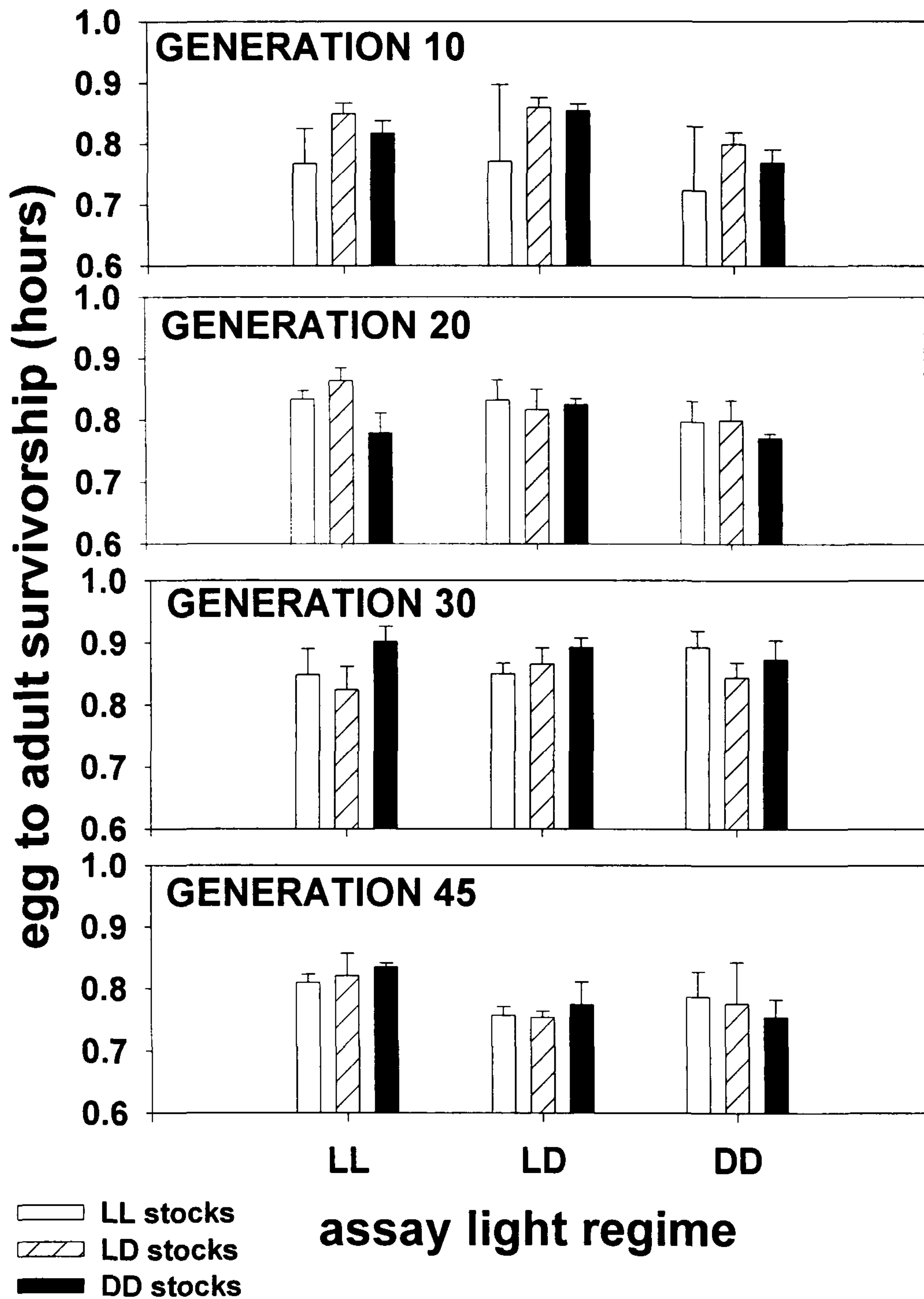

Fig. 6 Mean egg to adult survivorship of flies from LL, LD and DD stocks, assayed under LL, LD and DD regimes. Error bars are standard errors about the mean of the four replicate populations.

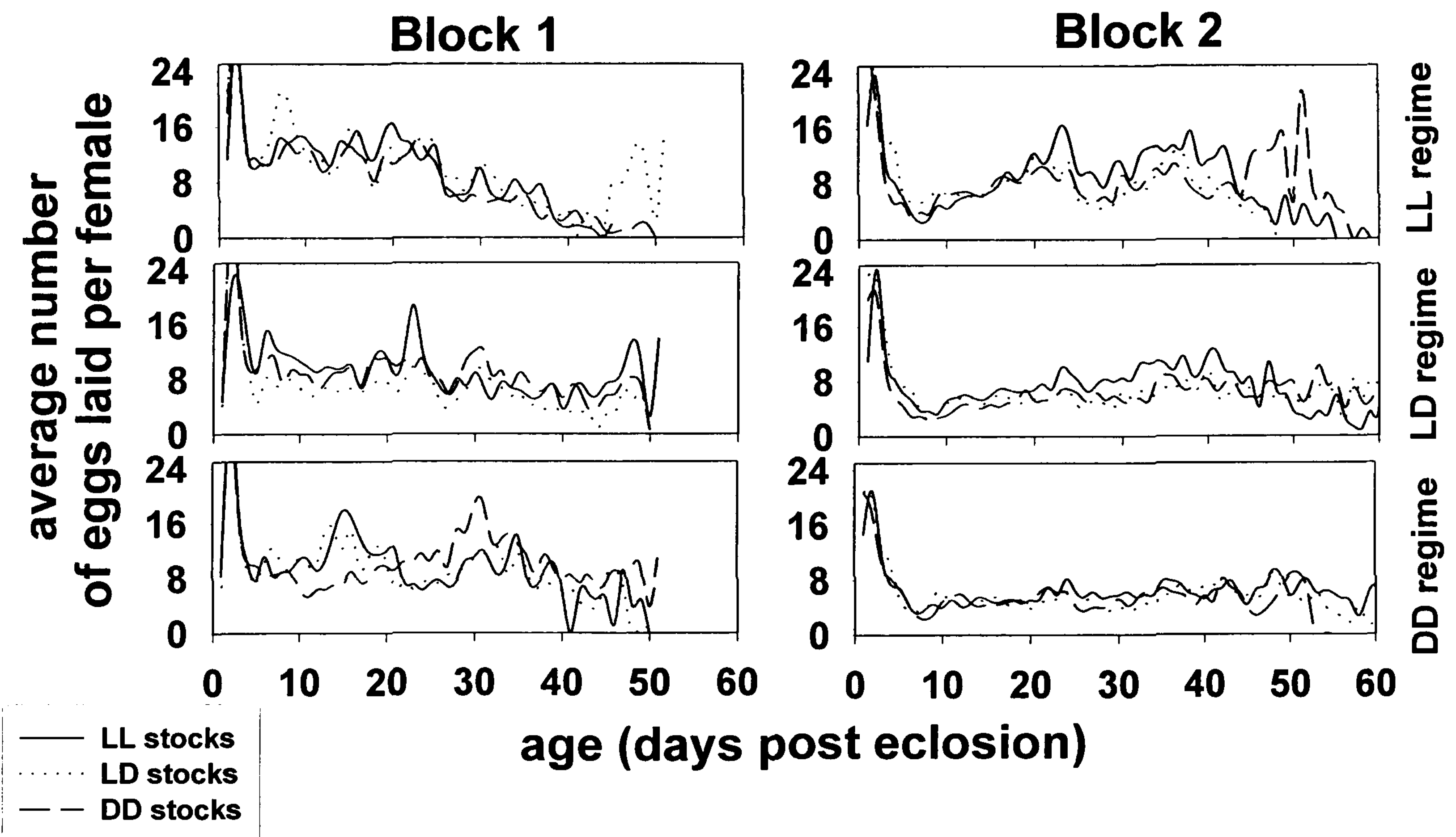

Fig. 7a Lifetime fecundity profile of females from LL, LD and DD stocks of blocks 1 (left panels) and 2 (right panels) assayed under LL (top panels), LD (middle panels) and DD (bottom panels) regimes.

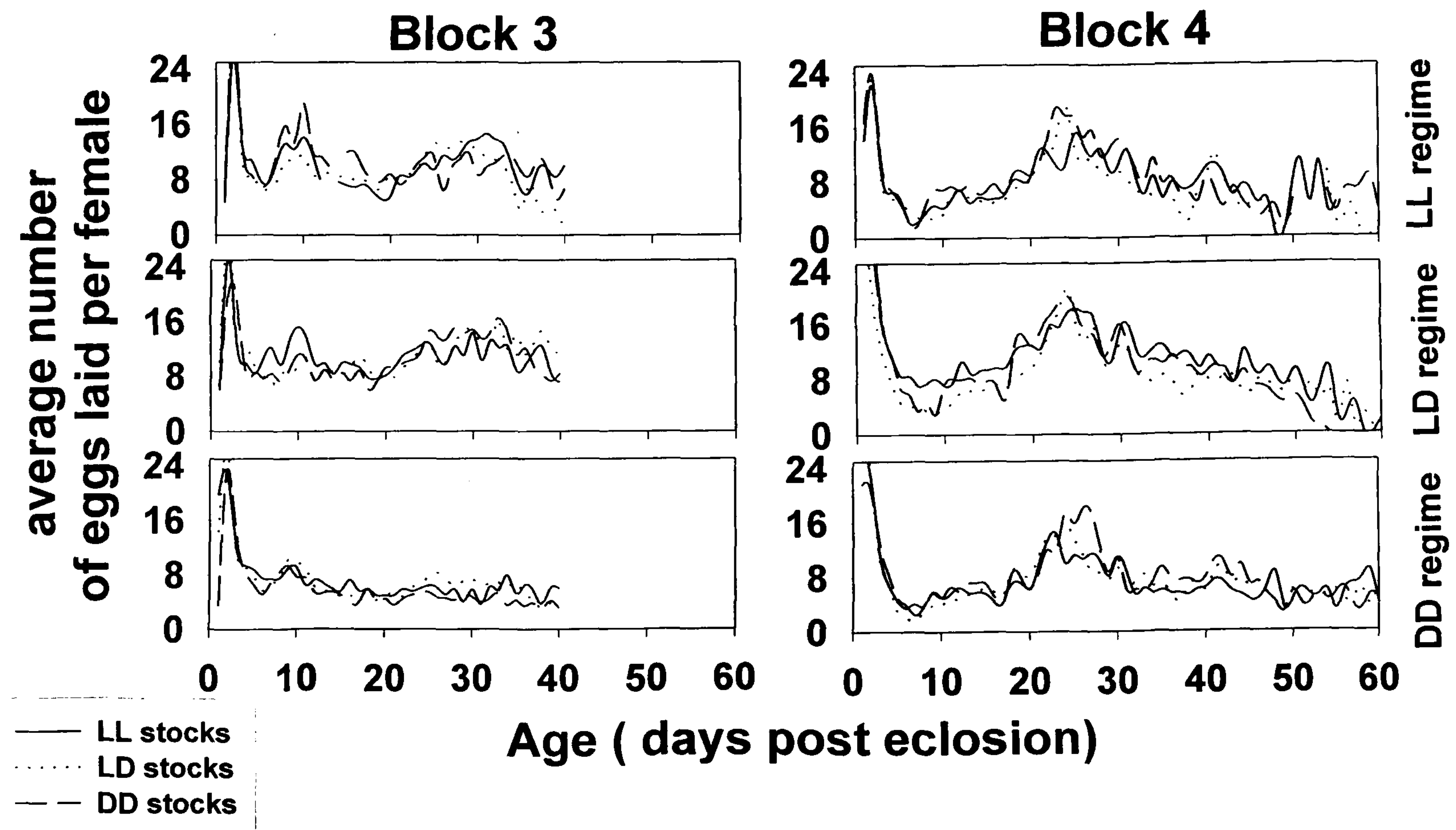

Fig. 7b Lifetime fecundity profile of females from LL, LD and DD stocks of blocks 3 (left panels) and 4 (right panels) assayed under LL (top panels), LD (middle panels) and DD (bottom panels) regimes.

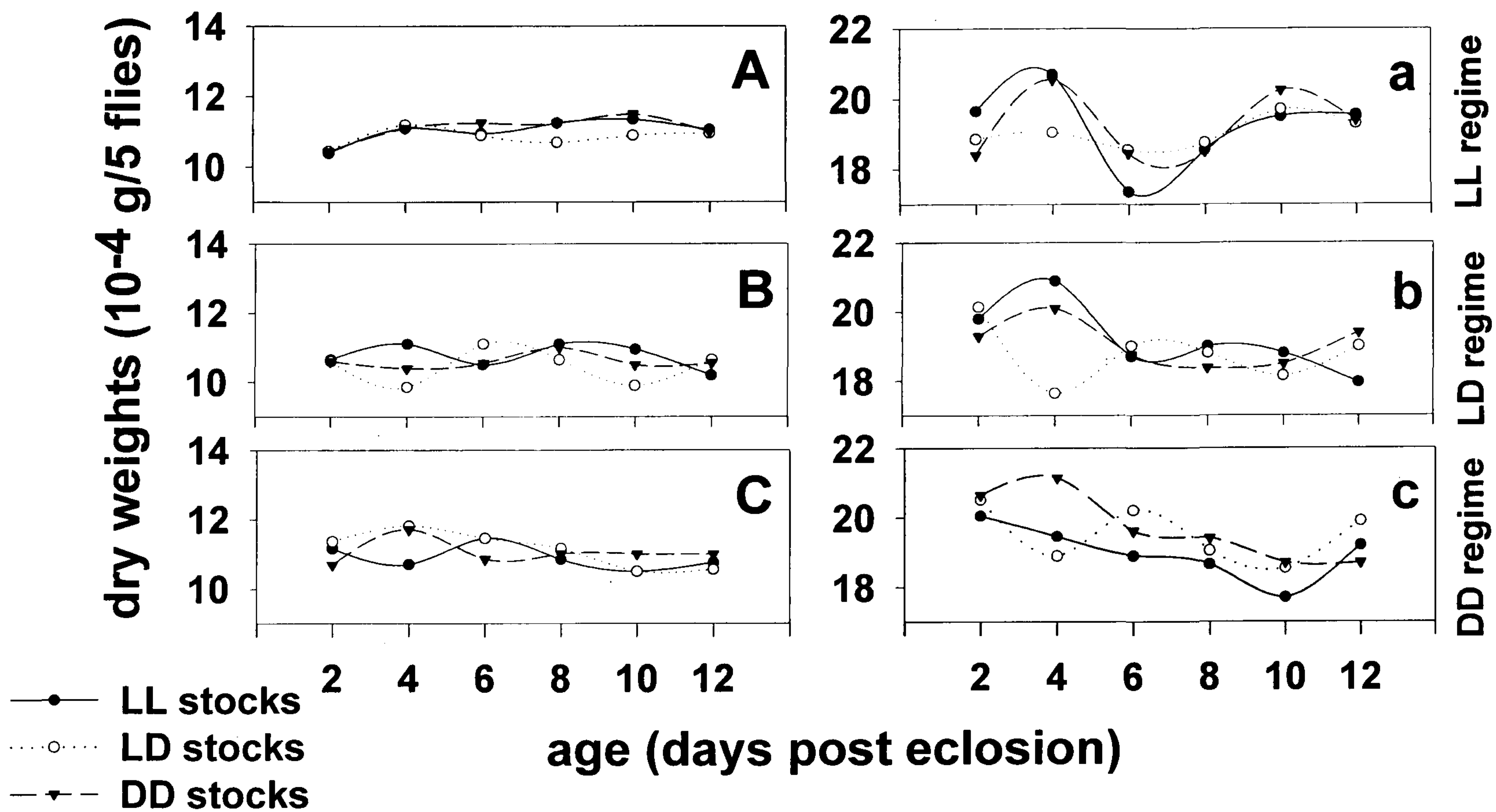

Fig. 8 Profile of adult dry weights assayed on different days for males (A, B, C) and females (a, b, c) averaged across four replicate populations of LL, LD and DD stocks in LL (A, a), LD (B, b) and DD (C, c) regimes.

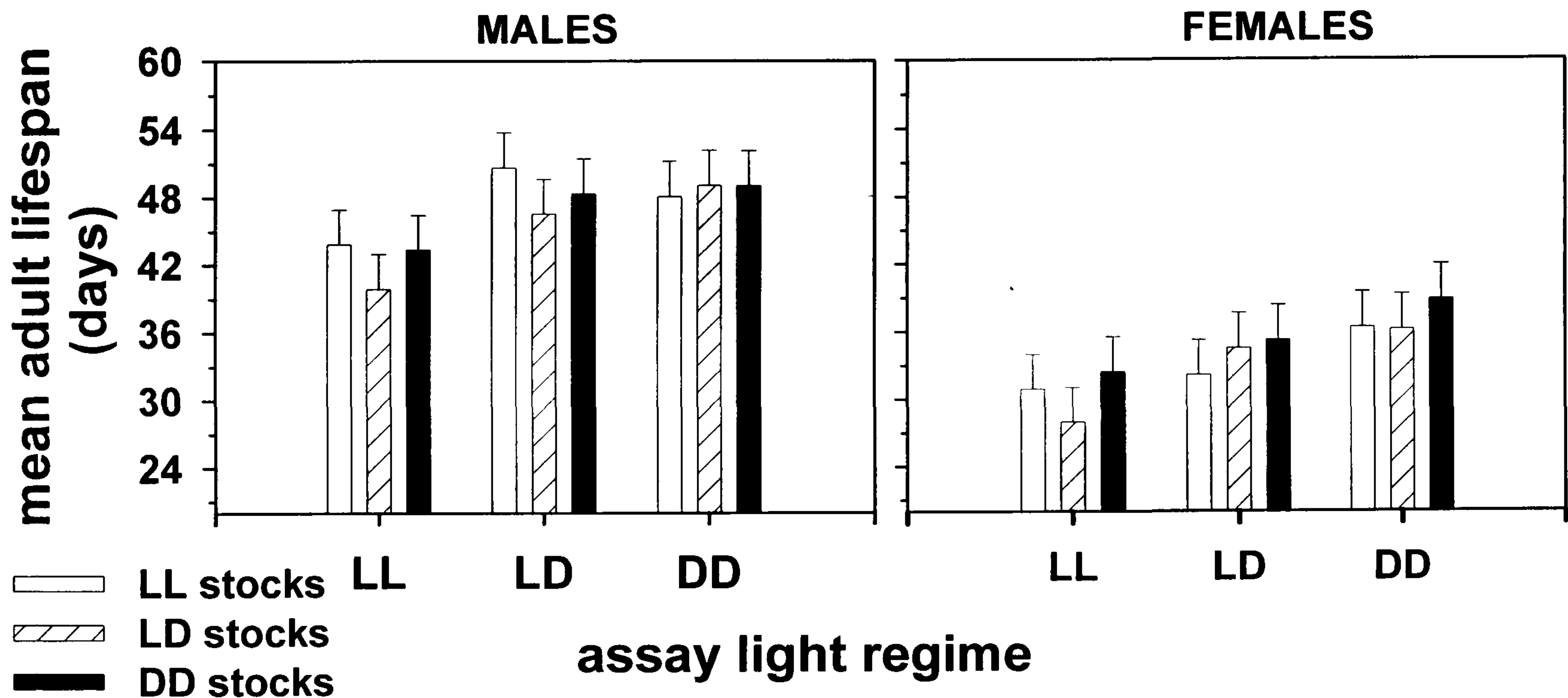

Fig. 9 Mean adult lifespan under LL, LD and DD regimes of males and females of LL, LD and DD stocks. Error bars represent 95% confidence intervals about the mean of four replicate populations in each assay, calculated using least squares estimates of the standard errors of the appropriate cell means in the randomised block ANOVA and can, therefore, be used for visual hypothesis testing.

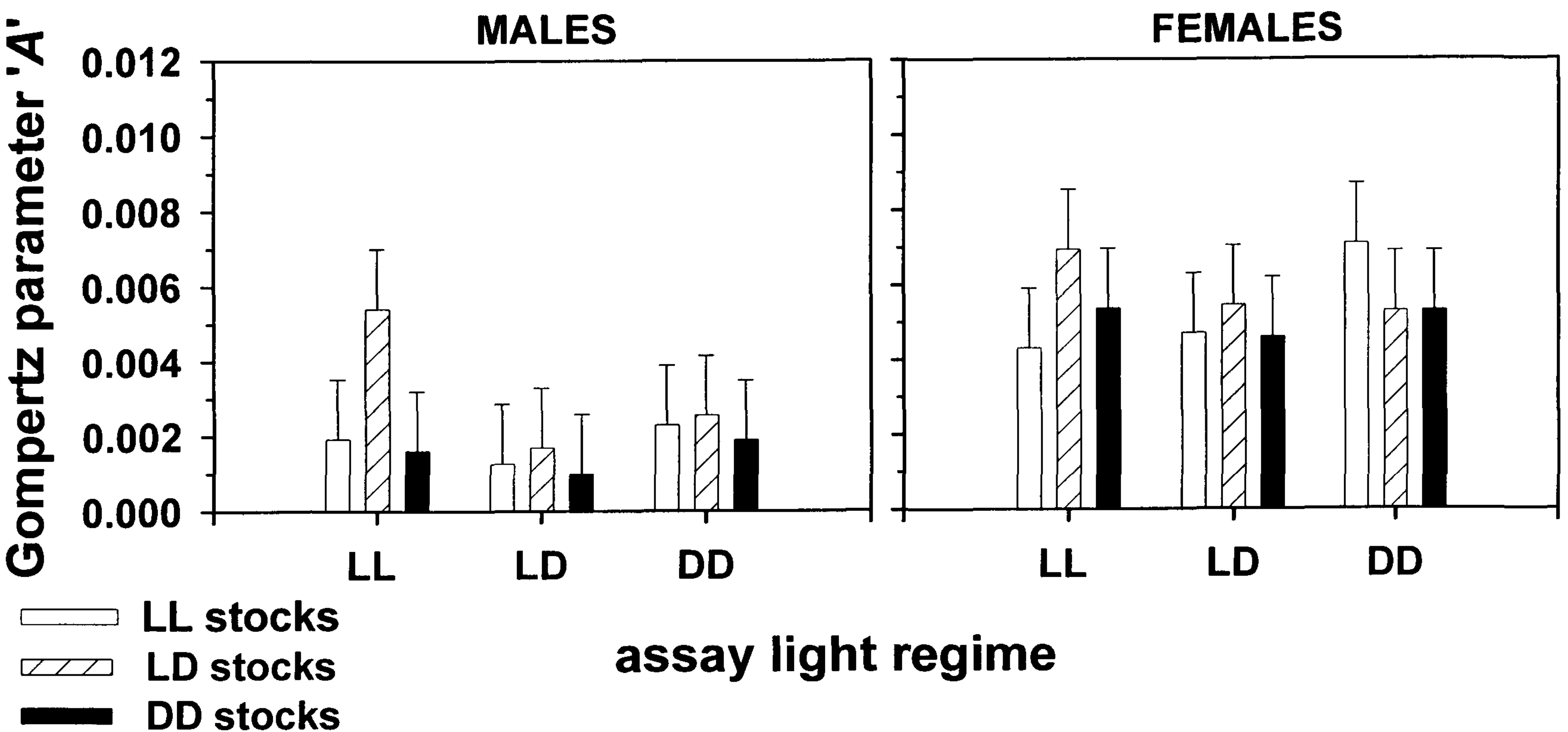

Fig. 10 Estimated values of age-independent mortality (Gompertz parameter *A*) under LL, LD and DD regimes of males and females of LL, LD and DD stocks. Error bars represent 95% confidence intervals about the mean of four replicate populations in each assay, calculated using least squares estimates of the standard errors of the appropriate cell means in the randomised block ANOVA and can, therefore, be used for visual hypothesis testing.

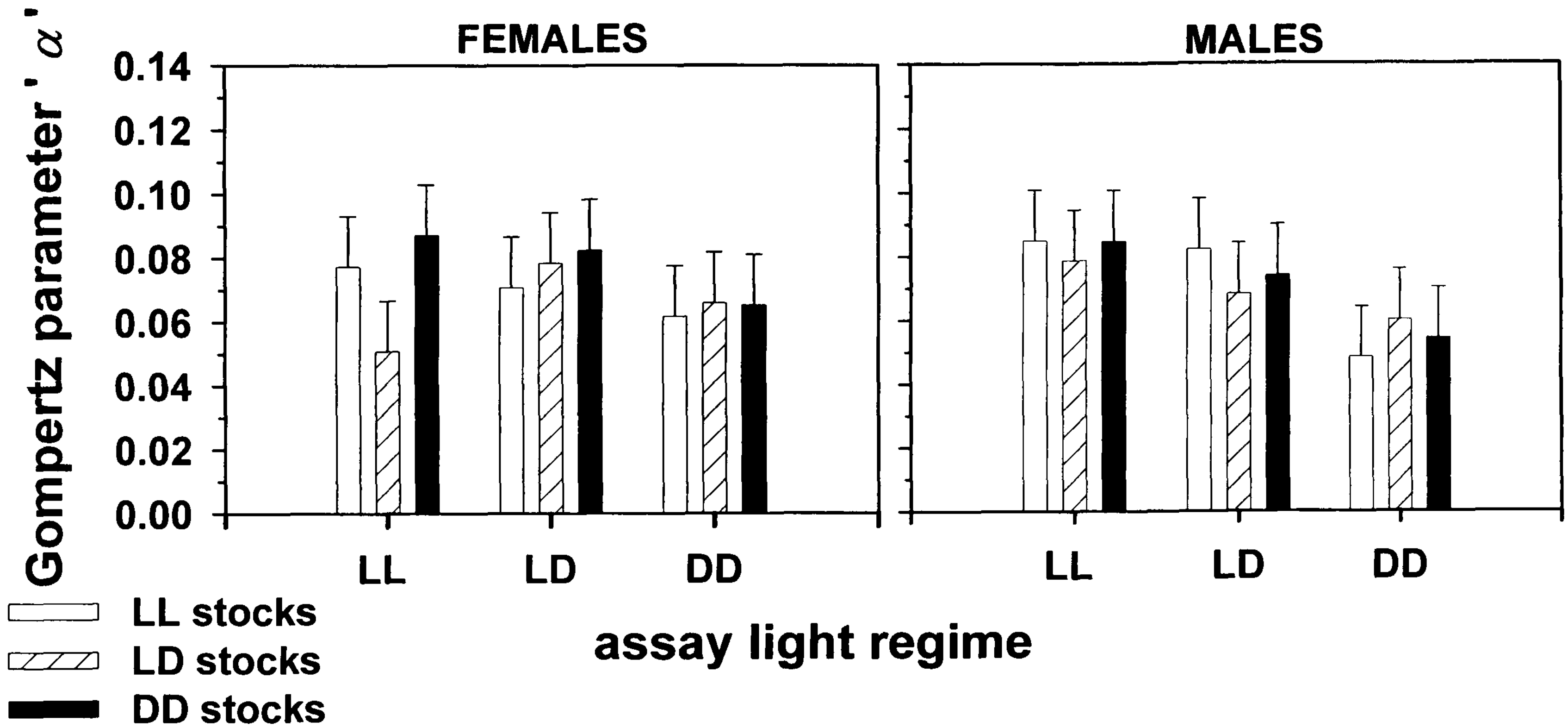

Fig. 11 Estimates of the Gompertz "rate of aging" (α) under LL, LD, and DD regimes of males and females of LL, LD and DD stocks. Error bars represent 95% confidence intervals about the mean of four replicate populations in each assay, calculated using least squares estimates of the standard errors of the appropriate cell means in the randomised block ANOVA and can, therefore, be used for visual hypothesis testing.

Table. 4 Results of analysis of variance (ANOVA) on mean egg to eclosion development time of males and females from four replicate populations from LL, LD and DD stocks assayed at generation 10, 21, 33 and 45 in LL, LD and DD regimes. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance and have not been presented here.

Effect	***df***	**MS**	***F***	***p*-level**
Generation (G)	3	8552.77	3.3117	0.0710
Assay light regime (L)	2	10238.9	274.793	**<0.0001**
Selection regime (R)	2	27.8264	1.5173	0.2929
Sex (S)	1	1612.07	142.517	**0.0012**
G × L	6	2585.56	31.769	**<0.0001**
G × R	6	24.1394	2.6966	**0.0478**
L × R	4	7.2693	1.3104	0.321
G × S	3	2.0365	0.2229	0.8781
L × S	2	3.2054	0.9589	0.4351
R × S	2	8.6938	2.5274	0.1599
G × L × R	12	8.7946	1.2467	0.2913
G × L × S	6	10.1103	4.7693	**0.0045**
G × R × S	6	1.8506	0.6017	0.7255
L × R × S	4	4.9379	0.5746	0.6864
G × L × R × S	12	1.9751	0.7544	0.6906

Table. 5 Results of analysis of variance (ANOVA) on mean egg to eclosion survivorship of flies from four replicate populations of LL, LD and DD stocks assayed at generation 10, 21, 33 and 45 in LL, LD and DD regimes. Since the analysis was performed on population means, the effects *of block, and interactions involving block, cannot be tested for significance.*

Effect	***df***	**MS**	***F***	***p*-level**
Generation (G)	3	0.217	4.786	**0.029**
Assay light regime (L)	2	0.0396	3.910	**0.082**
Selection regime (R)	2	0.0022	0.081	0.923
Block (B)	3	0.0352	--	--
G × L	6	0.0181	1.909	0.134
G × R	6	0.0201	0.633	0.703
L × R	4	0.0058	1.239	0.346
G × B	9	0.0454	--	--
L × B	6	0.0102	--	--
R × B	6	0.0265	--	--
G × L × R	12	0.0020	0.291	0.987
G × L × B	18	0.0095	--	--
G × R × B	18	0.0317	--	--
L × R × B	12	0.0047	--	--
G × L × R × B	36	0.0070	--	--

Table. 6 Results of analysis of variance (ANOVA) on mean dry weight of males and females from four replicate populations of LL, LD and DD stocks assayed under LL, LD and DD regimes on day 2, 4, 6, 8, 10, and 12 post eclosion. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance and have not been presented.

Effect	***df***	**MS**	***F***	***p*-level**
Block (B)	3	33.1981	--	--
Day (D)	5	3.6505	0.25315	0.9316
Assay light regime (L)	2	6.0823	2.0256	0.2127
Selection regime (R)	2	1.0023	0.2274	0.8031
Sex (S)	1	7486	466.8	**<0.0001**
D × L	10	3.859	2.349	**0.035**
D × R	10	2.3243	1.6437	0.1418
L × R	4	1.8931	2.3393	0.1142
D × S	5	4.6811	0.7699	0.5858
L × S	2	0.5495	0.9006	0.4549
R × S	2	0.3343	0.1317	0.8791
D × L × R	20	1.0005	1.1716	0.3095
D × L × S	10	1.1017	1.7198	0.122
D × R × S	10	1.8186	3.2119	0.0064
L × R × S	4	0.3471	0.5228	0.7210
D × L × R × S	20	0.4666	0.6862	0.8237

Table. 7 Results of analysis of variance (ANOVA) on coefficient of variation of dry weight on day 2, 4, 6, 8, 10, and 12 post eclosion. of males and females from four replicate populations of LL, LD and DD stocks assayed in LL, LD and DD regimes. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance.

Effect	*df*	**MS**	***F***	***p*-level**
Block (B)	3	0.0007	--	--
Assay light regime (L)	2	0.0004	0.1885	0.8329
Selection regime (R)	2	6.7E-05	0.0877	0.9172
Sex (S)	1	0.0055	5.3723	0.1033
B × L	6	0.0024	--	--
B × R	6	0.0008	--	--
L × R	4	9.8E-05	0.416	0.7941
B × S	3	0.0010	--	--
L × S	2	0.0016	1.6538	0.2679
R × S	2	0.0002	1.2994	0.3397
B × L × R	12	0.0002	--	--
B × L × S	6	0.0009	--	--
B × R × S	6	0.0001	--	--
L × R × S	4	0.0002	0.4723	0.7554
B × L × R × S	12	0.0005	--	--

Table. 8 Results of analysis of variance (ANOVA) on mean adult lifespan of flies from four replicate populations of LL, LD and DD stocks assayed at generation 10 under LL, LD and DD regimes. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance.

Effect	***df***	**MS**	***F***	***p*-level**
Block (B)	3	98.8335	--	--
Selection regime (R)	2	29.0022	1.1693	0.3725
Assay light regime (L)	2	276.638	11.395	**0.0090**
Sex (S)	1	2916.01	7.4306	0.0722
B × R	6	24.8027	--	--
B × L	6	24.2771	--	--
R × L	4	9.04726	1.1202	0.3921
B × S	3	392.4329	--	--
R × S	2	14.0691	1.1135	0.3879
L × R	2	15.5130	2.7834	0.1396
B × R × L	12	8.0766	--	--
B × R × S	6	12.6348	--	--
B × L × S	6	5.5734	--	--
R × L × S	4	7.8538	1.5543	0.2490
B × R × L × S	12	5.0528	--	--

Table. 9 Results of analysis of variance (ANOVA) on the age-independent mortality (Gompertz parameter A) of flies from four replicate populations of LL, LD and DD stocks assayed at generation 10 under LL, LD and DD regimes. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance.

Effect	*df*	MS	*F*	*p*-level
Block (B)	3	3.14E-05	--	--
Selection regime (R)	2	1.04E-05	1.4105	0.3147
Assay light regime (L)	2	9.05E-06	3.2936	0.1083
Sex (S)	1	0.000193	3.9020	0.1427
B × R	6	7.4E-06	--	--
B × L	6	2.75E-06	--	--
R × L	4	7.8E-06	3.6330	**0.0367**
B × S	3	4.94E-05	--	--
R × S	2	1.61E-06	0.5600	0.5984
L × R	2	2.28E-06	0.4877	0.6364
B × R × L	12	2.15E-06	--	--
B × R × S	6	2.87E-06	--	--
B × L × S	6	4.68E-06	--	--
R × L × S	4	1.59E-06	0.5258	0.7190
B × R × L × S	12	3.02E-06	--	--

Table. 10 Results of analysis of variance (ANOVA) on the Gompertz 'rate of ageing' (α) of flies from four replicate populations of LL. LD and DD stocks assayed at generation 10 under LL, LD and DD regimes. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance.

Effect	***df***	**MS**	***F***	***p*-level**
Block (B)	3	0.0008	--	--
Selection regime (R)	2	0.0003	0.9189	0.4486
Assay light regime (L)	2	0.0024	23.8251	**0.0014**
Sex (S)	1	3.94E-06	0.0332	0.8670
B × R	6	0.0004	--	--
B × L	6	0.0001	--	--
R × L	4	0.0004	1.9224	0.1713
B × S	3	0.0001	--	--
R × S	2	0.0002	1.0668	0.4014
L × R	2	0.0007	3.8507	0.0840
B × R × L	12	0.0002	--	--
B × R × S	6	0.0002	--	--
B × L × S	6	0.00018	--	--
R × L × S	4	0.0002	1.2620	0.3380
B × R × L × S	12	0.0002	--	--

generation of assay was seen, with flies in the 33rd generation assay having an overall greater survivorship in all three assay light regimes (Fig. 6, Table 5). Moreover, we did not see any significant main effect of the selection regime on the development time, survivorship or dry weights of these flies. The ANOVAs on dry weight at eclosion also did not reveal any significant effect of generation, selection regime or assay light regimes. The expected significant main effect of sex was seen, with males weighing less than females ($F_{1,3} = 720.3$; $p < 0.0001$). The selection regime × assay light regime × generation interaction was not significant for any of the three pre-adult components assayed (Tables 4, 5).

Assays of adult components of fitness

The ANOVAs on the data obtained from the lifetime fecundity assay revealed no significant main effect of either selection regime or assay light regime (Fig. 7 a, b). The interaction between selection regime and assay light regime was also not significant. The ANOVA on body weights of adults on day 2, 4, 6, 8, 10 and 12 did not reveal any significant main effect of selection or assay light regimes (Fig. 8, Table 6). A significant main effect of sex was seen, with females weighing more than males (Table 6). The interaction between day of assay × assay light regime was significant ($p < 0.04$). Multiple comparisons revealed that this was due to the fact that the relationship between dry weights in the different light regimes was different on different days post-eclosion. The ANOVA on the coefficient of variation also did not reveal any significant main effect or interactions between selection or assay light regimes (Table 7). The ANOVA on mean adult lifespan revealed a significant main effect of assay light regime (Fig. 9, Table 8). Multiple comparisons showed that mean adult lifespan of flies in LL regime was significantly shorter than that in LD and DD regimes (Tukey's test). This ANOVA did not show a significant main effect of sex ($p = 0.07$) although the mean adult lifespan of

females was 33.83 days and males was 46.56 days. The lack of significant effect of sex could be due to the fact that in one of the blocks (populations LL3, LD3 and DD3) the mean adult lifespan of females was greater than that of males in LL and DD regimes. The ANOVA on Gompertz parameter A did not reveal any significant main effect of either selection or assay light regime or of sex (Fig. 10, Table 9). The ANOVA on the Gompertz parameter α revealed a significant main effect of assay light regime (Fig. 11, Table 10). Multiple comparisons revealed that α of flies in DD regime was significantly less than that in LL and LD regimes (Tukey's test). No significant interactions were seen for the ANOVAs done on mean adult lifespan, or Gompertz parameters.

4.3 c Discussion

The lack of significant interaction between assay light regime and selection regime suggests that three sets of stocks have not diverged significantly from each other in terms of various pre-adult and adult fitness components assayed in the light regime in which they are routinely maintained. The effects of assay light regime on development time and adult lifespan are consistent with the effects observed in the baseline populations. The egg to adult development time of the three stocks was not detectably different after ~ 45 generations of selection under different light regimes. Two other pre-adult components of fitness, egg to adult survivorship and the body weight at eclosion, which were unaffected by the three assay light regimes LL, LD 12:12 h and DD in the baseline population remained unaltered in the three stocks even after selection for ~ 45 generations. We also did not detect any evolutionary change in adult components of fitness, such as lifetime fecundity, body weight and lifespan, after nearly 45 generations of selection.

Although we were unable to detect any statistically significant alterations in pre-adult and adult components of fitness in our populations that have been maintained in three different light regimes for about 45 generations, the differences in the circadian parameters of free running period and phase angle difference for locomotor activity rhythm and the phase angle difference of the eclosion rhythm clearly point to an evolutionary divergence of the LD stocks from the LL and DD populations. Such differences in circadian parameters in four different populations can occur only if changes in circadian rhythm parameters are associated with fitness differences in the three selection lines. It is possible that light regime specific differences in fitness among stocks exist but are not detected as being significant in our assays. Further studies by which relative fitness of these selected populations can be more accurately detected may help to understand the manner in which the differences in fitness components may have led to the observed differences in circadian parameters. Experiments involving stressful environments during development or in adult stages where individuals of the same population may compete among themselves or against a common competitor may also help to accentuate differences in fitness components among the populations reared in different selection regimes, rendering them more easily detectable.

Chapter 5

Developmental Plasticity Of Circadian Organisation

5.1 Introduction

The τ of a circadian rhythm is often regarded as a rigid characteristic of a species, with the τ of individual animals being approximately normally distributed around the species mean, usually with a fairly small variance (Moore-Ede et al., 1982). For the same group of individuals, the free-running period measured in constant darkness (DD) (τ_{DD}) and in constant light (LL) (τ_{LL}) typically differs (Pittendrigh, 1960; Aschoff, 1979). Moreover, however, there is evidence that the τ of a circadian pacemaker varies in response to various environmental conditions, often reflecting residual effects of prior environmental conditions experienced, typically referred to as "after effects" (Pittendrigh, 1960; Sokolove, 1975; Christensen, 1978; Page and Block, 1980). After effects have been observed in rhythms monitored under DD after the animals were previously exposed to LL, or to light/dark (LD) cycles of varying photoperiod length, but the results have not been unequivocal in experiments using different rodent species (Pittendrigh, 1960) and insects (Sokolove, 1975; Christensen, 1978). The after effects of LD cycles are speculated to be of some functional significance in helping organisms to perform various behavioural and physiological functions at appropriate times even when the environmental LD cycle is masked, for example due to cloud cover (Beersma et al., 1999). Systematic investigation of the effects of light regime experienced during early life stages on the circadian organisation later in life is also relevant to the comparison and interpretation of results from studies on adult circadian rhythms because such studies often differ from one another in the type of light regime the pre-adult organisms were reared in prior to the actual experiments.

Among insects, after effects of LL of varying intensities on τ_{DD} of the locomotor activity rhythm have been studied in *D. melanogaster*, and the kind of after effect seen was found to vary with *per* locus genotype (Konopka et al., 1989). In cockroaches, exposure of nymphal stages to

LD cycles of varying periodicity affects not only the τ, but also the sensitivity to a brief light pulse, as can be observed in terms of modified light pulse phase response curve (PRC) in the adults (Barrett and Page, 1989; Page and Barrett, 1989). In these studies, animals raised as pre-adults in LD 11:11 h had significantly shorter τ_{DD} and a reduced *delay* portion of the PRC, whereas those raised in LD 13:13 h had longer τ_{DD} and reduced *advance* portion of the PRC, relative to animals raised in LD 12:12 h. However, the PRC of animals raised as nymphs in DD did not differ significantly from those raised in LD 12:12 h. Animals raised as nymphs in LL were also found to have significantly longer τ_{DD} as adults, compared to animals raised as nymphs in DD (Page and Barrett, 1989). Circadian rhythms in adults have also been reported to be affected by photoperiod experienced in pre-adult stages in the cricket species *Gryllus bimaculatus* (Tomioka and Chiba, 1989 a, b).

Effects on adult rhythms of light/dark regimes experienced during the pre-adult stage have also been observed in *D. melanogaster* which, unlike cockroaches or crickets, undergoes complete metamorphosis wherein virtually all adult tissues are formed anew. In *Drosophila*, the circadian timekeeping mechanism is believed to function from the first larval instar (L1) onwards (Helfrich-Förster, 1995), and light pulses given during L1 can shift the phase of the adult eclosion and locomotor activity rhythms (Kaneko et al., 1997). A recent study on wild type (Canton S) and *per* mutant *D. melanogaster* reared in LL, in DD, and in 24 h LD cycles with different ratios of light/dark; 4:20 h; 6:18 h; 12:12 h; 18:6 h and 20:4 h, revealed that the light regime experienced during the pre-adult and early adult stages (egg, larval, pupal stages and first seven days as adult) affects τ_{DD} of adult locomotor activity rhythms (Tomioka et al., 1997). Earlier, Konopka et al (1989) reported that the τ_{DD} of the locomotor activity rhythm of per^S and per^L mutants of *D. melanogaster* (derived from a Canton-S strain) behaved in a reciprocal

manner when they underwent a transfer from DD to LL or LL to DD. Interestingly, the results of Tomioka et al (1997) are opposite to those of Konopka et al (1989): following rearing in LL during early stages of development, τ_{DD} was decreased in *per*$^{+}$, *per*^{S} and *per*^{L1} homozygotes, relative to τ_{DD} in control flies that were reared in DD throughout the pre-adult and adult stages. However, for the flies reared in LD 12:12 h, LD 8:16 h and LD 4:20 h, Tomioka et al (1997) did observe opposite responses of subsequently measured τ_{DD} to the light regime experienced in *per*^{S} and *per*^{L1} homozygotes, as had been earlier reported by Konopka et al (1989).

One drawback of the study by Tomioka et al (1997) was that their experimental design, as the authors note, confounds the effects of light regime experienced during pre-adult stages with that experienced during the first 7 days of adult life. Consequently, one cannot unequivocally ascribe the observed effects of light regime on τ_{DD} to the light regime experienced as pre-adults or early in adult life, and it may be that an interaction between light regime experienced at different life stages is responsible for the discrepancy between their results and those of Konopka et al (1989). Another potential problem in generalising from the results of Konopka et al (1989) and Tomioka et al (1997) is that in both studies the wild type strain (*per*$^{+}$ homozygotes) was Canton S. Since such laboratory populations are typically maintained at small population sizes and are often inbred, it is difficult to formally rule out the possibility, however implausible it may seem, that the after effects seen in the Canton S strain reflect some aspect of its genotypic constitution other than the fact that the flies are homozygous for the *per*$^{+}$ allele.

We conducted two experiments in which the effect of the light regimes experienced during the pre-adult (larval and pupal) and early adult stages on τ_{DD} of the adult locomotor activity rhythm was examined. We used the four replicate populations of *D. melanogaster*, LL (1..4) which have been maintained separately, i.e. without any gene flow among them, for over

600 generations (described in chapter 1). In these two experiments, individuals from these populations were exposed to LL, DD and LD 12:12 h from the egg through the pupal stages, and the τ_{DD} and the phase angle difference (ψ) of the locomotor activity of the adults were assayed. In the first experiment, the τ_{DD} of the locomotor activity of the freshly emerging adults was assayed in DD immediately after eclosion, while in the second experiment, the locomotor activity of flies emerging in the three regimes was first monitored in LD 12:12 h for fifteen days following which they were introduced into DD regime and the τ_{DD} of the locomotor activity of the adults was assayed for up to 15 days.

5.2 Materials and methods

For each experiment, eggs were collected from the running culture of each population in plexiglas cages ($25 \times 20 \times 15$ cm^3), by allowing females to lay eggs on food medium for about 2 hours. From each population, exactly 50 eggs each were collected into 24 vials (9.0 cm h $\times$ 2.4 cm dia) containing approximately 6 ml of banana-jaggery food. Eight vials from each population were kept under LL, 8 vials under LD 12:12 h, and 8 under DD.

The light phase in these treatments was achieved by means of fluorescent white light sources (approximate intensity 2.5 W/m^2 or 300 lux), whereas the dark phase was actually dim red light ($\lambda > 640$ nm), which facilitated observation and manipulation of flies without interrupting the dark phase. The DD in our experiments also refers to continuous dim red light ($\lambda > 640$ nm). In all three light regimes, the vials were closely monitored once pupae darkened. When peak eclosion occurred, flies that eclosed within a 4-5 h window were collected and males and females separated. The virgin flies thus obtained were set up individually in glass tubes (80 mm h, 6 mm dia) with sugar crystals at the bottom and cotton wicks moistened with water at the

upper end (26 flies per sex per population per rearing light regime). The up and down movement of the flies was monitored by two pairs of infra red (IR) emitters and sensors which were placed perpendicular to one another in such a manner that when a fly cut the IR beams, the event was recorded by a computerised recording and display system. Activity was recorded in 5 min bins and each fly was monitored for up to 15 days in the first experiment and for up to 30 days in the second experiment.

The τ_{DD} of locomotor activity was estimated for each fly separately from the slope of a least squares regression line fit to the time of onset of activity for at least 6 consecutive days. The values of τ_{DD} for flies reared in all three light regimes for each sex were then used as data in a mixed model analysis of variance (ANOVA) in which replicate populations were treated as random blocks, and assay (with or without exposure to LD cycle during early adult stage), pre-adult light regime and sex as fixed factors crossed with block; post hoc comparisons were done using Tukey's test. The ψ of locomotor activity rhythm for "onset of activity" in LD 12:12 h, was estimated by averaging the time interval between onset of activity and "lights on" for about 10 consecutive days. The values of ψ for onset of activity for the flies of both sexes, reared in all three regimes were used in a mixed model ANOVA. Replicate populations were treated as random blocks while pre-adult light regime and sex were fixed factors crossed with block. All statistical analyses were implemented using STATISTICA™ for Windows Release 5.0 B (StatSoft Inc, 1995).

5.3 Results

The τ_{DD} of the locomotor activity rhythm in flies reared under different light regimes during the pre-adult stages ranged between 22.5 to 25.5 h (Fig. 1, Tables 1, 2). The ANOVA on

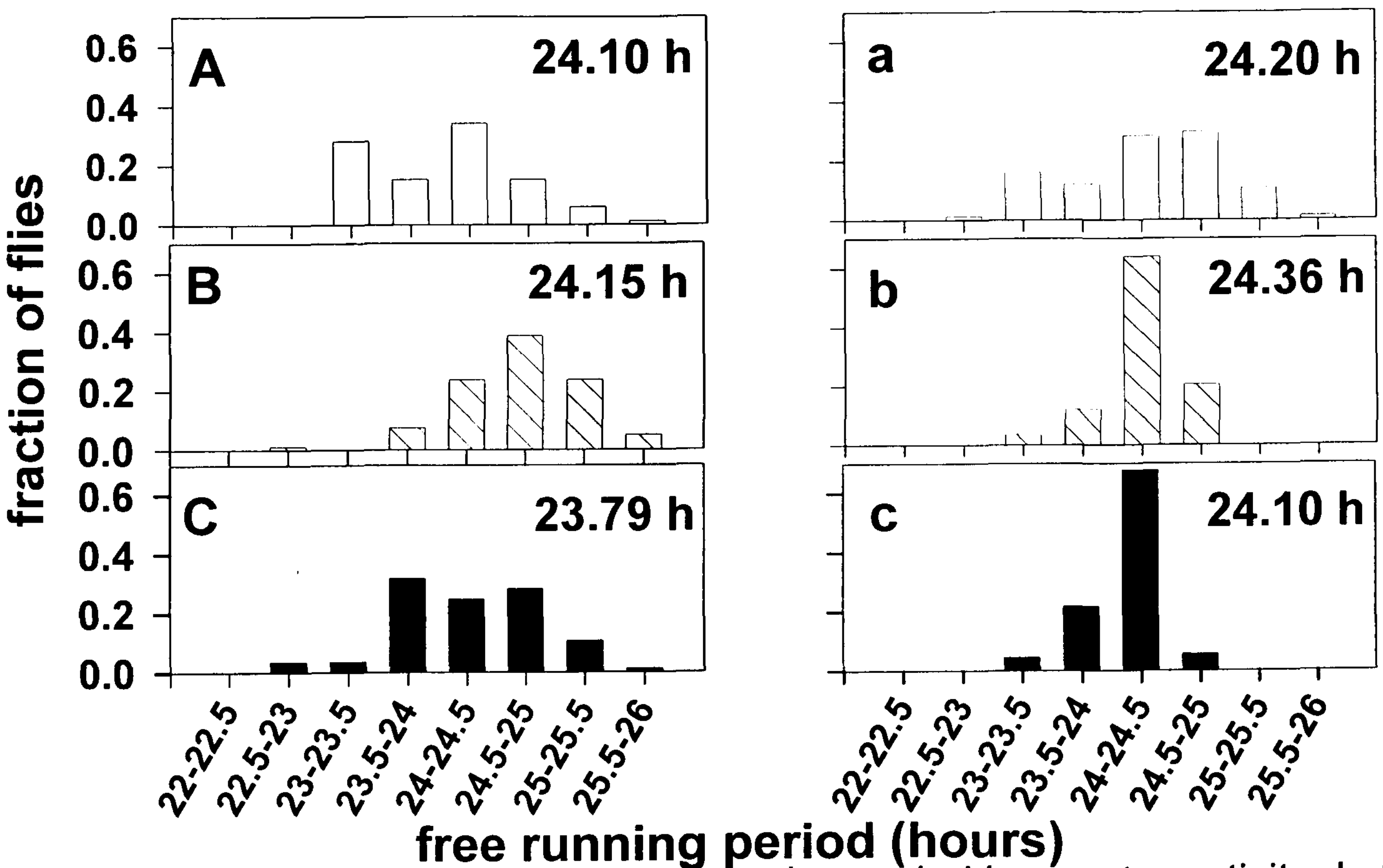

Fig. 1 Frequency distribution and mean free running period locomotor activity rhythm in DD of flies (both males and females) reared in different light regimes (A, a - LL regime; B, b - LD regime; C, c - DD regime). (A-C) flies were assayed directly after eclosion, (a-c) flies were assayed after exposure to LD12:12 h regime for 15 days.

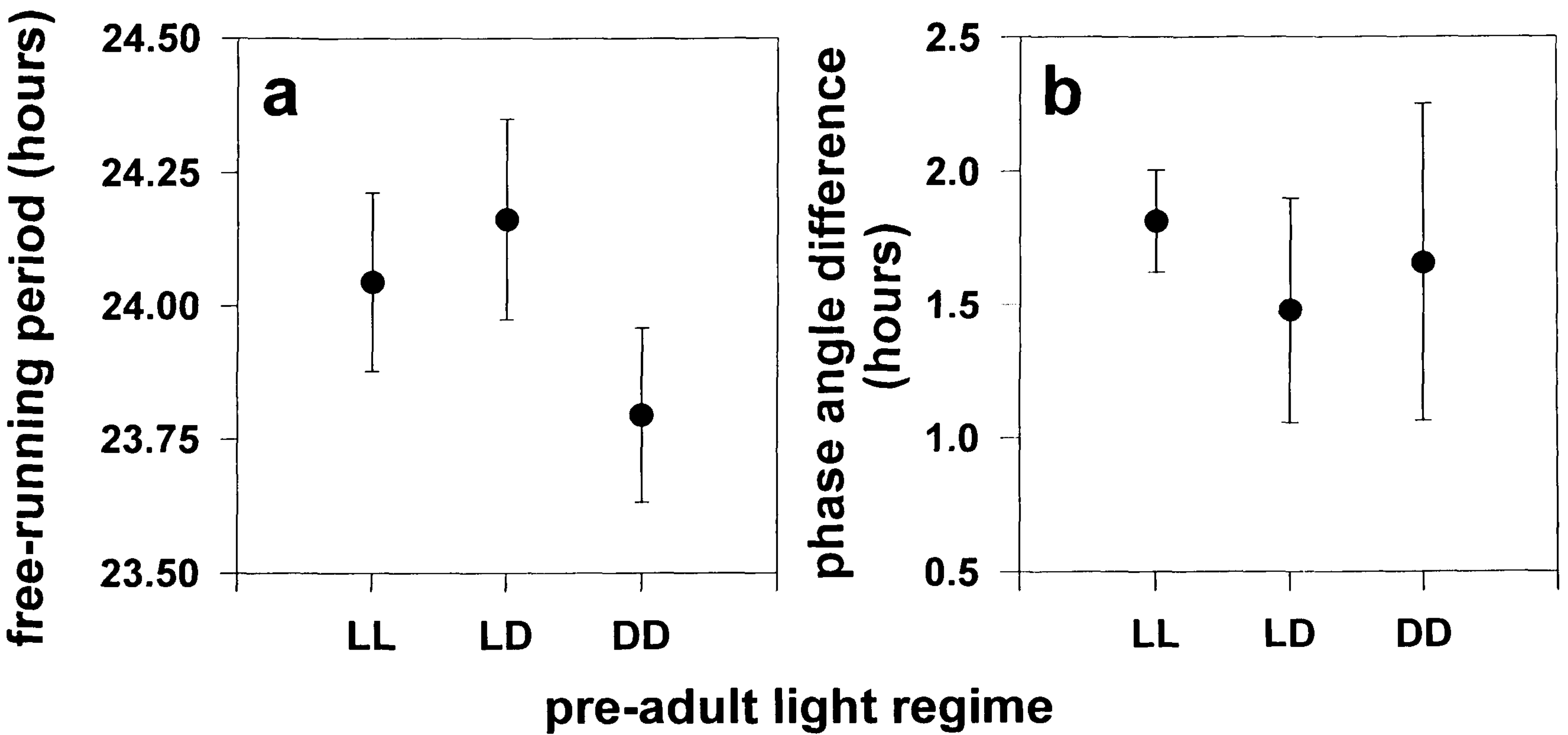

Fig. 2 Circadian rhythm parameters of adult locomotor activity rhythm of flies reared as pre-adults under constant light (LL), light/dark cycle (LD) and constant darkness (DD).

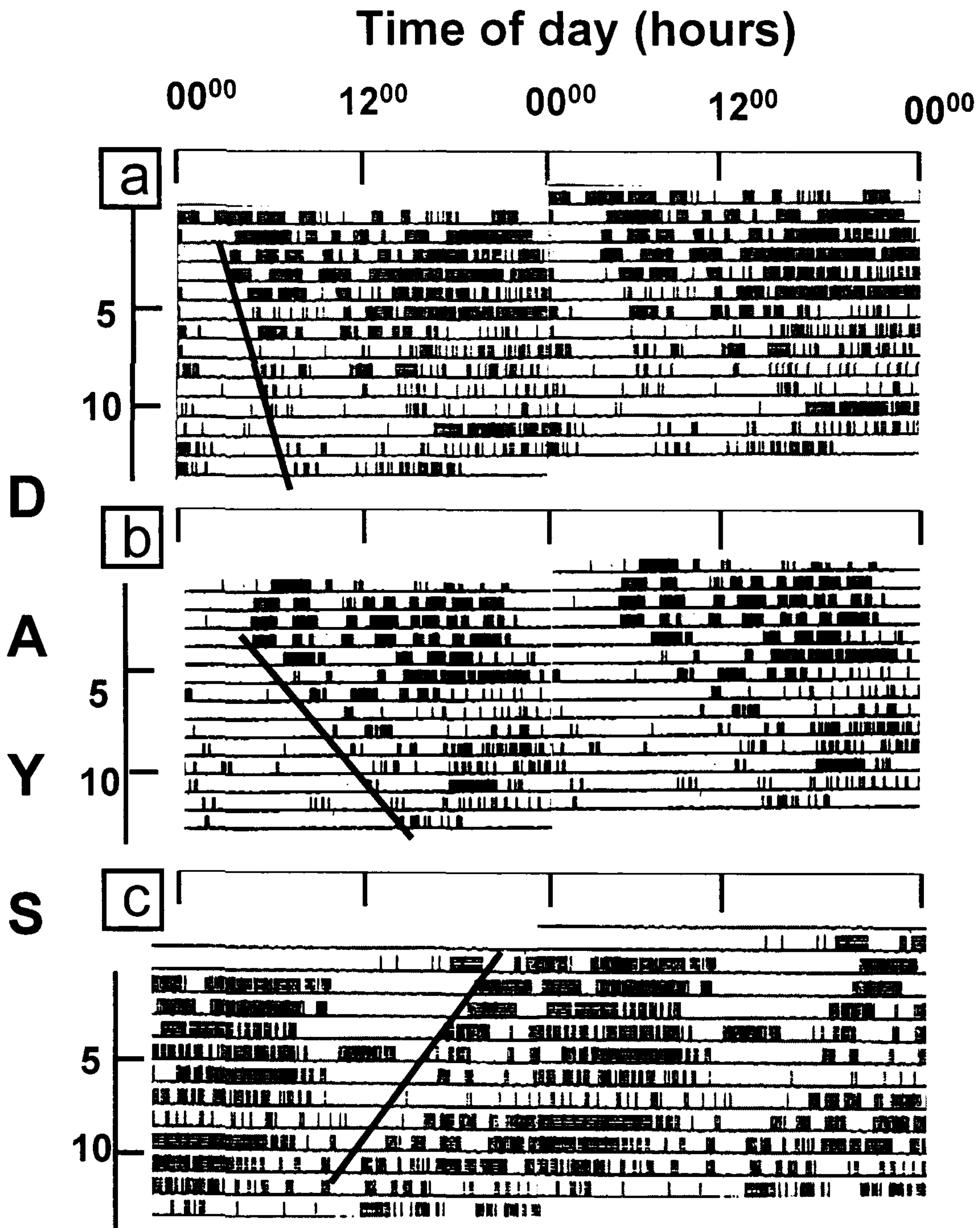

Fig. 3 Representative locomotor activity records of male flies reared as pre-adults in different light regimes assayed in DD regime. (a) reared in LL and exhibits a free running period of 24.33 h. (b) reared in LD 12:12 h and exhibits free running period of 24.78 h. (c) reared in DD and exhibits free running period of 23.24 h. Abscissa shows time of day while ordinate shows number of days. Thick bars indicate activity, while horizontal lines indicate rest.

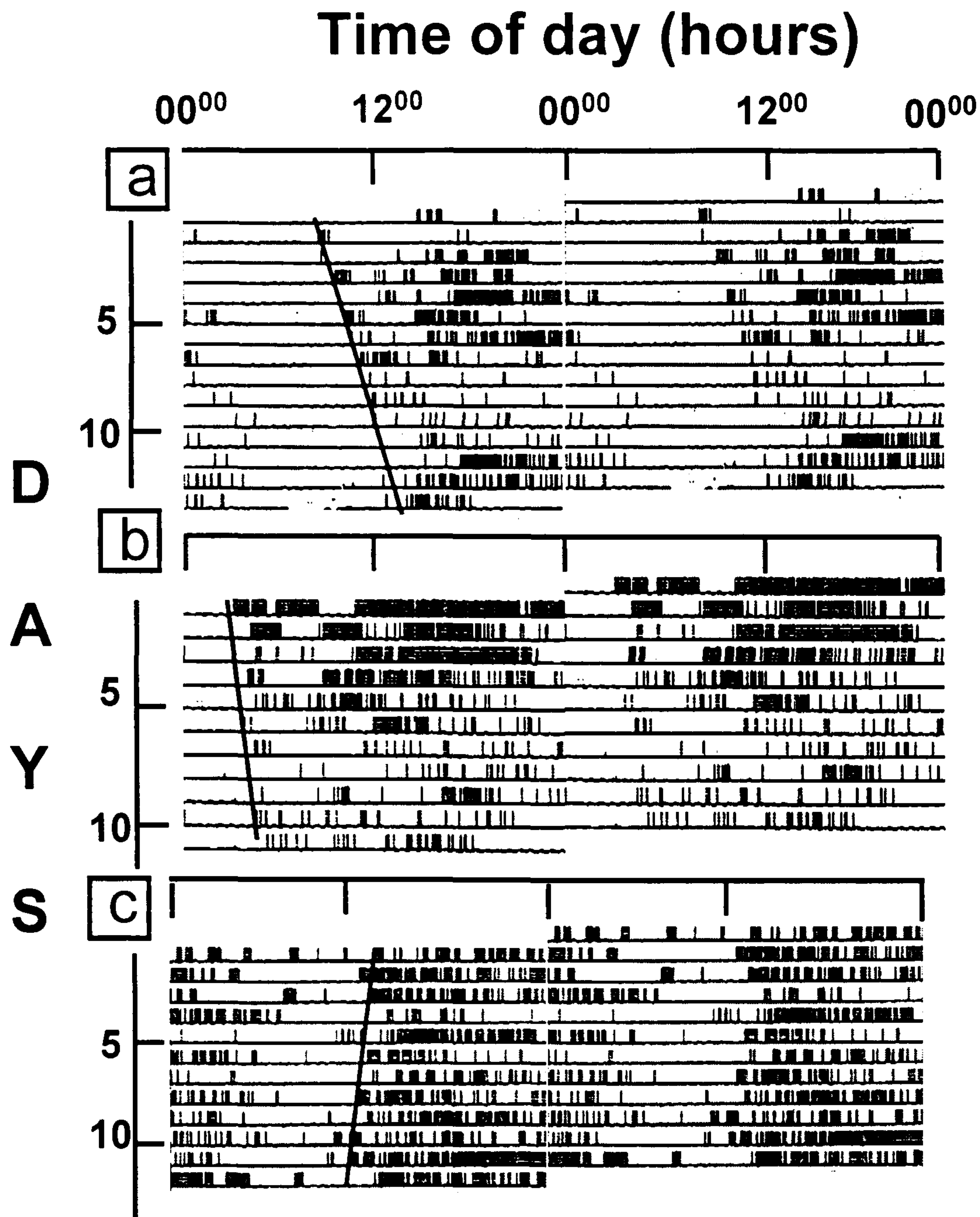

Fig. 4 Representative locomotor activity records of female flies reared as pre-adults in different light regimes assayed in DD regime. (a) reared in LL and exhibits a free running period of 24.22 h. (b) reared in LD 12:12 h and exhibits free running period of 23.71 h. (c) reared in DD and exhibits free running period of 23.6 h. Other details as in Fig. 3.

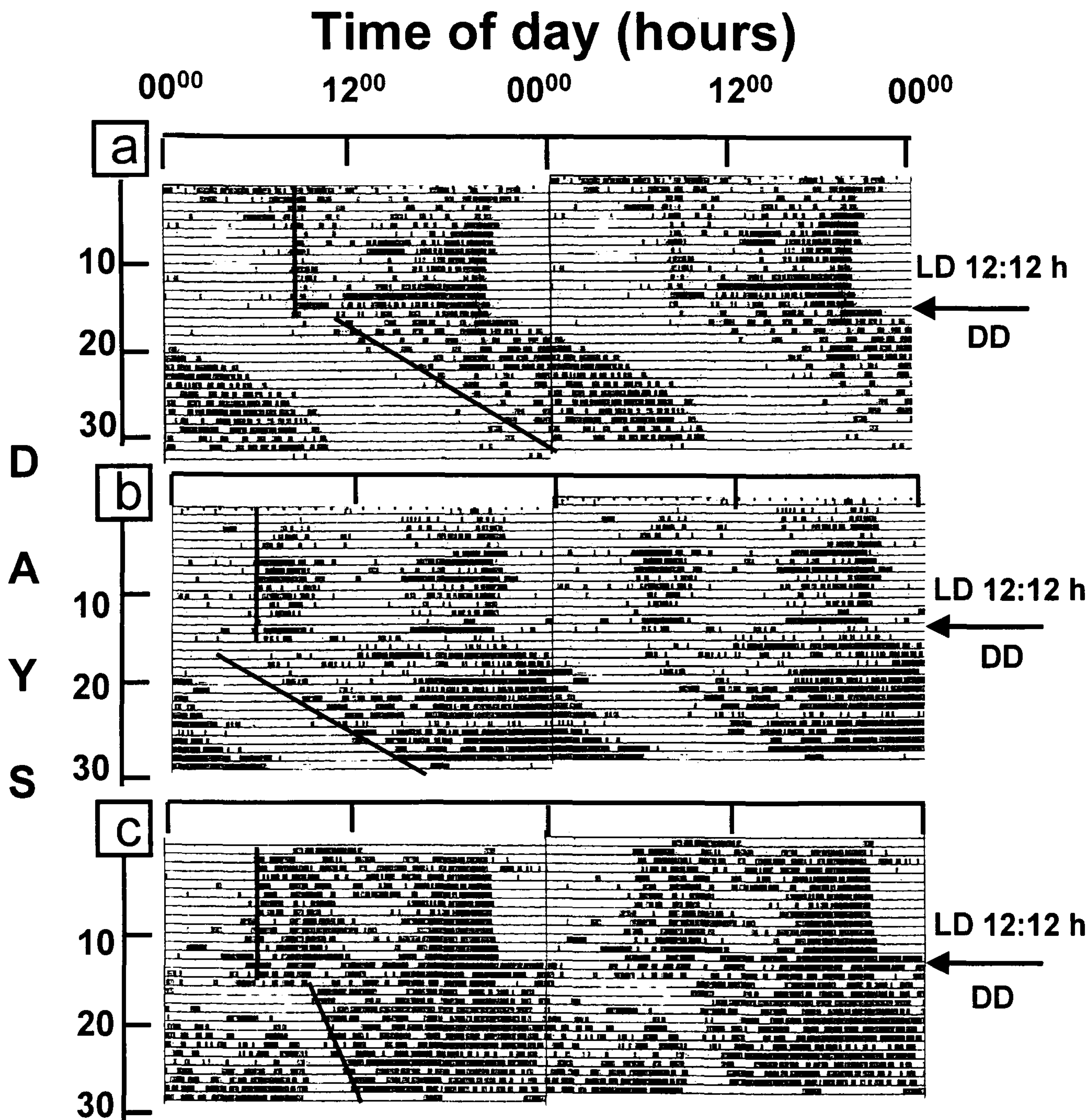

Fig. 5 Representative locomotor activity records of male flies reared as pre-adults in different light regimes assayed for first 15 days in LD regime and next 15 days in DD regime.(a) reared in LL and exhibits a phase angle difference of 0.00 h, free running period of 24.91 h. (b) reared in LD 12:12 h exhibits a phase angle difference of +02.10 h, and exhibits free running period of 24.78 h. (c) reared in DD exhibits a phase angle difference of +02.10 h and free running period of 24.19 h. Other details as in Fig. 3.

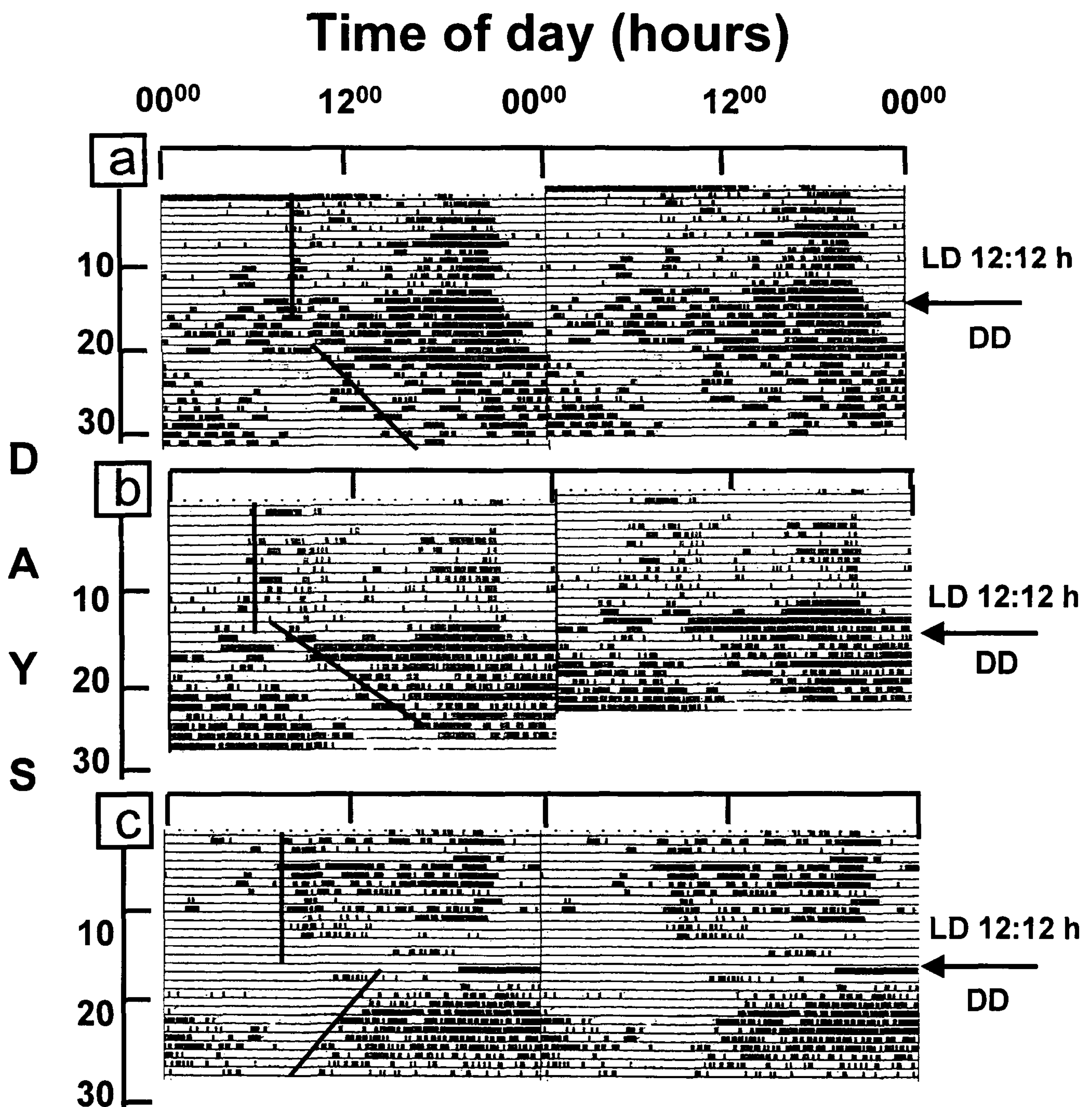

Fig. 6 Representative locomotor activity records of female flies reared as pre-adults in different light regimes assayed for first 15 days in LD regime and next 15 days in DD regime. (a) reared in LL and exhibits a phase angle difference of -0.29 h, free running period of 24.46 h. (b) reared in LD 12:12 h exhibits a phase angle difference of +03.00 h, and exhibits free running period of 24.97 h.(c) reared in DD exhibits a phase angle difference of -0.50 h and free running period of 23.66 h. Other details as in Fig. 3.

Table. 1 Mean free-running period (τ) of the locomotor activity rhythm of adult flies of both sexes, raised as pre-adults in three different light regimes (LL, LD 12: 12 h, and DD) and assayed in constant darkness after eclosion (DD). The confidence intervals (C.I.) are based on the variation among the four replicate populations (LL1, LL2, LL3 and LL4).

Populations of flies	Mean free-running period (τ) in hours of male flies when raised as pre-adults in			Mean free-running period (τ) in hours of female flies when raised as pre-adults in		
	LL	**LD**	**DD**	**LL**	**LD**	**DD**
LL 1	24.336	24.485	23.946	24.577	24.317	23.770
LL 2	24.330	24.489	24.173	24.161	24.219	23.882
LL 3	23.800	23.868	23.450	24.288	24.145	23.834
LL 4	23.712	23.805	23.610	23.594	23.911	23.699
Mean	**24.044**	**24.162**	**23.795**	**24.155**	**24.148**	**23.796**
95 % C.I.	**0.533**	**0.599**	**0.519**	**0.656**	**0.275**	**0.127**

Table. 2 Mean free-running period (τ) of the locomotor activity rhythm of adult flies of both sexes, raised as pre-adults in three different light regimes (LL, LD 12: 12 h, and DD). After eclosion their locomotor activity rhythm was monitored in LD 12:12 h regime for first 15 days of the adult life and then in constant darkness (DD) for another 15 days. The confidence intervals (C.I.) are based on the variation between all the four replicate populations.

Populations of flies	The free-running period (τ) in hours of male flies when raised as pre-adults in			The free-running period (τ) in hours of female flies when raised as pre-adults in		
	LL	**LD**	**DD**	**LL**	**LD**	**DD**
LL 1	24.086	24.319	24.127	24.158	24.35	24.033
LL 2	24.195	24.225	24.018	24.197	24.50	24.083
LL 3	24.457	24.425	24.109	23.760	24.225	24.187
LL 4	24.556	24.379	24.241	24.149	24.450	24.030
mean	**24.3232**	**24.3371**	**24.1238**	**24.0658**	**24.3813**	**24.0833**
95 % C.I.	**0.350**	**0.137**	**0.145**	**0.326**	**0.193**	**0.117**

Table. 3 Results of mixed model ANOVA on the τ of the locomotor activity of individual males and females raised in three light regimes (LL, LD and DD) as pre-adults for the two assays (with and without prior entrainment). Replicate populations were treated as random blocks, and the assay, light regime and sex as fixed factors crossed with block. Since the analysis was performed on population means, the effects of block, and interactions involving block, cannot be tested for significance.

Effect	***df***	**MS**	***F***	***p*-level**
Assay (A)	1	0.492	1.870	0.265
Block (B)	3	0.130	--	--
Light regime (L)	2	0.389	43.104	**<0.0001**
Sex (S)	1	0.008	0.735	0.454
A × B	3	0.2623	--	--
A × L	2	0.046	1.437	0.309
B × L	6	0.009	0.302	--
A × S	1	0.041	--	0.620
B × S	3	0.011	0.306	--
L × S	2	0.008	--	0.747
A × B × L	6	0.032	--	--
A × B × S	3	0.137	5.338	--
A × L × S	2	0.0496	--	**0.047**
B × L × S	6	0.026	--	--
A × B × L × S	6	0.009	--	--

τ_{DD} revealed significant main effects of block (replicate population) and light regime experienced during the pre-adult stages, but not of type of assay (with or without exposure to LD cycles), or sex (Table 3). Post hoc comparisons using Tukey's tests revealed that mean τ_{DD} of the locomotor activity rhythms of the flies raised as pre-adults in DD (23.95 ± 0.035 h; mean ± S.E.) was significantly shorter than that of flies raised as pre-adults in LL (24.15 h ± 0.07 h; mean ± S.E.) which was, in turn, shorter than flies reared as pre-adults in LD (24.26 ± 0.06 h; mean ± S.E.) ($p < 0.05$ for all three comparisons) (Figs. 1, 2 a, 3-6). The marginal significance of the assay × light regime × sex interaction (Table 3) could be driven by the fact that females raised in LL from population LL3 in the second experiment had lower mean τ_{DD} as compared to those raised in DD regime (Table 2). The ANOVA on ψ did not reveal any significant main effect of pre-adult light regime ($p = 0.88$) (Fig. 2 b).

5.4 Discussion

Our finding that τ_{DD} of the adult locomotor activity of flies raised as pre-adults in LD 12:12 h is significantly greater than that of flies raised in DD (Figs. 1-6) is in concordance with the observations of Tomioka et al (1997) for *per*^{S} and *per*$^{+}$ homozygotes. Konopka et al (1989) reported that the free-running period of *per*^{S} homozygotes increased by about 0.6 h, whereas the period of *per*^{L1} homozygotes decreased by about 1 h after they were transferred from DD to LL. The reverse was reported to occur when these flies are subjected to LL to DD transfer. Unfortunately, only the effects of LL and DD regimes during early adult stages were examined in the study by Konopka et al (1989), ruling out a broader comparison across studies using *D. melanogaster*. Tomioka et al (1997) reported that, following rearing in LL, τ_{DD} was decreased in *per*$^{+}$, *per*^{S} and *per*^{L1} homozygotes, relative to τ_{DD} in control flies that were reared in DD

throughout the pre-adult and adult stages. However, for the flies reared in LD 12:12 h, LD 8:16 h and LD 4:20 h, the responses of subsequently measured τ_{DD} to the light regime experienced in per^{S} and per^{L1} homozygotes were opposite. The main problem in generalising the observations made in both these experiments and that of ours is that besides differences in the experimental protocols, the flies used in our studies are different from those used by Konopka et al (1989) and Tomioka et al (1997). The difference shorter τ_{DD} of the locomotor activity rhythm of the flies raised in DD as compared to those raised in LL observed in our experiments, is also in agreement with observations of Barrett and Page (1989) on cockroaches. In addition the results of our experiments further suggests that that both males and females are influenced by the pre-adult light regimes in a similar manner as no statistically significant interaction between sex and light regime was observed (Table 3).

The lack of a significant main effect of assay or interaction between assay (with or without exposure to LD 12:12 h) and the light regime experienced during pre-adult stages (LL, LD 12:12 h and DD) (Table 3) suggests that the pattern of larval light regime effects on the value of τ_{DD} of the locomotor activity rhythm is the same even after flies have been subjected to LD cycles during early adult stage. However, the experiment confounds any possible age-specific effects on τ_{DD} with assay, because in the experiment in which flies were not exposed to LD cycles during early adult stages τ_{DD} was assayed for the first 15 days of adult life, while in the other experiment τ_{DD} was assayed starting from the 16th day of adult life. We also observed that the phase relationship between the onset of locomotor activity and the lights on in the LD cycle were not significantly different among the flies reared as pre-adults in LL, LD 12:12 h and DD (Figs 2 b, 5, 6). This suggests that although manipulating the developmental light conditions can modify the τ_{DD} of the circadian clocks, the phase-angle difference, which may play a key role in

an organism's survival in field conditions, remains unchanged. Further experiments in which the phase response curves to light stimuli are plotted may reveal whether the pre-adult light regime influences the sensitivity of the circadian organisation that controls locomotor activity rhythm in these flies.

Most laboratory strains used for genetic analyses are maintained at rather small population sizes (often for many years) and, consequently, are likely to be highly inbred. One problem with generalizing from the results of any phenotypic manipulation of inbred populations is that one is never sure to what extent fortuitous fixation of particular alleles at some locus in that particular strain contributes to any observed response. Similarly it is also not clear to what extent responses to phenotypic manipulations observed in inbred lines are representative of the kinds of response one is likely to see in larger outbred populations, especially if the species is a normally outbreeding one (Rose et al., 1996; Mueller, 1997; Reznick and Ghalambor, 1999; Harshman and Hoffmann, 2000; Mueller and Joshi, 2000), a problem that is relatively unappreciated in the literature on chronobiology (Sheeba et al., 2000). Given, moreover, that *Drosophila* is a commonly used model organism in chronobiology, it is of obvious interest to have some knowledge of the effects that pre-adult rearing conditions have on "wild type" individuals from large outbred populations with a large amount of standing genetic variation. Unlike experiments of Konopka et al (1989) and Tomioka et al (1997), which were done on laboratory populations of wild type and *per* mutant Canton S strains of *D. melanogaster,* which are likely to have been highly inbred, our studies were carried out using four independent outbred populations. These four populations share a common ancestry but have been maintained as separate populations without any gene flow among them for over 600 generations. The fact that all four populations show a similar trend in the pattern of larval light regime effects on the

τ_{DD} of locomotor activity rhythm further strengthens the argument that the observed pattern of developmental modifications in the circadian phenotype is not merely because of fortuitous fixation of alleles at certain loci that influence the overt circadian rhythm.

The *Drosophila* circadian pacemaker is believed to be composed of at least two interlocked feedback loops: one consisting of the cycling of mRNA production of a gene ***dclk*** and another that involves the cycling of the mRNA of two other genes, *per* and *tim*. Under LD cycles, the transcripts of *per*, *tim* and *dclk*, and their protein products PER, TIM and dCLK, show robust oscillation within the cells of *D. melanogaster* (Sehgal et al., 1994; Bae et al., 1998; Darlington et al., 1998; Dunlap, 1999). Entrainment to LD cycles is achieved by light-dependent degradation of the protein TIM. The level of TIM falls rapidly at 'lights-on', thus preventing the formation of PER-TIM heterodimers required for the negative feedback loop to function. Instead, TIM gets phosphorylated by another protein DBT thus causing a fall in the level of PER-TIM heterodimers. This allows another heterodimer dCLK-CYC to bind to the E-box of the promoter region of *per* and *tim* genes and transcription is initiated once again. Thus light is involved in resetting the phase of the circadian oscillator (Zeng et al., 1996).

In LL, the levels of PER abundance and phosphorylation are suppressed and low levels of PER are constitutively expressed (Price et al., 1995). It has also been observed that a minimum length of 6-8 hours of darkness is required for the feedback loop to function (Qui and Hardin, 1996). Hence, in the *Drosophila* circadian clock the lengthening of τ after LL can be due to degradation of TIM by light, which would result in longer time being required to build up sufficient amount of PER-TIM heterodimer. In DD, in the absence of light induced degradation of TIM, the PER-TIM heterodimer formation and its subsequent nuclear entry occurs at shorter intervals than that in LD or LL regimes. Thus, based on current understanding of the *Drosophila*

circadian oscillator, we suggest that lengthening or shortening of the overt rhythm assayed after pre-adult exposure to different light regimes may be a reflection of changes in the molecular mechanisms underlying overt circadian rhythms.

The overall effects of light regime on the τ_{DD} of locomotor activity rhythm in *Drosophila* may be mediated by interactions between the light regimes experienced during the pre-adult and early adult stages. If so, the fact that the pre-adult light regimes in the study by Konopka et al (1989) was LD 12:12 h, 25° C and LL, 22 ° C and that the study by Tomioka et al (1997) confounds light regime effects at different life stages, may together perhaps provide a resolution of the discrepancy between these two studies. In both our experiments the flies were reared in three different light regimes (LL, LD 12:12 h and DD) only during the pre-adult stage and, therefore, the modification in the free-running period can be ascribed to the influence of light regimes experienced during only the pre-adult stages. However, factorial experiments with different combinations of pre-adult and early adult light regimes may yield a better understanding of any interactions between light regimes experienced at different life stages.

Chapter 6

Multi-oscillatory Circadian Organisation Of Eclosion, Locomotor Activity And Oviposition Rhythms In *D. melanogaster*

6.1 Introduction

Circadian rhythms can be observed at various levels of biological organisation and complexity, ranging from mRNA concentrations within a cell, to the patterns of eclosion in populations of fruit flies (Scully and Kay, 2000). Moreover, in several organisms, there is evidence for multiple oscillators controlling different circadian rhythms. In the marine dinoflagellate *Gonyaulax polyedra*, which exhibits circadian rhythms in photosynthesis, cell aggregation, superoxide dismutase production, phototaxis, bioluminescence and cell division, two oscillatory sub-systems sensitive to different wavelengths of light, one controlling aggregation and the other controlling bioluminescence, have been demonstrated (Roenneberg, 1994, 1996). The locomotor activity of the arctic ground squirrel *Spermophilus undulatus* showed a break up of the activity band into two components, each of which free-ran with different periods when kept in constant light for several cycles (Pittendrigh, 1960), and a similar phenomenon was observed in hamsters kept in constant light for several days (Pittendrigh, 1974). In pinealectomised birds, several separate oscillators in an individual could be independently entrained to light/dark cycles (Takahashi and Menaker, 1982).

In many insects, the activity-rest, feeding, and mating cycles have been shown to exhibit circadian periodicity (Saunders, 1982). In addition to these individual level rhythms, insects circadian rhythmicity at the level of the population has also been observed in phenomena like eclosion, pupation and egg laying (Pittendrigh, 1966; Rensing and Hardeland, 1967; Gillett, 1972; Saunders, 1982). In the flesh fly *Sarcophaga argyrostoma*, at least three distinct oscillators are believed to regulate the initiation of larval wandering, diapause induction, and pupal eclosion rhythms, respectively (Saunders, 1986). Adult locomotor activity, the deposition of cuticular growth layers on thoracic apodemes, and the duration of larval wandering prior to pupariation

are also likely to be controlled by distinct oscillators (Saunders, 1986). The view emerging from these studies is that different cellular functions, or groups of functions, are likely to be controlled by separate circadian oscillators, and that these oscillators are coupled such that they influence each other, making up a multi-oscillator system (Roenneberg, 1996; Scully and Kay, 2000; Zordan et al., 2000).

In *D. melanogaster*, circadian rhythmicity has been reported at various hierarchical levels of biological organisation. A number of genes like *per*, *tim*, *clock*, and *cry*, exhibit oscillations in the concentration of mRNA and/or their protein products (Zordan et al., 2000). In individual flies, locomotor activity and oviposition have been observed to be rhythmic (Helfrich, 1985; Sheeba et al., 2001 a), and at the level of the population, eclosion is also known to follow a circadian rhythm (Pittendrigh and Skopik, 1970; Sheeba et al., 1999 a). The best studied circadian rhythms in *Drosophila* are those of eclosion and locomotor activity (Helfrich-Förster, 1996). Although endogenous control of oviposition has been demonstrated in a number of insects (Minis, 1965; Skopik and Takeda, 1980; Numata and Matsui, 1988), there was no clear evidence for endogenous origin of this rhythm in *D. melanogaster* until very recently (McCabe and Birley, 1998; Sheeba et al., 2001 b). In *D. melanogaster*, oviposition is believed to be an outcome of at least two physiological processes: vitellogenesis and egg retention. Vitellogenesis and egg retention are periodic in light/dark (LD) 12:12 h, with maximal egg retention just before the peak of oviposition, and the rhythm in vitellogenesis is claimed to persist in constant darkness (DD) (Allemand, 1976 a). It has been assumed that vitellogenesis, which is controlled by a circadian oscillator, could play a major role in the expression of free-running oviposition rhythms under constant conditions (King et al., 1956; Allemand, 1976 a, b; Allemand and Boulétreau-Merle, 1989). Most overt rhythms studied in *D. melanogaster* are accompanied by *per* mRNA cycling in

tissues like the gut, malphigian tubules, male reproductive system and corpora cardiaca (Siwicki et al., 1988; Zerr et al., 1990; Frisch et al., 1994; Hardin, 1994; Konopka et al., 1994, Plautz et al., 1997), except in the case of ovaries where *per* mRNA cycling has not been observed (Hardin, 1994) even though oviposition follows a clear circadian rhythm.

In a detailed study using *D. pseudoobscura,* the free-running period (τ), and the phase response curves (PRCs) of the eclosion and locomotor activity rhythms were found to be different, suggesting that separate circadian pacemakers control these two rhythms in that species (Engelmann and Mack, 1978). To the best of our knowledge, such detailed studies have not been carried out on *D. melanogaster*, although a difference in τ of eclosion and locomotor activity rhythms has been reported (Helfrich, 1985). Interestingly, the oviposition rhythm in *Drosophila* has not been used to investigate the possible role of multiple oscillators in controlling circadian rhythms, even though the lack of cycling of *per* mRNA level in the ovary of *D. melanogaster* (Hardin, 1994) suggests that oviposition may be under the control of a circadian oscillator other than *per*. It would therefore be interesting to investigate whether oviposition is controlled by oscillator(s) independent of those that control locomotor activity and eclosion rhythms in *D. melanogaster*.

In this chapter, I describe the results of two sets of experiments which were aimed at (a) examining the multiple oscillatory control of three overt rhythms of *D. melanogaster,* and (b) investigating whether differences in phasing of one overt rhythm (eclosion rhythm) are reflected in the clock parameters of another (oviposition rhythm).

6.2 Examining the multiple oscillatory control of three overt rhythms of *D. melanogaster*

We chose one population level rhythm (eclosion), and two individual level rhythms (locomotor activity and oviposition), the underlying molecular mechanisms of which have been fairly well studied (Helfrich-Förster, 1996; Scully and Kay, 2000). Although eclosion and locomotor activity rhythms have been investigated in some detail in the context of multioscillatory control of circadian rhythms in *Drosophila*, our main goal was to compare the circadian parameters of these two rhythms with those of the relatively less studied oviposition rhythm. In this section, I present the results of experiments on one of our laboratory populations (LD1, described in detail in chapter 1) which has been reared in LD cycles for ~35 generations. The eclosion, oviposition and locomotor activity rhythms in these flies were assayed in DD and in LD 12:12 h, and the τ and ψ of these rhythms were recorded and compared with one another.

6.2 a Materials and methods

The experimental flies were sampled from a large ($N \sim 1600$ breeding adults), outbred, laboratory population of *D. melanogaster* that has been reared in LD 12:12 h for ~ 35 generations (population LD1, described in chapter 1). Eclosion rhythm was assayed by collecting eggs at high densities (~300 eggs per vial) into vials (9 cm height × 2.4 cm diameter) containing ~6 ml of food medium. Twelve such vials each were introduced into both DD and LD 12:12 h regimes. When adults began to eclose, the vials were monitored every 2 h and any eclosing adults were collected, and their number and sex recorded. This process was continued for 10 consecutive days, or until most of the pupae had eclosed (Fig. 1). Oviposition rhythm of females that emerged during the peak of eclosion was assayed for 10 consecutive days in LD 12:12 h and

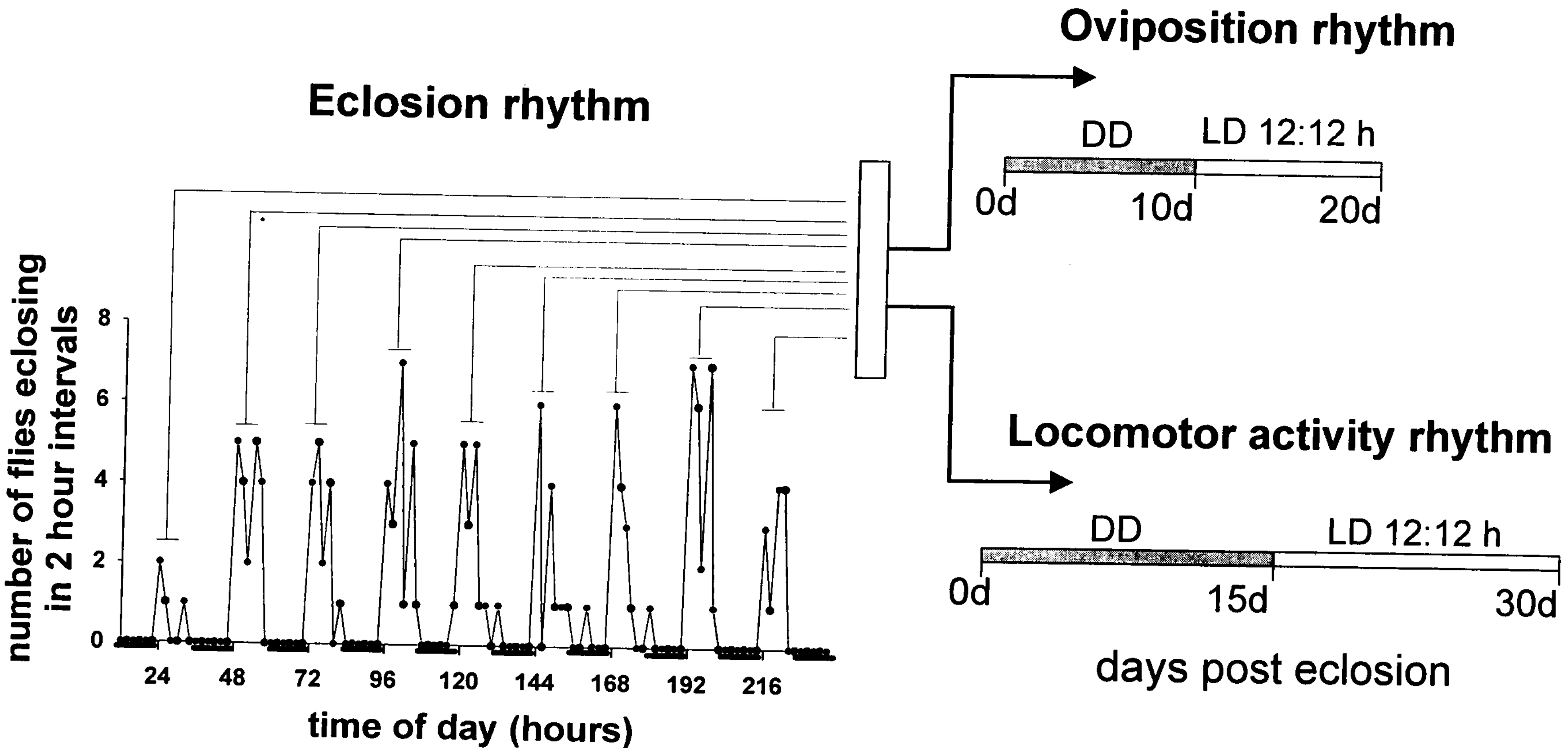

Fig. 1 Schematic representation of the experimental protocol followed to assay the rhythms in eclosion, oviposition and locomotor activity . Flies that emerged within the peak of eclosion rhythm were used to assay the circadian parameters τ and ψ of oviposition and locomotor activity rhythms under DD and LD 12:12 h regimes respectively. The horizontal dark bands denote the dark phase of the LD cycle.

DD regimes as described in chapter 2. Twenty-four male-female pairs were assayed per light regime. Locomotor activity rhythm was assayed in LD 12:12 h regime for 15 days and then in DD regime for 15 days using individual virgin male and female flies (n = 25) that eclosed during the peak of eclosion. The flies were transferred to the locomotor activity-monitoring setup within 24 – 48 h after eclosion. The protocol for assaying locomotor activity was as described earlier in chapter 2.

The τ of locomotor activity rhythm was estimated by fitting a least squares linear regression to the onset of activity for at least 6 consecutive days. The τ of eclosion and oviposition rhythms were calculated by subjecting the time series data obtained from each vial in the eclosion rhythm assay, and each female in the oviposition rhythm assay, to Fourier spectral analysis using STATISTICA ™ (Statsoft Inc, 1995). Statistical significance of observed peaks in the periodogram was tested using the technique of Siegel (1980). The ψ of eclosion rhythm in LD 12:12 h was estimated as the average time interval, over 10 consecutive days, between peak eclosion and 'lights-on'. The ψ of oviposition rhythm in LD 12:12 h was estimated as the average time interval, over 10 consecutive days, between peak oviposition and 'lights-on', while the ψ of locomotor activity rhythm in LD 12:12 h, was estimated by averaging the time interval between onset of activity and 'lights-on' over 10 consecutive days. Separate one-way Analyses of Variance (ANOVA) were carried out on the ψ values obtained in order to test for any significant effect of the type of rhythm (eclosion, locomotor activity and oviposition) on ψ. The mean τ of eclosion, locomotor activity and oviposition rhythms were compared using Students t-test, because there was no variation for τ of eclosion rhythm among replicate vials, ruling out the use of ANOVA for the comparison of the three rhythms. All statistical analyses were done using STATISTICA ™ (Statsoft Inc, 1995)

6.2 b Results

The τ of eclosion, locomotor activity and oviposition rhythm assayed in DD regime were significantly different from one another ($p < 0.03$ for all three comparisons; Students t-test) (Fig. 2 a, 3, 4 a-c, 5), with the τ of eclosion rhythm being the least, and that of the oviposition rhythm being the greatest. The τ of the eclosion rhythm in DD was observed to be identical for all the vials used in the assay. All the three rhythms (eclosion, locomotor activity and oviposition) were found to entrain to LD 12:12 h (Fig. 4 a-c, 6, 7) with a period not significantly different from 24 h. The mean ψ of eclosion rhythm was found to be -4.43 ± 0.49 h (Fig. 8) (mean ± 95% C.I.), while that of oviposition rhythm was -10.495 ± 2.03 h (Fig. 8) (mean ± 95% C.I.) and of locomotor activity was $+3.64 \pm 1.28$ h (mean ± 95% C.I.) (Fig. 4 a-c). One way ANOVA on the ψ of all three rhythms revealed a significant effect of type of rhythm ($F_{2,37} = 123.91, p < 0.001$), and multiple comparisons revealed that ψ of all three rhythms significantly differed from one another ($p < 0.001$ for all three comparisons; Scheffe's test) (Fig. 2 b, 8). While the locomotor activity rhythm phase leads 'lights-on' of the LD 12:12 h cycle, the eclosion rhythm phase lags 'lights-on', whereas the oviposition rhythm is almost 180° out of phase compared to eclosion and locomotor activity rhythms (Fig. 8).

6.2 c Discussion

Mutations in several rhythm related genes of *D. melanogaster*, like *per*, *tim*, and *disco*, affect the rhythms in locomotor activity and eclosion in a similar manner (Konopka and Benzer, 1971; Dushay et al., 1989). Moreover, the τ of oviposition and locomotor activity rhythms of *per* mutants and wild type *D. melanogaster* have been found to be significantly positively correlated, suggesting that the *per* gene influences the circadian rhythmicity of both activity and oviposition

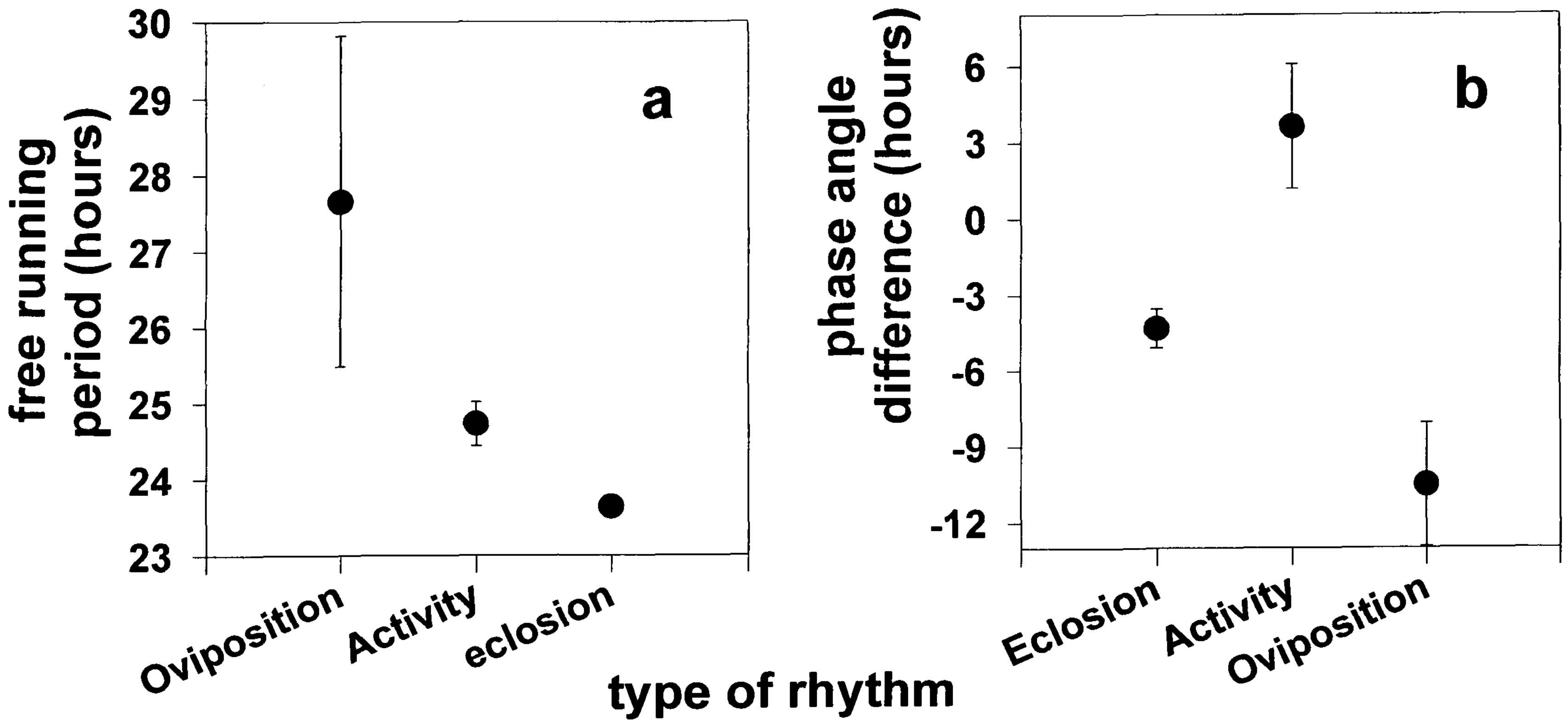

Fig. 2 (a) Mean free running periods in DD of eclosion, locomotor activity and oviposition rhythms. (b) Mean phase angle difference of eclosion, locomotor activity and oviposition rhythms under LD 12:12 h regime. Error bars represent 95% confidence intervals and can be used for visual hypothesis testing.

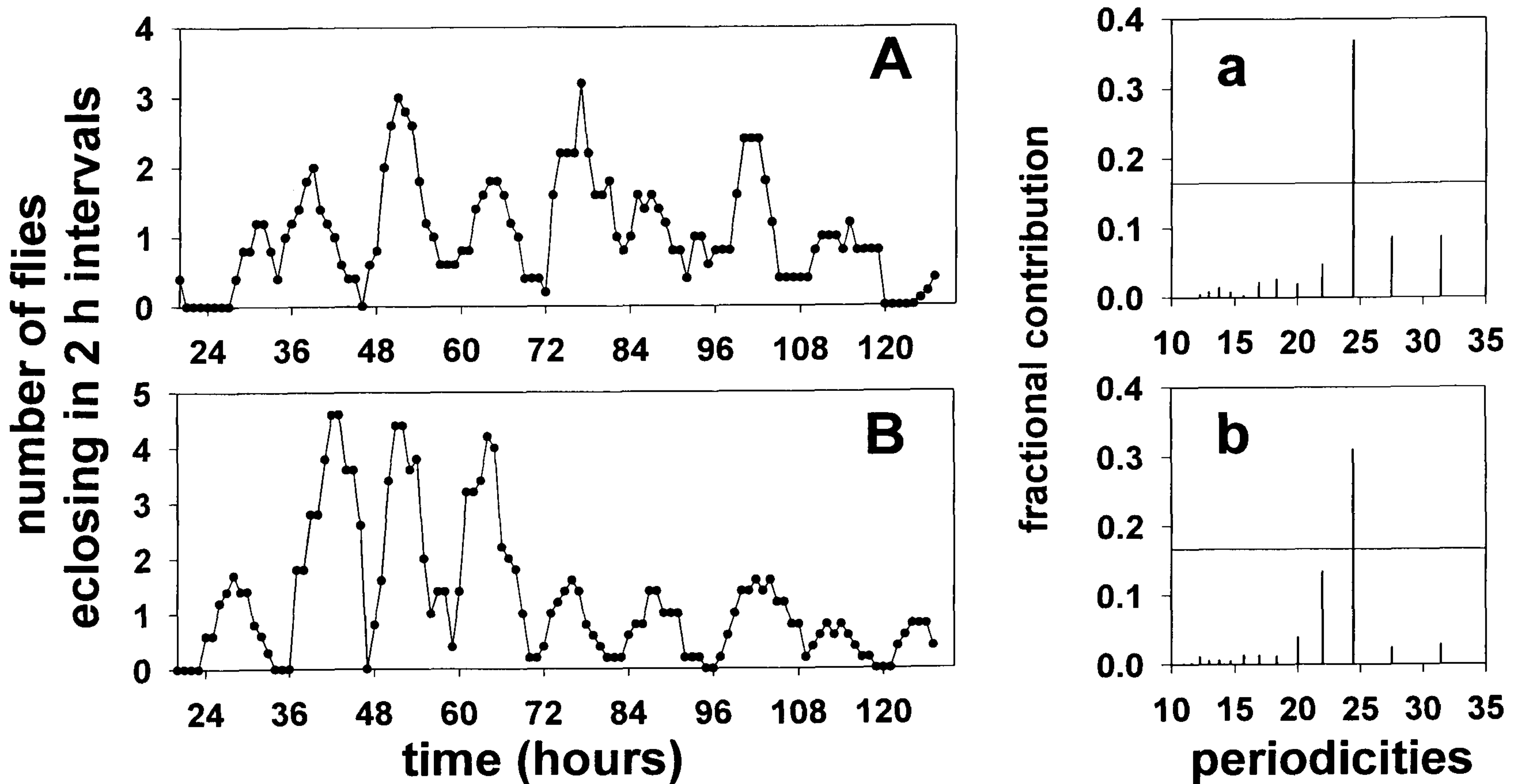

Fig. 3 Time series data of number of number of eclosing flies in two representative vials in continuous darkness (A, B). The corresponding periodograms show a significant contribution of 23.6 h periodicity (a, b).

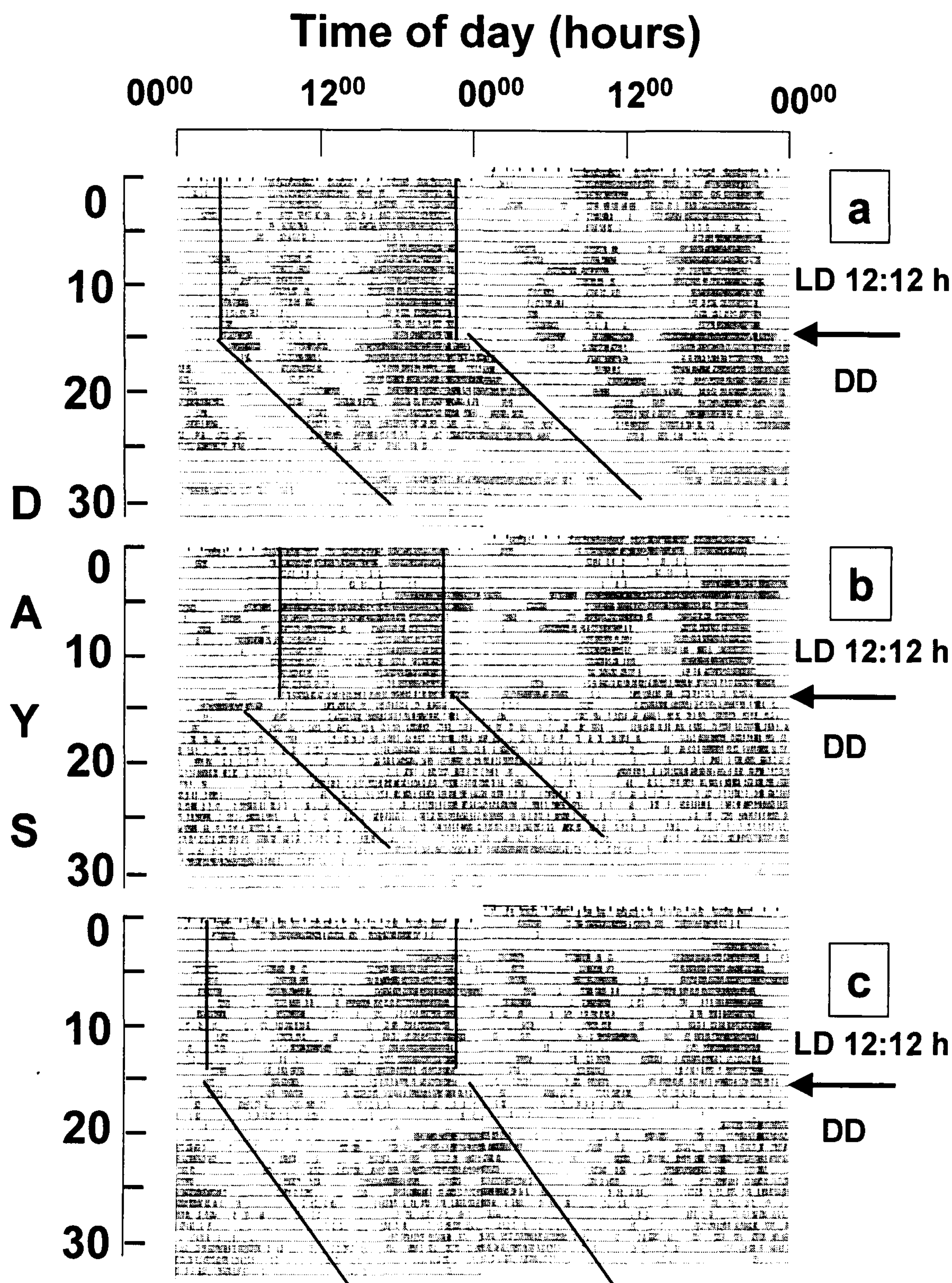

Fig. 4 Locomotor activity records of three representative individual flies kept for the first 15 days in LD regime, followed by DD regime for the next 15 days. Lights were switched on at 08.00 h and switched off at 20.00 h in the LD regime. Phase angle difference, measured as time interval between onset of activity and 'lights on' was (a) +4.77 h (b) +0.077 h and (c) +5.88 h. Free running period was (a) 24.59, (b) 24.66 and (c) 24.25 h.

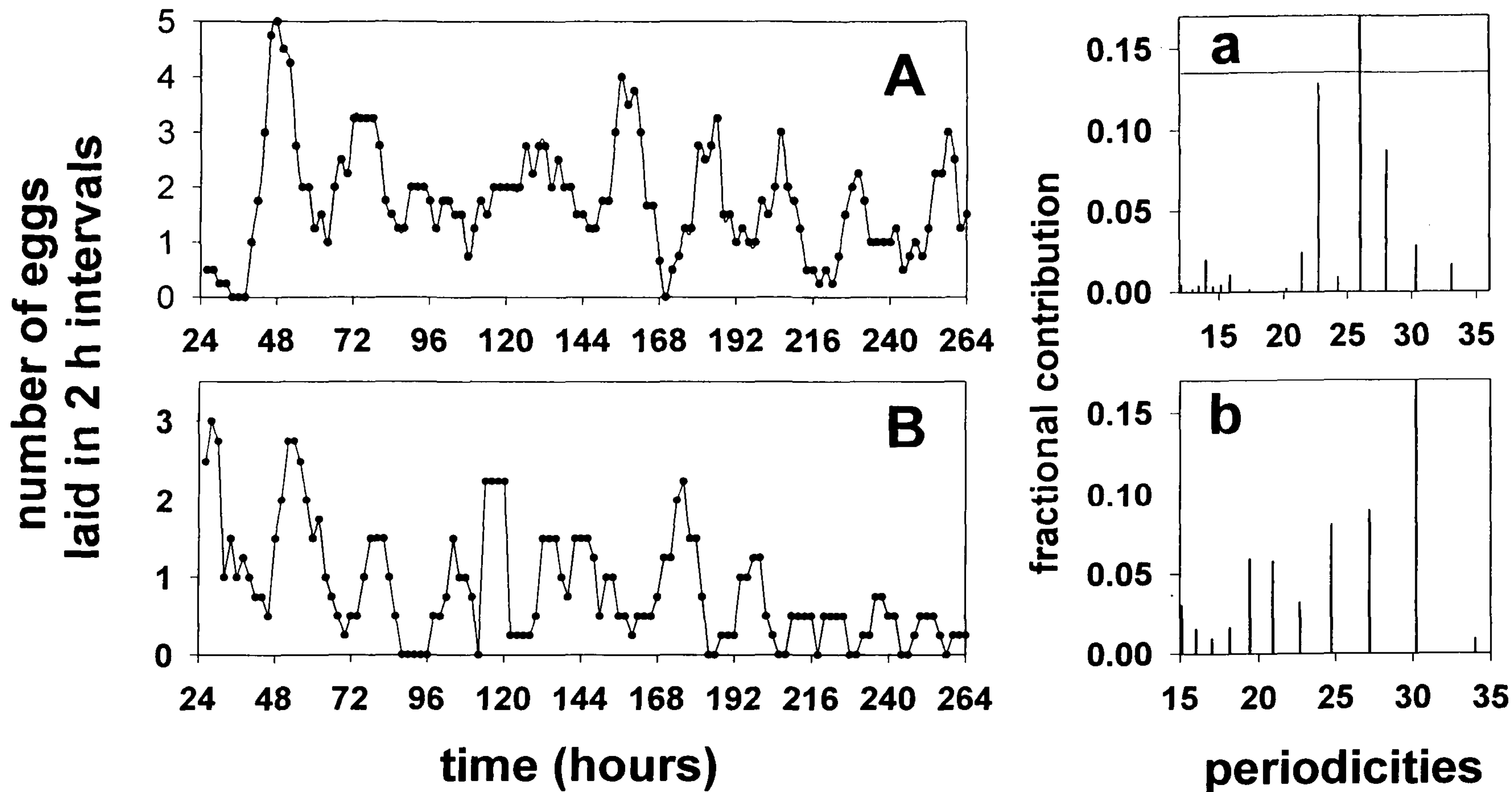

Fig. 5 Time series data of number of eggs laid by two representative females in continuous darkness (A, B). The corresponding periodograms show a significant contribution of 26 h and 30.22 h periodicity (a, b).

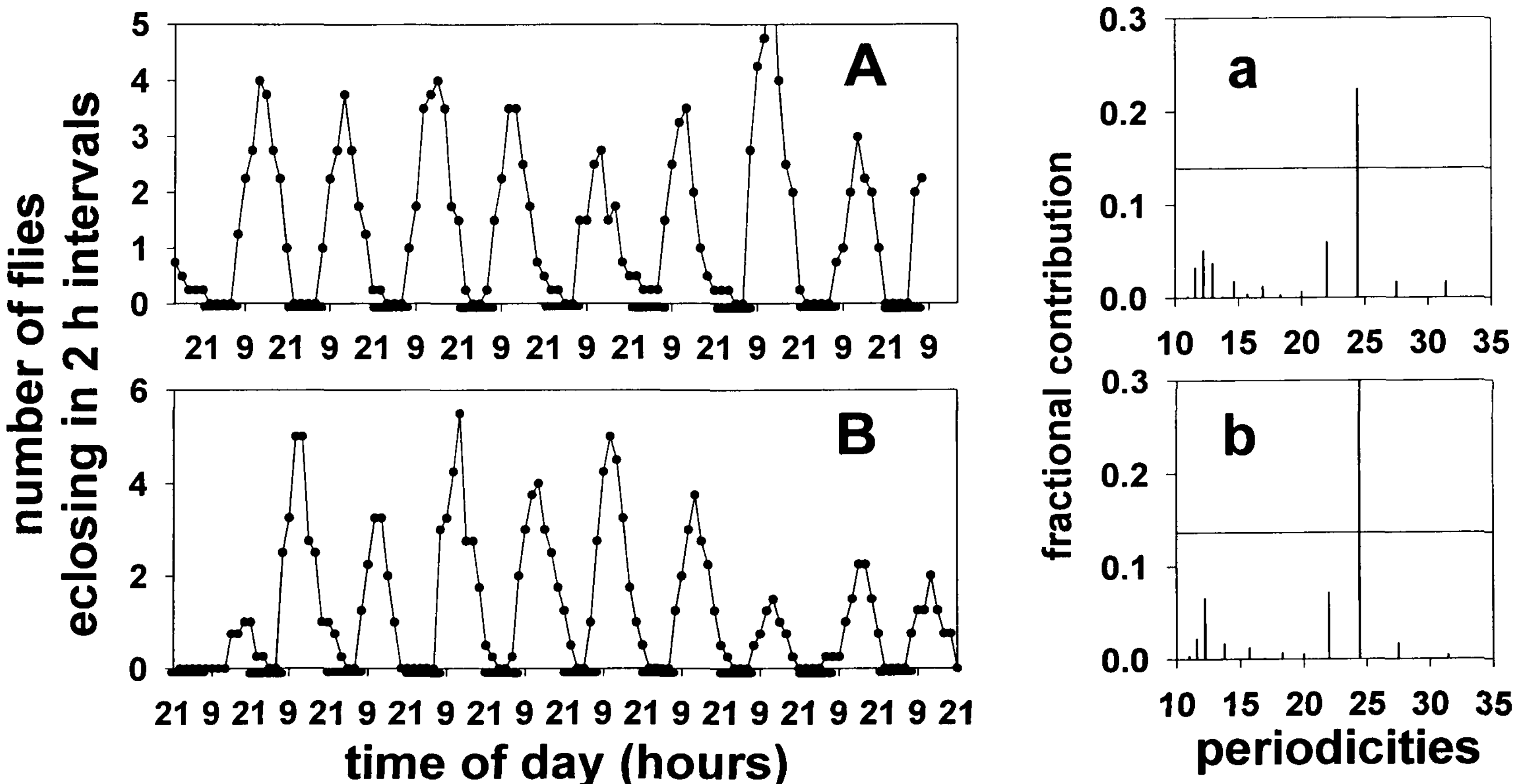

Fig. 6 Time series data of eclosion rhythm of two representative vials in LD regime (A, B). The corresponding periodograms show that eclosion in the two vials occured with 24 h periodicity (a, b). Lights were switched on at 08.00 h and switched off at 20.00 h (dark bands represent the dark phase of the LD cycle).

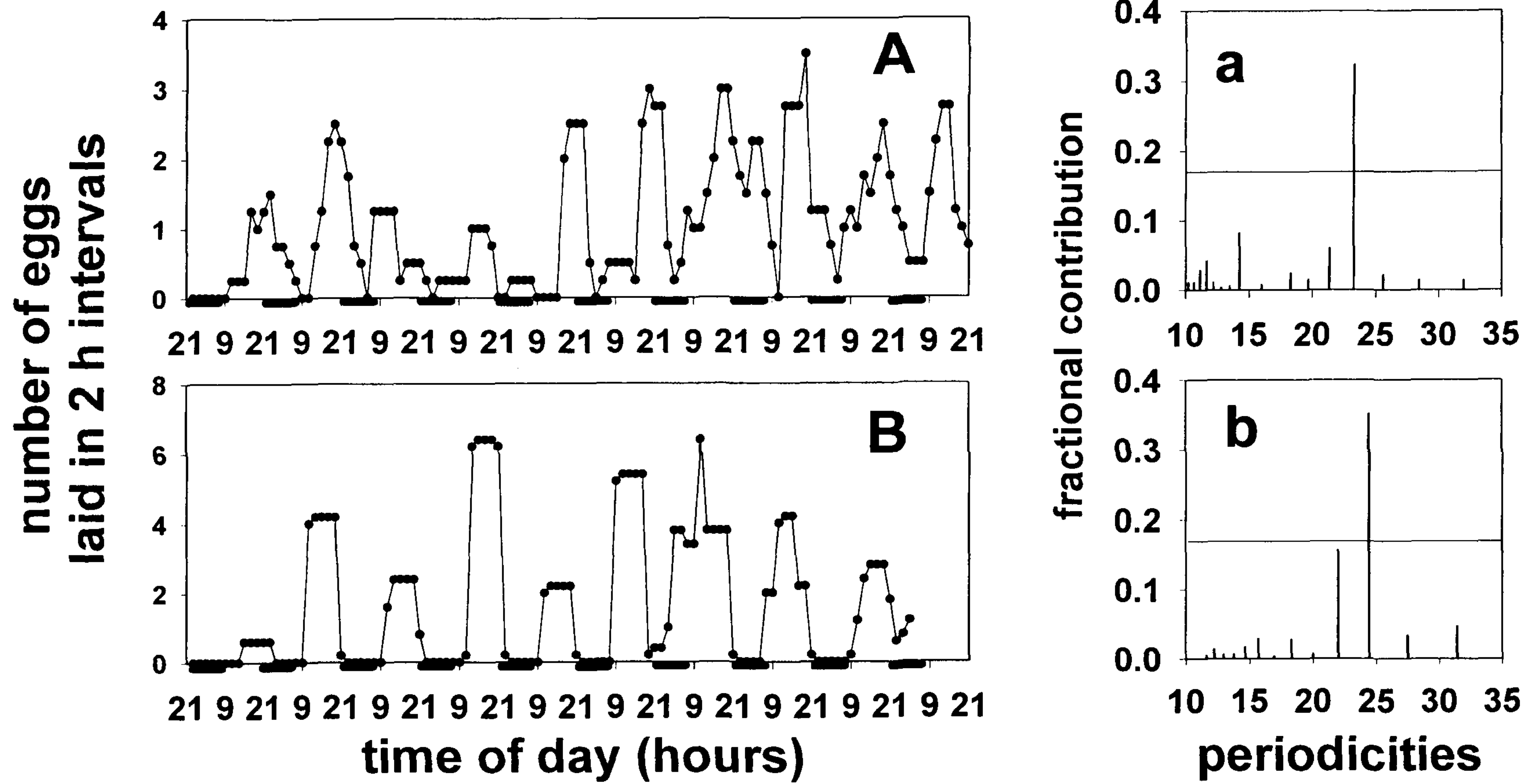

Fig. 7 Time series data of oviposition rhythm of two representative females in LD regime (A, B).The corresponding periodograms show that oviposition of the two flies occured with period close to 24 h (a, b). Lights were switched on at 08.00 h and switched off at 20.00 h (dark bands represent the dark phase of the LD cycle).

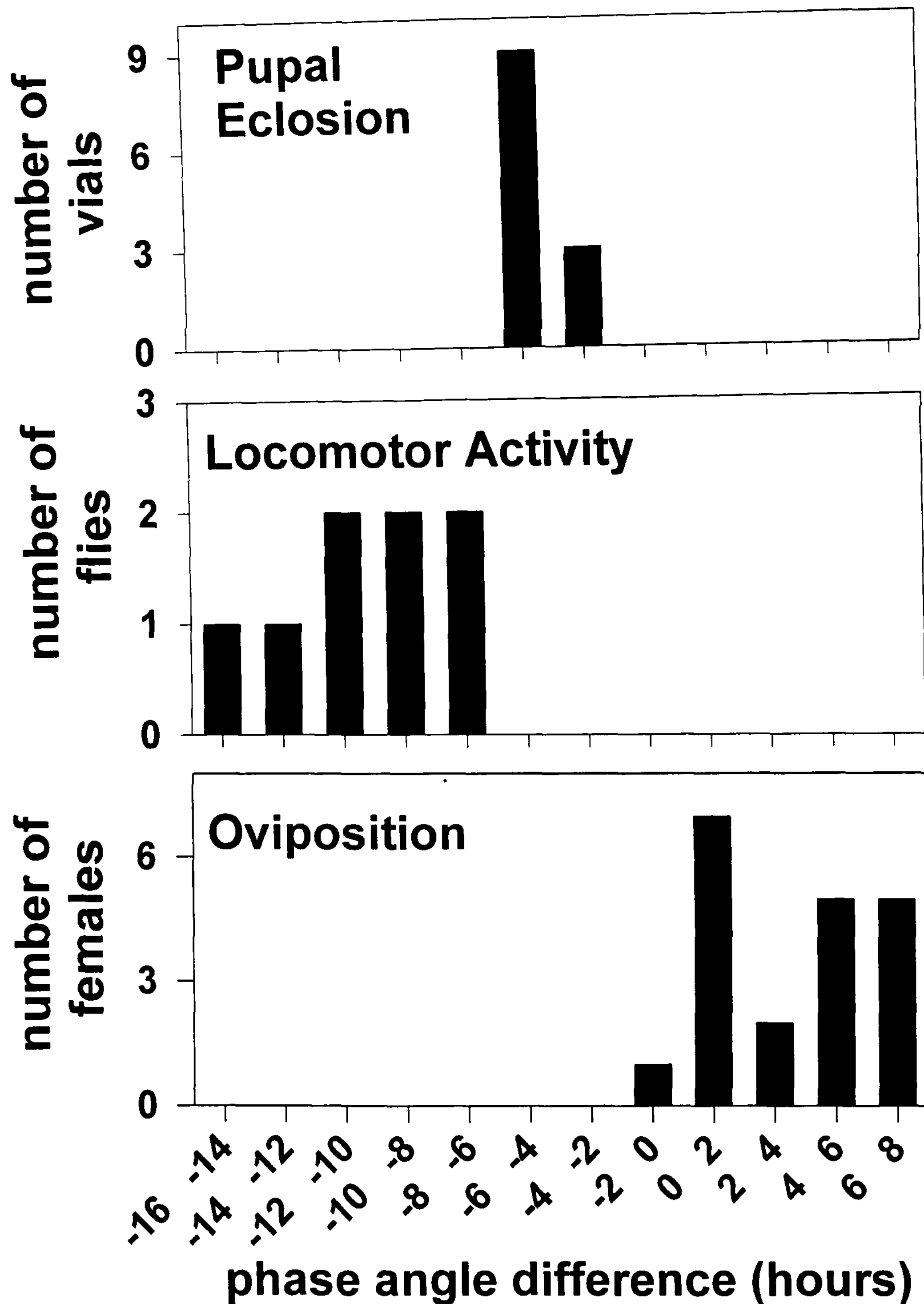

Fig. 8 Frequency distribution of the phase angle difference in LD 12:12 h for eclosion, oviposition and locomotor activity rhythms. The x-axis represents class intervals with a range of 2 h starting from -16 *h to* +8 *h*.

in a similar manner (McCabe and Birley, 1998). Yet, some mutations like *ebony* and *lark* affect only one of the two rhythms of eclosion and locomotor activity (reviewed in Jackson, 1993). While *ebony* mutants show arrhythmic adult locomotor activity and normal eclosion rhythm, *lark* mutants have arrhythmic eclosion rhythm and periodic locomotor activity (Newby and Jackson, 1991, 1993), suggesting that these mutations may be affecting clock-controlled genes (ccgs) rather than a central pacemaker. However, a study using *D. pseudoobscura* revealed that the τ and the PRC of eclosion and locomotor activity rhythms were different, suggesting that separate circadian pacemakers control these two rhythms (Engelmann and Mack, 1978).

The τ and ψ of the eclosion and locomotor activity have also been reported to be different in *D. melanogaster* (Helfrich, 1985). The results of our experiments on *D. melanogaster* also demonstrate that τ and ψ of the eclosion, locomotor activity and oviposition rhythms are significantly different from each other. Immunohistochemical studies of the expression of *per* in the brain during the larval and pupal stages show that a small group of neurons in the lateral frontal brain close to the medulla, termed the lateral neurons (LNs) express *per* in a rhythmic fashion (Helfrich-Förster, 1996). A small portion of the ventral lateral neurons (LNvs) with small stomata show *per* cycling throughout metamorphosis starting from first instar onwards. The LNvs with large stomata, which develop when 50-60% of metamorphosis is complete, show weak cycling in *per* until eclosion. Dorsal LNds and glial cells show cyclic *per* expression after about 50% of pupal development. These results suggest that perhaps only a certain subset of neurons (LNvs with small stomata) determines pupal eclosion, whereas all the LNs and the glial cells control adult activity rhythms (Helfrich-Förster, 1996). Therefore the observed differences in the rhythm parameters of the eclosion and the locomotor activity rhythms may be due to the

progressive change in the composition of the physiological machinery that regulates these two rhythms.

In one of the first attempts to probe the possible endogenous control of the oviposition rhythm in *D. melanogaster*, groups of flies that were reared in DD were found to be arrhythmic when assayed in DD (Allemand, 1977). Moreover, a transfer from LD cycle of 12:12 h to DD did not elicit rhythmicity in oviposition, although a rhythm in vitellogenesis was found to persist in studies that used groups of females. The lack of rhythmicity in oviposition in DD observed by Allemand (1977) could be due to the fact that oviposition was assayed on groups of females. In groups, inter individual variation in periodicity could mask the group rhythm as each individual may exhibit peak oviposition at a different phase under DD regime. This is a likely explanation, given that we have demonstrated circadian oviposition rhythm in individual females of *D. melanogaster* assayed in DD regime (chapter 2), and have also shown that no evidence of rhythmicity is seen when data from individual females are pooled for analysis (chapter 2; Sheeba et al., 2001 b).

The mRNA of the *period* gene, as well as its protein product PER, exhibit circadian oscillation in various tissues in the body of *D. melanogaster* (Hardin, 1994) and these oscillations have been shown to reflect the overt circadian rhythms exhibited by organisms using various *per* mutant flies. The level of *per* mRNA and its protein product do not, however, show any oscillation in the ovarian tissues (Hardin, 1994; Plautz et al., 1997). Moreover, PER is primarily found in the cytoplasm in the ovary, whereas in all other body tissues studied, it is found in the nucleus (Hardin, 1994). Current understanding of the molecular basis of circadian rhythmicity in *Drosophila* suggests that nuclear entry of PER, after it has formed a heterodimer with another protein TIM, is essential for the generation of circadian rhythmicity. If *per* is the

only gene regulating circadian rhythm in ovaries. the failure to detect PER in the nucleus of ovarian tissues would imply a complete loss of circadian rhythms in ovarian functions. The fact that we observed circadian rhythm in oviposition in *D. melanogaster* suggests that the pattern of expression of the *per* gene in ovarian tissues is unique, and some genes other than *per* may play a role in the generation of the oviposition rhythm. However, it is more likely that oviposition rhythm is controlled by the central nervous system and not by the ovarian tissues. This can be tested by assaying oviposition rhythm in the flies that express *per* only in the brain.

The results of our experiments demonstrate that the τ and the ψ, of eclosion, oviposition and locomotor activity were significantly different from each other. Overall, our results, together with those from other studies, suggest that although eclosion, locomotor activity and oviposition rhythms in *D. melanogaster* share a central pacemaker (of which *per-tim* feedback loop is a component), there may be differences in the output pathways originating from a central pacemaker. Based on our observations and previous reports we conclude that separate circadian oscillators control the three rhythms (eclosion, locomotor activity and oviposition), although they may share some elements in the regulatory pathways.

6.3 Are differences in phasing of one overt rhythm (eclosion) reflected in the rhythm parameters of another (oviposition) rhythm?

The *Drosophila* eclosion rhythm is the most well studied population rhythm so far, and several early experiments have shown that although eclosion is an event that occurs only once in the lifetime of an organism, its timing is controlled by an ongoing oscillation in the developmental process (Pittendrigh and Skopik, 1970). The timing of eclosion in a population of pupae raised under LD cycles is rhythmic, with well-defined peaks occurring close to dawn. This rhythm free-runs in DD with a free running period (τ) close to 24 h, provided the individuals are exposed to a stimulus during any stage of their development, either in the form of transfers from LL to DD or LD to DD or even in the form of a brief non-recurrent light pulse. The *phase* of the circadian cycle at which the circadian pacemaker that controls eclosion rhythm receives a stimulus determines the time of eclosion (Pittendrigh and Skopik, 1970). Pittendrigh and Bruce (1957) postulated that the circadian system controlling eclosion in *Drosophila* is composed of two separate coupled oscillators; the driving 'A' oscillator and the driven or 'B' oscillator. The A oscillator or the pacemaker is temperature compensated and light entrainable, while the B oscillator which directly influences the physiology of the overt rhythm is controlled by the A oscillator and is neither temperature compensated nor light sensitive. The B oscillator maintains a stable phase relationship with the external periodic environment because the A oscillator is entrained by the light/dark cycle and it, in turn, entrains the B oscillator. It is the phase of the B oscillator that finally controls the phase of eclosion (Pittendrigh and Bruce, 1957; Pittendrigh et al., 1958). The results of many subsequent experiments with *Drosophila*, birds and mammals support the coupled oscillator model (Chandrashekaran, 1967; Pittendrigh, 1967; Binkley and Mosher, 1987; Sharma et al., 2000).

When a population of *D. melanogaster* eggs is introduced into LD 12:12 h cycles, a large fraction of flies eclose within a narrow window of time (~ 4 h) almost immediately after 'lights-on', referred to as the 'gate' of eclosion, whereas only a small fraction of them eclose during the rest of the LD cycle (Sheeba et al., 1999 a). It is, therefore, pertinent to investigate the nature of circadian organization in the flies that eclose within the gate (WG) and outside the gate (OG) of eclosion, because the differences between the WG and OG flies in the phase of eclosion could be ascribed to one or more of the following.

(i) The A oscillators of the WG and OG flies are in phase, but the B oscillators are 180° out of phase.

(ii) The OG group of flies contains a greater fraction of arrhythmic flies, or flies with periodicity beyond the range of entrainment, as compared to the WG flies.

(iii) The circadian parameters (τ and ψ) for the WG and the OG flies are different.

In order to investigate the nature of the circadian organization of the WG and OG flies, we examined the egg laying rhythm of individual flies from these two groups in constant darkness (DD) and light/dark cycles of 12:12 hours (LD 12:12 h). The τ in DD and the ψ of the egg laying rhythm in LD 12:12 h were compared between the WG and OG flies.

6.3 a Materials and methods

The population of flies used in this study was the same as described in Section 6.2 of this chapter. From the running culture, ~ 300 eggs were collected into vials containing ~ 6ml of banana-jaggery food medium. Forty such vials were introduced into LD 12:12 h ('lights-on' at 08^{00} h and 'lights-off' at 20^{00} h) and monitored for eclosing adults. On the first 2 days after the flies began to eclose, the number of flies eclosing in each vial was recorded at 2-h intervals.

Among all the two hour intervals, the maximum number of flies were seen to eclose between $09^{00} - 11^{00}$h and, therefore, that interval was designated as the 'gate of eclosion' (Fig. 9). The flies that eclosed during this period on the first day, and on subsequent days, were designated as being within the gate (WG) while those that eclosed between $21^{00} - 05^{00}$ h were designated as being outside the gate (OG). WG and OG flies were collected in this manner for eight consecutive days. From among these flies in each group, male-female pairs were introduced into vials containing ~ 3 ml of food medium, with 24 such pairs being set up for both WG and OG flies and under each of two regimes: LD 12:12 h or DD. Fluorescent white light sources were used in the light phase of the LD regime, whereas dim red light ($\lambda > 640$ nm) was used for DD and the dark phase of the LD regime. At 2-h intervals, the flies were transferred to fresh food vials and the number of eggs laid within that interval was counted, yielding a time series of two hourly egg production for each individual fly. This procedure was carried out for ten days in each light regime. In case of death or escape of males, they were replaced using flies from a cohort of the same age that was maintained in the respective light regime as backups.

The periodicity of the egg laying rhythm in both light regimes was estimated by first smoothing the raw time series data by taking a moving average of 4 data points (which is equal to 8 h) and then subjecting the data to Fourier spectral analysis using STATISTICA ™ (Statsoft Inc, 1995). Statistical significance of observed peaks in the periodogram was tested using the technique of Siegel (1980). The WG and OG flies in DD regime were then classified as having one period (single), more than one period (multiple), or having no significant periodicity (none). A 3×3 contingency table was used to test whether the distribution of flies exhibiting single, multiple and no period differed among the two types of flies. The phase angle difference (ψ) was determined for each fly by measuring the interval of time between the peak in oviposition on

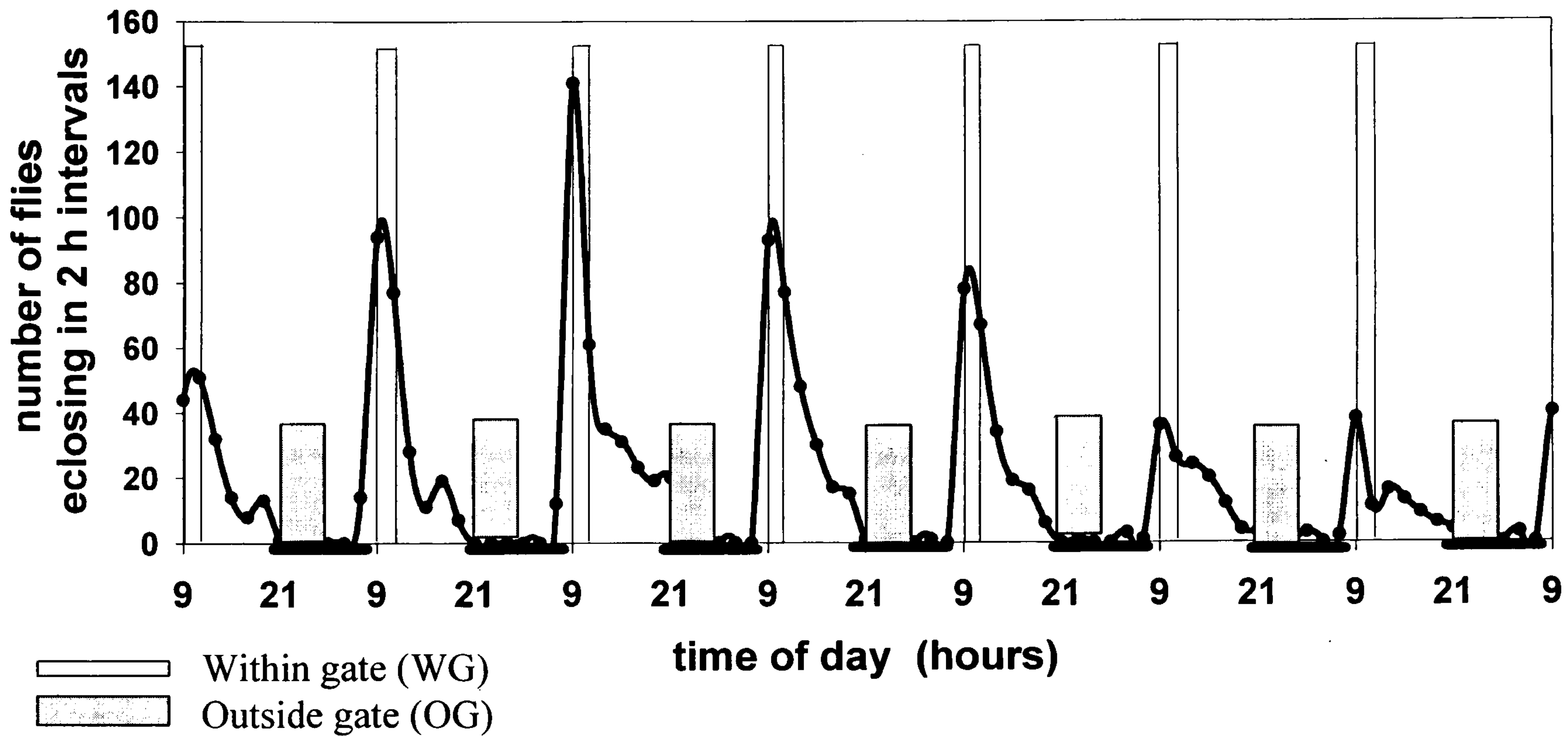

Fig. 9. Schematic representation of the experimental protocol followed to sample flies that emerged within (WG) and outside (OG) the gate of eclosion in an LD 12:12 h regime with 'lights-on' at 08^{00} h and 'lights-off' at 20^{00} h. The time interval between 09^{00} and 11^{00} was considered as the gate of eclosion. The black line denotes the number of flies eclosing in 2 hour intervals. The open and stippled bars indicate the time at which the OG and WG flies were sampled. The horizontal dark bands denote the dark phase of the LD cycle.

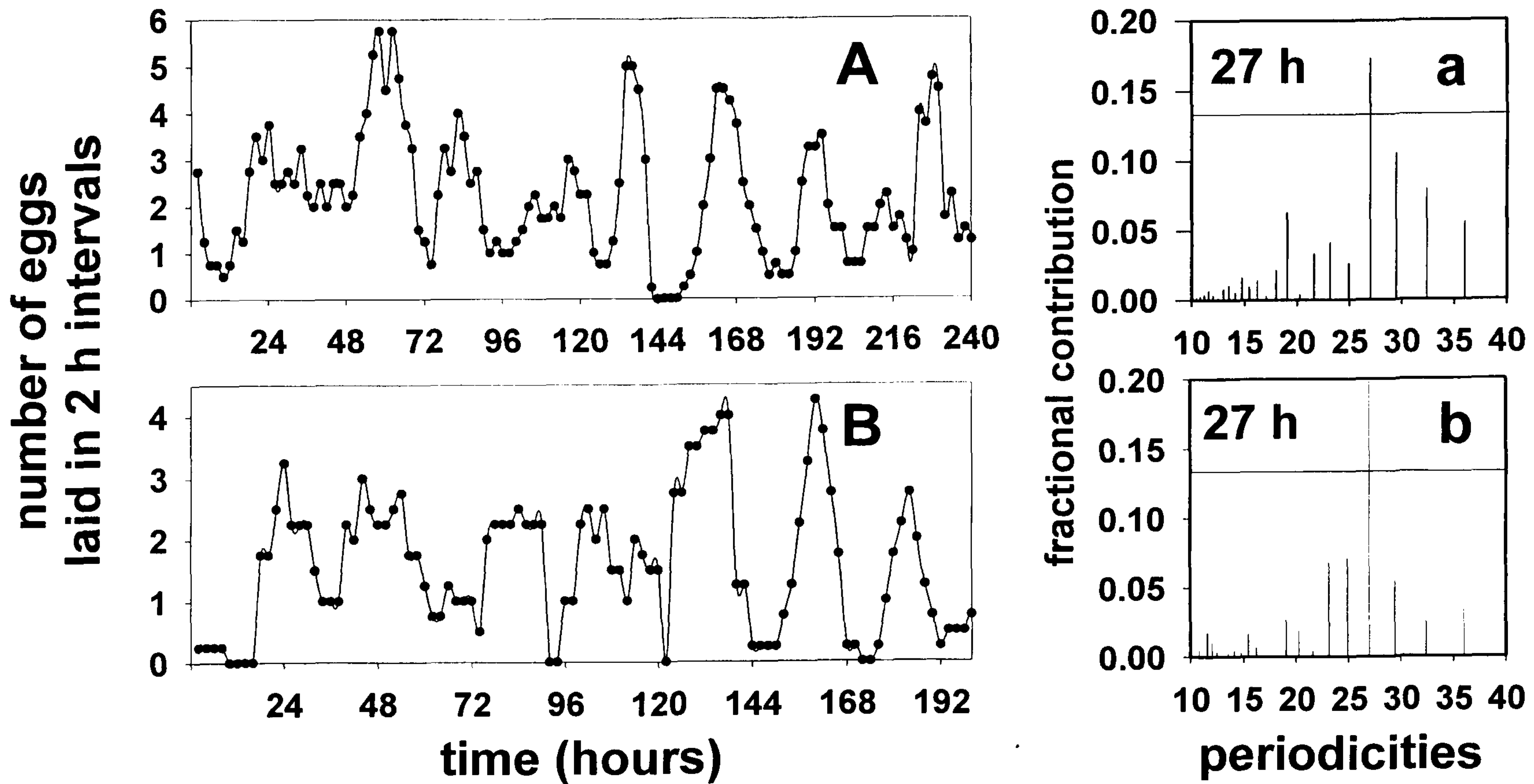

Fig. 10 Time series data of oviposition rhythm of two WG flies in DD regime (A, B). Corresponding periodograms show that the two flies exhibit oviposition rhythm with single significant period of 27 h (a, b).

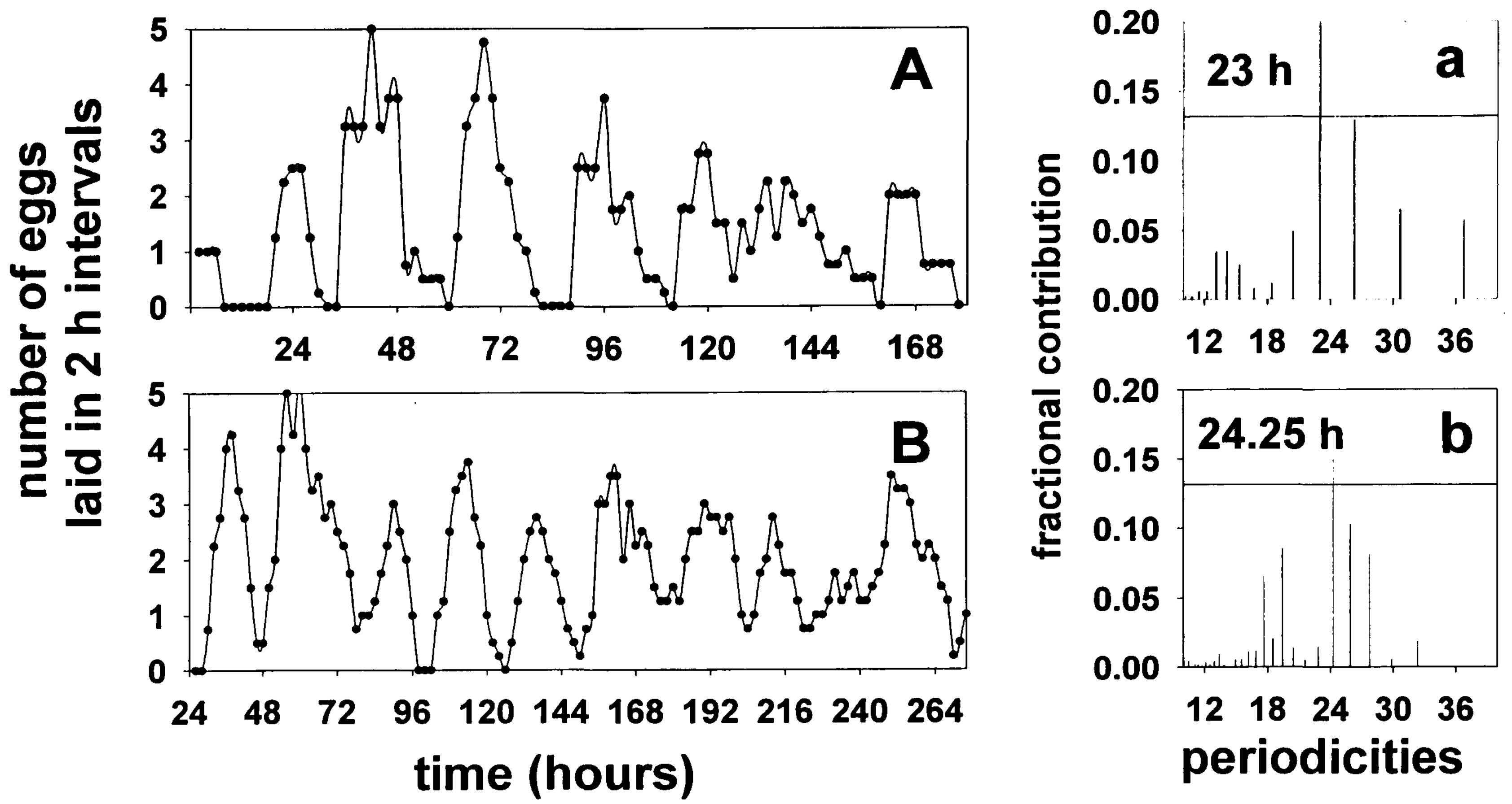

Fig. 11 Time series data of the oviposition rhythm of two OG flies in DD regime (A, B). Corresponding periodograms show that the two flies exhibit oviposition rhythm with single significant periods of 23 h and 24.5 h respectively.

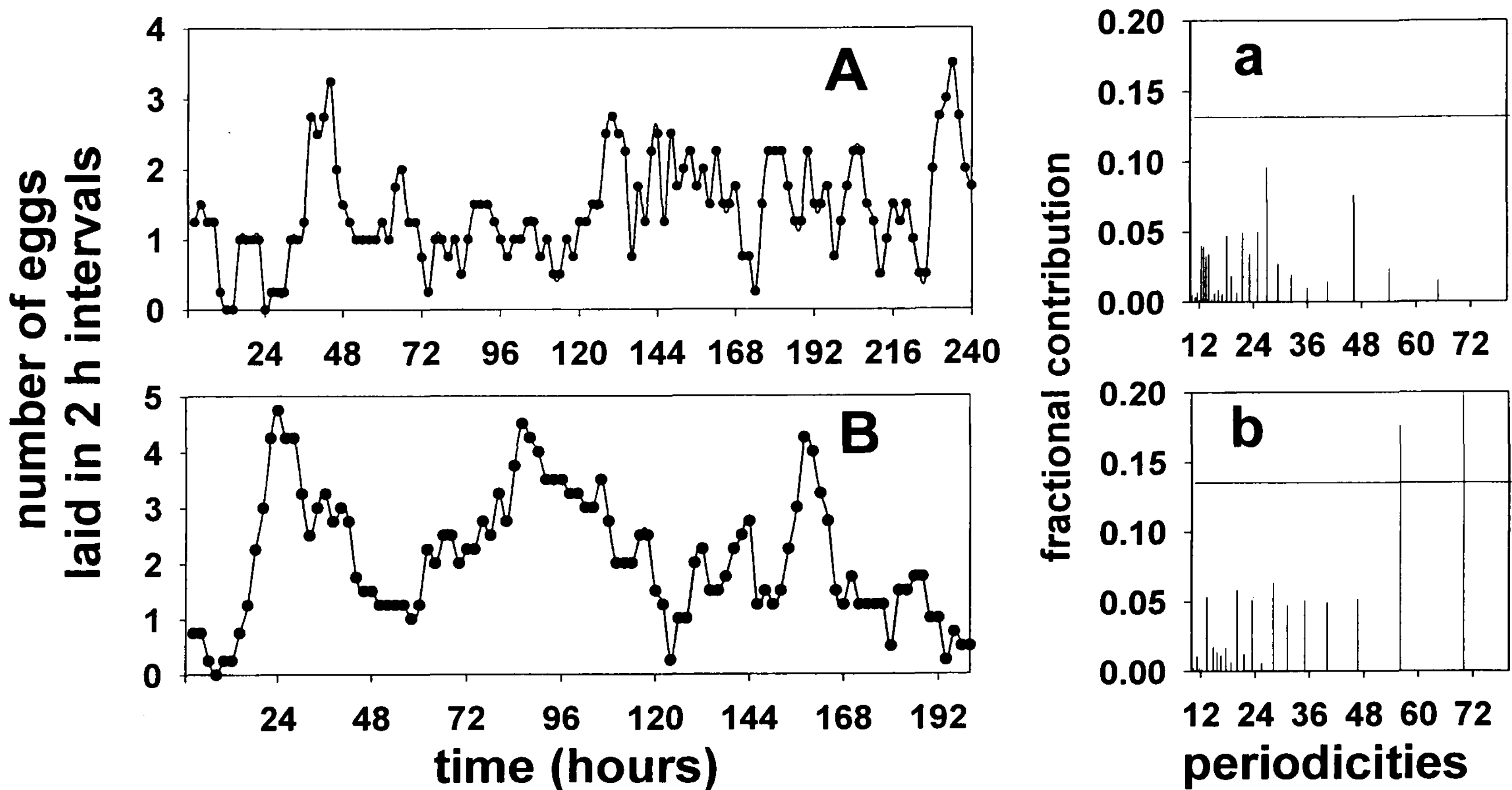

Fig. 12 Time series data of the oviposition rhythm of two WG flies in DD regime (A, B). Corresponding periodograms show that (a) exhibits no significant period, while (b) exhibits multiple periodicities.

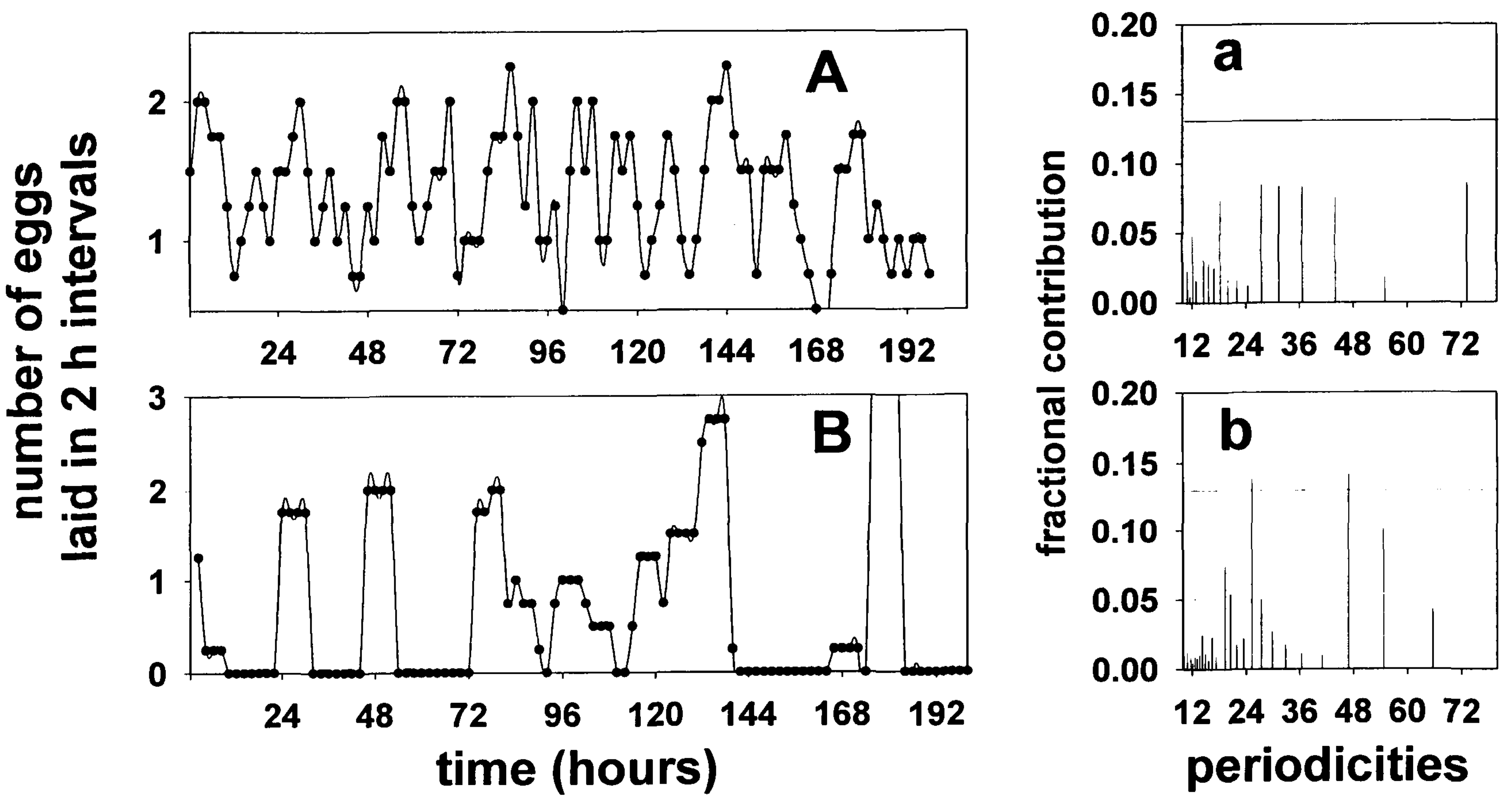

Fig. 13 Time series data of the oviposition rhythm of two OG flies in DD regime (A, B). Corresponding periodograms show that (a) exhibits no significant period, while (b) exhibits multiple periods.

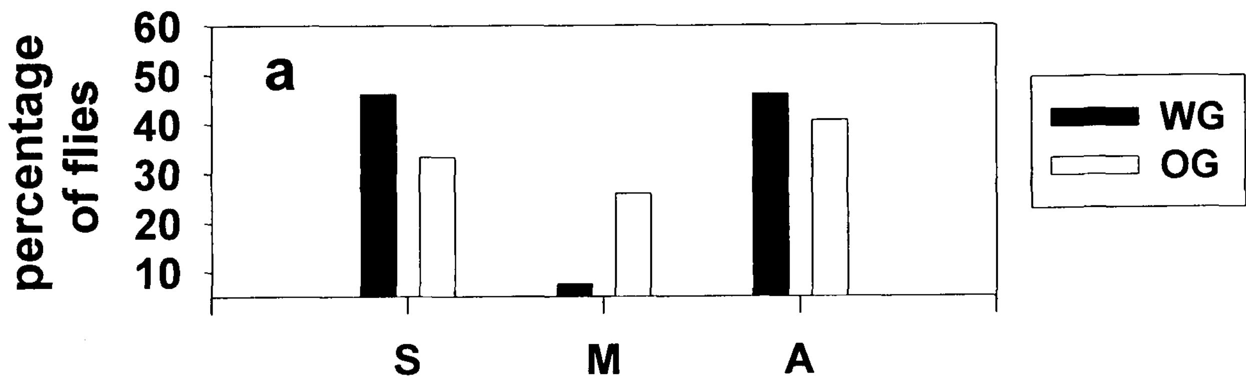

Fig. 14 (a) Fraction of within gate (WG) and outside gate (OG) flies exhibiting single period (S), multiple periods (M), and no significant period (A) in oviposition rhythm under DD regime. Fig. 14 (b) Mean free-running period and (c) phase angle difference of oviposition rhythm of WG and OG flies under DD and LD 12:12 h regimes respectively.

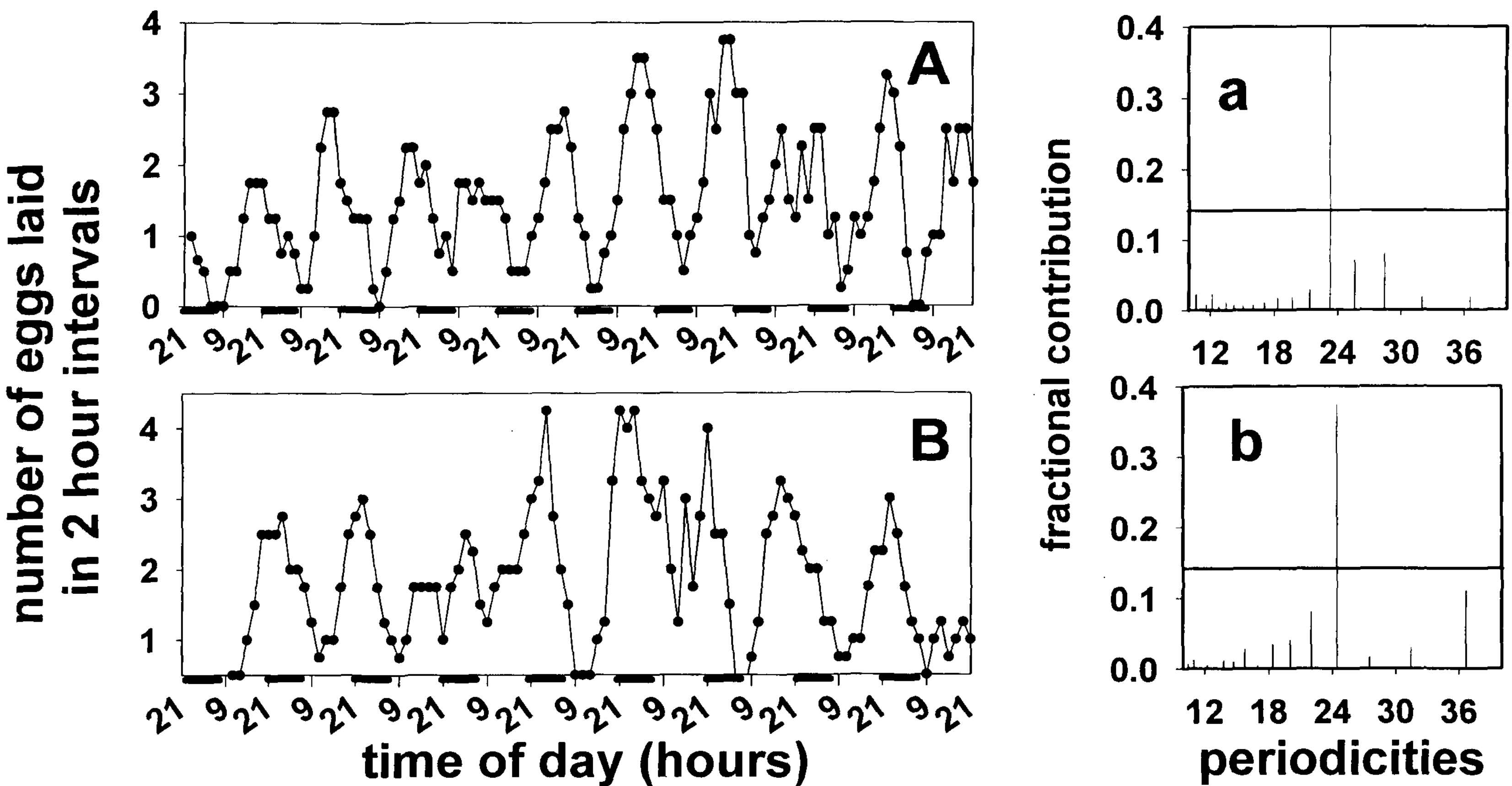

Fig. 15 Time series data of oviposition rhythm of two WG flies in LD regime (A, B). Corresponding periodograms show that the two flies exhibit oviposition rhythm with periods close to 24 h. Lights were switched on at 08.00 h and switched off at 20.00 h (dark bands indicate the dark phase of the LD cycle).

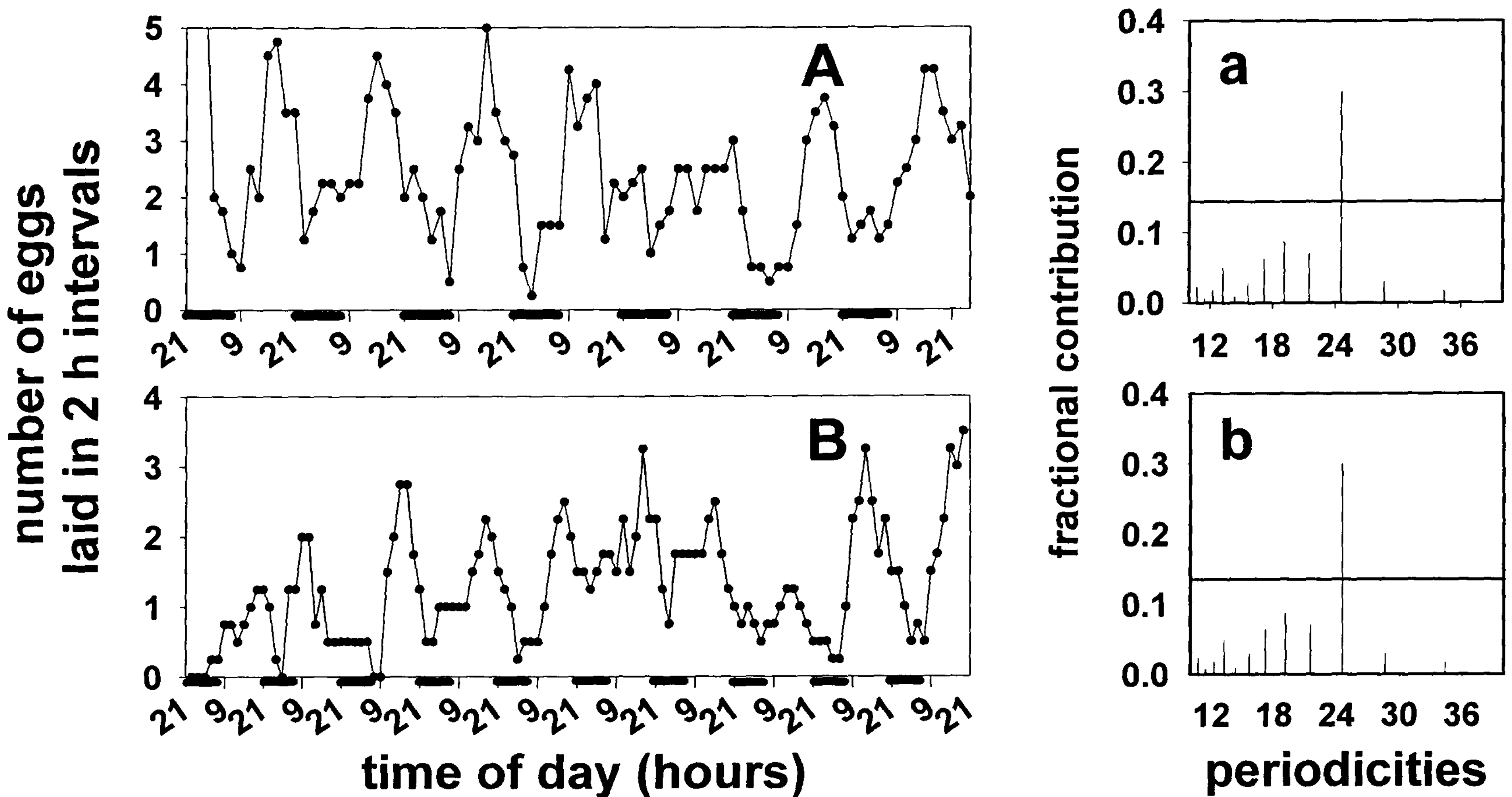

Fig. 16 Time series data of oviposition rhythm of two OG flies in LD regime (A, B). Corresponding periodograms show that the two flies exhibit oviposition rhythm with periods close to 24 h. Lights were switched on at 08.00 h and switched off at 20.00 h (dark bands indicate the dark phase of the LD cycle).

Table. 1 Percentage of within gate (WG) and outside gate (OG) flies exhibiting single period, multiple period and no significant periodicity in oviposition under DD and LD regimes.

	DD		LD	
	Within gate	Outside gate	Within gate	Outside gate
Single	46.15	33.33	60.87	50
Multiple	7.69	25.92	4.35	13.64
None	46.15	40.74	26.1	31.82

Table. 2 Periodicity of oviposition rhythm of within gate (WG) and outside gate (OG) flies exhibiting a single period, under DD and LD regimes.

Single period (hours)			
DD		LD	
Within gate	Outside gate	Within gate	Outside gate
27.42	21.88	24	24
27	22.4	24	24
26.54	24.6	24	24
21.6	18.91	21.33	24
27	23	22.84	24
23.6	24.9	26.57	20.8
26	24.25	28.44	24
24.4	22.55	24	22
30.4	23.2	24	19.69
24.43		24	24
34.28		24	24
32		24	
		29.14	
		24	

each day and the time of 'lights-off' in the LD 12:12 h cycle, and calculating the mean difference over ~ 10 days. The WG and OG flies were compared for mean τ in DD and mean ψ in LD 12:12 h using *t*-tests. The mean periodicity in oviposition under LD 12:12 h was also compared using *t*-test, so as to rule out any differences in entrainment between the WG and OG flies.

6.3 b Results

While nearly half the number of flies assayed in both regimes were rhythmic with a single significant period, a small fraction (7 –26%) showed multiple significant periodicities, and the remainder were arrhythmic (Fig. 10, 11, 12, 13, Table 1). The Chi-square (contingency table) analysis revealed that the distribution of the fraction of flies exhibiting a single period, multiple periods and no significant period in WG flies was significantly different from the OG flies (χ^2 =13.33, df=2, p< 0.001). A relatively larger fraction of the OG flies (~ 26%) showed multiple period of oviposition in DD compared to a smaller fraction of about 7% of the WG flies (Fig. 14 a). A larger fraction of the WG flies (~ 46%) exhibited oviposition rhythm with single significant period in DD, compared to the OG flies (~ 33%) (Fig. 14 a). The fraction of arrhythmic individuals was similar among the WG and OG flies (~ 46% and 41% respectively) (Fig. 14 a, Table 1).

The τ in DD of oviposition rhythm for the WG flies showing a single period (mean ± S.D.: 27.06 ± 3.63 h) was found to be significantly greater than the τ in DD of the OG flies (mean ± S.D.: 22.85 ± 1.80 h) (Fig. 14 b, Table 2). The period of oviposition rhythm of the WG flies showing a single period in LD (mean ± S.D.: 24.59 ± 2.08 h) did not differ significantly (p > 0.05) from that of the OG flies showing a single period (mean ± S.D.: 23.13 ± 1.57 h), suggesting that entrainment to a 12:12 h LD cycle is occuring to a similar degree in both WG and

OG flies (Table 2). The ψ of oviposition rhythm in LD 12:12 h between the WG (mean ± S.D.: 1.5 ± 2.43 h) and OG (mean ± S.D.: 3.94 ± 1.28 h) flies showing a single period was also found to be significantly different ($p = 0.025$) (Fig. 14 c).

6.3 c Discussion

The periodicity of the WG flies exhibiting oviposition rhythm with a single significant period in LD 12:12 h cycle was not significantly different from that of the OG flies ($p > 0.05$). Therefore, the majority of the OG flies do not appear to be individuals with a period beyond the limits of entrainment. However, the distribution of the fraction of individuals exhibiting oviposition rhythm with single, multiple and no significant period was significantly different among WG and OG flies (Fig. 14 a, Table 1), and the τ_{DD} of the WG flies exhibiting oviposition rhythm with single significant period was also significantly greater than that of the OG flies (Fig. 14 b). We also found that ψ in LD 12:12 h cycles for WG flies was significantly less than the OG flies (Figs. 15, 16). The experimentally observed relationship between τ_{DD} and ψ for the oviposition rhythm of WG and OG flies are consistent with theoretically predicted and experimentally observed relationship between τ and ψ for other organisms (Pittendrigh and Daan, 1976; Sharma et al., 1998). These results suggest that the WG and the OG flies are different with respect to the circadian system that controls their oviposition rhythm. However, since the oviposition rhythm is expressed only in the adult stage, the circadian oscillators regulating it could be distinct from those regulating the eclosion rhythm, especially as differences between 'larval' and 'adult' clocks have been observed in many insects (Nayar and Sauerman, 1971; Engelmann and Mack, 1978; Saunders, 1979; Hall, 1995; Hardin and Siwicki, 1995; Helfrich-Förster, 1995). As described in Section 6.2 of this chapter, we have also obtained

data which suggest that the different circadian oscillators control oviposition and eclosion rhythms in *D. melanogaster* (Sheeba et al., 2001 a). The observed differences in the circadian parameters of the clock controlling oviposition rhythm between the WG and OG flies could be said to reflect the differences in the circadian parameters of the clock controlling eclosion rhythm only if the regulatory mechanisms of these two rhythms share some common pathways. We however do not know yet whether such differences in the circadian clocks are due to ontogenetic modification of the same circadian system or whether the clocks controlling eclosion and oviposition rhythm have a different origin. The WG and OG flies differ in terms of the ψ of their eclosion rhythm and in terms of the τ_{DD} and ψ of the oviposition rhythm. This suggests that there is some interdependence between the eclosion and oviposition rhythms of the WG and OG flies. Taking into account the findings of our experiments described in the previous section of this chapter that two different circadian oscillators control eclosion and oviposition rhythms, the results described in this section suggest that the eclosion and oviposition rhythms may, nevertheless, share some common pathways in the circadian regulatory mechanism of these two rhythms.

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