Role of Social Interactions in Modulating Circadian Clocks of *Camponotus* Ants and *Drosophila melanogaster*

Thesis

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By

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Dedicated to...
ALL MY TEACHERS

THESIS DECLARATION

I declare that the work presented here in my thesis titled "**Role of Social Interactions in Modulating Circadian Clocks of *Camponotus* Ants and *Drosophila melanogaster***" is to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university. The results presented here are due to investigations carried out by me in Evolutionary and Organismal Biology Unit under the supervision of Prof. Vijay Kumar Sharma.

References made to work of other researchers have been duly acknowledged. I understand that though the list of references is long, it is not exhaustive. Any omission made is not deliberate but as a consequence of misestimation.

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CERTIFICATE

This is to certify that the work described in the thesis entitled "Role of Social Interactions in Modulating Circadian Clocks of *Camponotus* Ants and *Drosophila melanogaster*" is the result of investigations carried out by Mr. Shahnaz Rahman Lone in the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064, under my supervision, and that the results presented in the thesis have not previously formed the basis for the award of any diploma, degree or fellowship.

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Summary:

Many organisms use predictable changes in light, temperature, and humidity in the environment to anticipate their own cyclic behaviours and metabolic processes. Such anticipatory behaviours are critical for the adaptation to fluctuating environmental conditions, thus requiring timing systems which can measure passage of time and can help in keeping track of local time. Many organisms are known to use circadian clocks to time behavioural and physiological process, and make use of a variety of environmental time cues including cycles of light/dark (LD), temperature, food and social interaction, to keep pace with local time. Although LD cycles are considered to be the key time cue (Zeitgeber) for circadian timing systems of many organisms, there is growing evidence to suggest that non-photic signals such as temperature, food and social can serve as Zeitgeber. Notwithstanding the importance of cyclic social cues in circadian timekeeping process this issue has received relatively less attention. This served as the primary motivation for the studies described in my thesis. Here, I present the results of my studies aimed at understanding the role of social cues in modulating circadian clocks using two ant species *Camponotus compressus* and *Camponotus paria*, and fruit flies *Drosophila melanogaster*.

Social insects employ division of labor as a strategy to execute various tasks in the colony, where different castes are made to perform specific tasks at a different time of the day and year in a well orchestrated manner. It would be interesting to know how individuals living inside the colony with thousands of individuals of different castes display synchronized behaviours in-tune with the local environment. For this we have studied the circadian locomotor activity rhythm of workers and virgin queens of the

species *C. paria*. We find that daily interaction with workers is able to synchronize the circadian clocks of workers and queens, both in pair-wise as well as group-wise interactions. The outcome of cyclic social interaction is context dependent; when two workers cyclically interact, they consider that interval to be their subjective day, but when workers interact with a colony of workers and queens, that duration is considered to be subjective night. These results thus suggest that individuals of the ant species *C. paria* who live in underground nests keep track of local time by socially interacting with foragers who shuttle in and out of the colony. The results of this study are described in the second chapter of my thesis.

Subsequently my studies were aimed at testing if circadian clocks of fruit flies *D. melanogaster* (not known to have any kind of social organization) can be entrained by cyclic social interactions. We asked if cycles of presence and absence (PA) of conspecifics is able to entrain circadian timing system of *Drosophila*. To do so, males living under constant darkness (DD) (hosts) were presented with single male visitors daily for 12 hr. The results suggest that PA cycles do not entrain circadian activity rhythms of *Drosophila*. The outcome was not altered when male hosts were presented with female visitors suggesting that PA cycles of either sex are ineffective in bringing about entrainment of circadian clocks in *D. melanogaster*. However, we found that while PA cycles are unable to entrain circadian clocks, under certain conditions when the clocks of the hosts is labile, daily cyclic presence of visitors cause measurable change in the phase of subsequent free running rhythm. This suggests that PA cycles of conspecifics alters circadian clock phase but does not entrain the rhythm. Therefore, our study suggests that fruit flies *D. melanogaster* probably do not use social cues as their

primary Zeitgeber (time cue) for the entrainment of circadian clocks even though social cues are capable of altering its phase.

To further elucidate the role of social cues as Zeitgeber for *Drosophila* circadian clocks, we studied the effect of social interaction on the phase and period of circadian locomotor activity rhythm, which forms the subject matter of the fourth chapter of my thesis. We first estimated the percentage contribution of each interacting partner of socially interacting individuals on the cumulative rhythmic behaviour of the pairs. We also studied the effect of multi-individual interactions on the rhythmic behaviour of groups, and estimated phase synchrony among individuals of different strains maintained in both genetically homogenous and heterogeneous groups. Although it is known that social interactions improve phase synchrony among socially interacting individuals, we asked if such social interactions have the ability to synchronize highly phase desynchronized flies. We found that social interactions significantly alter phase and period of the rhythm. Individuals living in groups display greater phase synchrony than those living solitarily. Social synchronization is olfaction mediated because flies with compromised olfactory ability ($Or83b^{0}$) were unable to synchronize each other's clocks. These results thus suggest that social cues synchronize circadian clocks of *Drosophila* by modifying their phase and period.

Forager ants that shuttle between colonies and outside are able to entrain the circadian rhythms of workers and virgin queens confined to the colony. The fifth chapter of my thesis deals with the extent of synchrony in circadian clocks of males and queens of two species ants (night active *C. compressus* and day active *C. paria*). The *C. paria* queens emerge out of their colony for mating flights about 45 minutes before *C.*

compressus queens. Furthermore, we find that virgin males and queens of *C. paria* become active earlier than those of *C. compressus*, and their activity rhythm is less precise compared to *C. compressus*, who show startle response to lights-off and greater precision in activity rhythm. The virgin queens of *C. paria* are positively phototactic, while those of *C. compressus* are negatively phototactic. However, after mating *C. paria* queens become negatively phototactic, while *C. compressus* queens remain negatively phototactic. Following mating queens of both species undergo significant reduction in activity peaks. In addition *C. paria* queens become poor in anticipating lights-off and *C. compressus* queens poor in displaying startle response to lights-off. These ants (*C. compressus*) undergo age related changes in their circadian rhythm; activity profiles of virgin queens change from being unimodal to bimodal, and their evening activity peak undergoes reduction. However, such age related changes in activity rhythm are not seen in mated queens. These results thus suggest that queens of *Camponotus* ants experience reproductive state, and age related changes in their circadian behaviours, perhaps to meet new challenges posed by their post-mating phase of life.

Drosophila males and females follow elaborate courtship rituals before the actual act of copulation, which is reflected in terms of enhanced night time activity and loss of night time sleep in heterosexual couples. Olfaction plays a major role in such interactions; however, we do not yet know specifically which receptor(s) are involved. Further, the role of circadian clock neurons in the rhythmic regulation of socio-sexual interactions (SSI) is not yet fully understood. In the sixth chapter I report the results of our study where we assayed the locomotor activity and sleep-wake patterns of male-male (MM), female-female (FF), and male-female (MF) couples from several wild type and

mutant strains of *Drosophila* with an aim to identify specific olfactory receptor(s), and circadian clock neurons involved in the rhythmic regulation of SSI mediated circadian phenotypes in MF couples. The results indicate that *Or47b* receptors are necessary for SSI in MF couples, as ablation or silencing of these receptors have severe impact on the phenotype. Further, ventral and dorsal lateral (LN_v and LN_d) clock neurons appear to be dispensable for the rhythmic regulation of SSI. However, dorsal neuron (DN) and/or peripheral oscillators in *Or47b* expressing neurons are involved.

We know that courtship interactions peak in the night, as a result heterosexual couples display enhanced night time activity. However, we do not know if such induced nocturnal activity persists after the flies are separated. In the seventh chapter we report the results of our study of the long-term consequence of socio-sexual interactions as a result of co-habitation of males and females on the circadian clocks. Males show decrease in evening activity peak followed by a decrease in morning peak, and an increase in night time activity. Females show increase in evening activity peak, but unlike males their night time activity undergoes reduction. The circadian period of males lengthens following SSI. SSI mediated after-effects on circadian rhythms are clock dependent as loss of function mutants and wild type flies made to interact under continuous light conditions do not display such after-effects. Flies with electrically silenced Pigment Dispersing Factor (PDF) - positive neurons show partial SSI mediated reduction in activity, while those with ablated CRYPTOCHROME (CRY) - positive cells do not display SSI mediated after-effects. Such after-effects in circadian activity rhythm are olfaction mediated as $Or83b^0$ mutants and flies with ablated *Or83b* neurons do not display them. These results suggest that socio-sexual interactions between males and

females modulate circadian clocks in fruit flies *D. melanogaster*, and that such effects are clock-dependent and olfaction-mediated.

Following mating, ant queens lay eggs which normally develop in dark underground nests. Organization of a social insect colony depends upon timely development of certain individuals and their capacity to influence rate of development by bringing essential resources to the colony. I was interested in studying if environmental LD conditions play any role in modulating the rate at which ants develop as pre-adults. To address this we assayed pre-adult development time of two species of *Camponotus* ants under different light conditions. This forms the eighth chapter of my thesis. The results suggest that it is possible to modulate the rate of pre-adult development of ants by altering the environmental light conditions.

In summary, the results of my studies suggest that development and circadian timing systems of two related species of *Camponotus* ants are plastic and that social interactions plays a major role in timing rhythmic behaviours. Our studies on *Drosophila* suggest that circadian clocks are modulated by SSI, and that mediating signals is olfactory in nature. Such interactions cause long lasting after-effects on circadian clocks which persists long after the flies are isolated. While some circadian pacemaker neurons may be involved in the regulation of SSI, it appears to be largely under peripheral oscillator control. Unlike ants, social interactions among same sex couples do not cause measurable change in the circadian clocks of *Drosophila* to enable entrainment, suggesting that social cues may not serve as a primary Zeitgeber. However, social interactions are critical for ants as they synchronize members of the group and keep them in-tune with the external environment.

Contents

Chapter 1

Introduction

1.1 Introduction to circadian rhythms

Circadian clocks are endogenous time keeping systems whose period is largely protected against changes in ambient conditions. Nevertheless, they can be fine-tuned by environmental time cues (Zeitgebers) in a way that the period becomes indistinguishably close to 24 hr, and acquires a unique and reproducible phase-relationship with the environmental cycles (Daan and Aschoff, 2001). These clocks drive a variety of behavioural and physiological rhythms in a wide range of organisms from bacteria to humans, and help organisms in anticipating daily and seasonal events in their environment (Dunlap et al., 2004; Allada and Chung et al., 2010). Circadian clocks are believed to be of great adaptive value to organisms living under periodic as well as constant conditions as they enhance their fitness by coordinating various behavioural and physiological processes to external environmental and internal metabolic cycles (Ouyang et al., 1998; Sharma, 2003a). The earliest step to characterize the rhythm generating/regulating mechanisms at the gene level was taken in 1971 by Konopka and Benzer, when they found that three alleles of a newly discovered gene *period* (*per*) - per^L (long period of ~ 29hr), per^S (short period of ~ 19hr) and per^0 (arrhythmic phenotype) have impact on the activity/rest and adult emergence rhythms (Konopka and Benzer, 1971). Following the identification of *per* gene, other clock genes such as *timeless* (*tim*), *clock (clk)*, *cycle* (*cyc*), and *Cryptochrome* (*cry*) in *Drosophila*; *frequency (frq)* and *white collar* (*wc-1*,*wc-2)* in Neurospora; and *KaiA, KaiBC* in cyanobacteria; *Clock (Clk)*, *Period1-3* (*Per1-3*), *Bmal1*, and *Cry1-2* in mammals were identified (Shearman 2000;

Johnson 2001; Cheng et al 2001; Allada and Chung, 2010; Dunlap et al., 2004). Extensive research focusing on the role of clock genes and the possible ways in which they may generate a near 24 hour rhythm, helped in the general acceptance of a model involving transcriptional and translational feedback loops. These studies led to the proposal that in most organisms, circadian rhythms are the overt expression of endogenous oscillations regulated by Transcriptional-Translational feedback Loops (TTL) involving transcripts and proteins of clock genes that themselves cycle in circadian fashion. Some of the clock proteins inhibit their own transcription at certain time of the day after which the concentration of negative feedback elements start dropping off due to action of degradation complexes (Allada and Chung, 2010; Dunlap et al., 2004; Hardin, 2005). Heterodimers of transcriptional activators (e. g., CLOCK-BMAL1 in mammals and CLOCK-CYC in *Drosophila*) bind to the E-box in the promoter region of the clock genes to cause transcription. The transcripts are then translated and the protein products build up in the cytoplasm to reach a critical level that enables their entry into the nucleus to cause repression of their own transcription thus completing the negative feedback loop (Hardin, 2005; Allada and Chung, 2010; Dunlap et al., 2004). The feedback loops have time delay mechanisms involving kinases such as CASEIN KINASE Iε CK1ε) / DOUBLETIME (DBT), GLYCOGEN SYNTHASE KINASE 3β (GSK3β)/ SHAGGY (SGG), and CASEIN KINASE 2 (CK2) (Allada and Chung, 2010), and other similar post-translational processes which ensure that the length of TTL cycle is near 24 hr (Meyer et al., 2006). However, findings from more recent studies suggest that circadian rhythms can be generated without transcriptional feedback. For example, post-translational modifications alone were sufficient in generating circadian oscillations in

cyanobacteria, and in eukaryotic cells such as unicellular pico-eukaryotic alga *Ostreococcus tauri* and mammalian erythrocytes (Nakajima et al., 2005; O'Neill and Reddy, 2011 ; O'Neill et al., 2011).

Although in many metazoans rhythmic expression of clock genes is seen through out the body, their expression in certain tissues (usually in the brain) is often critical for normal rhythms at the behavioral and physiological level. Moreover there is evidence to suggest that the basic architecture of circadian timing system in many organisms is multi-oscillatory, comprising of master pacemakers located in the brain or elsewhere in the central nervous system and peripheral oscillators located throughout the body (Shibata and Tominaga, 1991; Damiola et al., 2000; Giebultowicz, 2000; Hall, 2003). In fruit flies *D. melanogaster* around 150 cells in the fly brain show cyclic expression of core clock genes and proteins (Kaneko et al., 1997; Kaneko and Hall, 2000; Helfrich-Förster, 2003, 2004), and hence are referred to as the clock neurons and the neural circuitry that they create is referred to as the circadian pacemaker circuit. The 150 neurons are divided into six neuronal groups on the basis of their location in the brain and clock proteins that they express – Pigment Dispersing Factor (PDF)-positive small and large ventral lateral neurons (sLN_v and ILN_v), PDF-negative 5^{th} sLN_v, dorsal lateral neurons (LN_d), three groups of dorsal neurons (DN1, DN2 and DN3), and lateral posterior neurons (LPN) (Kaneko and Hall, 2000; Helfrich-Förster, 2003, 2004; reviewed in Sheeba et al., 2008). Among the DNs, DN2 subset is the smallest having only 2 neurons in each hemisphere and DN3 is the largest with ~40 neurons. These neurons can be further classified on the basis of the expression of clock genes such as the CRY-positive, PDF-positive, and other neurotransmitter expressing clock neurons (Renn et al., 1999, Johard et al., 2009).

Modern genetic tools have helped in finer dissection of the *Drosophila* circadian pacemaker circuit. With the advent of binary GAL4-UAS expression system (which makes use of a yeast transcriptional activator-GAL4) it became possible to regulate the expression of any gene of choice in a tissue specific manner (Duffy, 2002), providing a great opportunity of high level of spatial and temporal control of gene expression to the investigator. This enabled circadian rhythm researchers to assign function(s) to subsets of clock neurons in the circuit by selectively ablating of cells. Furthermore, electrical silencing or hyperexciting these neurons by expressing altered forms of ion channel proteins (Nitabach et al., 2002; White et al., 2001), or by blocking synaptic transmission (Sweeney et al., 1995; Kaneko et al., 2000) followed by behavioural and immunohistochemical analyses showed that normal electrical activity of the LN_v subset of clock neurons is necessary for them to function as pacemakers of the circuit. Down regulation (Nawathean et al., 2005) or overexpression of the core clock genes such as *tim* and *per* specifically in the circadian pacemaker circuit resulted in arrhythmicity at the cellular and behavioral levels (Blanchardon et al., 2001; Yang and Sehgal, 2001), indicating the importance of subsets of these cells and the role of specific genes in specific tissues.

Other studies targeted the peripheral tissues or part of central clock neurons using dominant negative forms of CLK and CYC (*CLK*Δ and *CYC*Δ) to further dissect out the role of these proteins in the peripheral cells (Tanoue et al., 2004). Using a repressor of GAL4 called GAL80 further spatial and temporal specificity of neuronal targets was achieved allowing researchers to examine the effect of speeding up or slowing down of the clock machinery in subsets of pacemaker cells and testing for its effect on rest of the

circuit (Stoleru et al., 2004, 2005, 2007; Grima et al., 2004; Sheeba et al., 2008). Further sophistication of the GAL4-UAS binary expression system by the use of Mosaic Analysis with Repressible Cell Marker (MARCM) (Lai and Lee, 2006; Wu and Luo, 2006) is likely to yield further insights into the development and functioning of neurons in the *Drosophila* pacemaker circuit.

In recent years the importance of peripheral oscillators being as important as the central oscillators in maintaining certain physiological and behavioural rhythms is being recognized. Peripheral oscillators regulate either independently (e.g., in antennae) or in consultation with (e.g. prothoracic gland) the central pacemaker a large variety of rhythms (Giebultowicz, 2000; Myers et al., 2003; Tanoue et al., 2004). The PDF-positive sLN_v neurons help in anticipating lights-on and are referred to as morning clock neurons. Further these neurons along with other clock neurons such as LN_d and 5^{th} PDF-negative sLN_v are responsible for the anticipation and timing of the evening activity peak and hence referred as evening neurons (Stoleru et al., 2004, Grima et al., 2004). The dorsal neurons are known to respond to changes in environmental temperature, and help in synchronsation of *Drosophila* clocks to temperature cycles (Busza et al., 2007; Miyasako et al., 2007; Picot et al., 2009; Zhang et al., 2010, Zhang et al., 2010). In addition, lateral posterior neurons (LPNs) also participate in temperature entrainment (Miyasako et al., 2007). More recent studies suggest that DN1s are also light receptive (Zhang et al., 2010; Zhang et al., 2010). The above studies suggest that different neurons in the circadian pacemaker circuit may be responsible for performing specific functions; however, the circuit itself is plastic (Sheeba et al., 2008).

Peripheral oscillators in *Drosophila* are located through out the fly body including organs such as malpighian tubules, antennae, prothoracic glands, and oenocytes fat bodies (Plautz et al., 1997; Giebultowicz, 2000; Myers et al., 2003; Tanoue et al., 2004; Krupp et al., 2008; Xu et al., 2008). Some peripheral oscillators work efficiently only in coordination with the central pacemaker, while others work more or less independently (Myers et al., 2003; Xu et al., 2008; Tanoue et al., 2004). The peripheral oscillators can be directly entrained by time cues such as light, temperature, food, and social interactions (Giebultowicz, 2000).

Circadian oscillators (central or peripheral) respond to environmental time cues to schedule biological events at a unique time of the day determined through an interaction between circadian oscillators and environmental cycles (Allada and Chung, 2010; Roenneberg et al., 2003). This is achieved when circadian oscillators respond to external time cues in a discriminate and time dependent manner (Pittendrigh and Daan, 1976; Daan, 2000; Roenneberg et al., 2003). This process is referred to as entrainment and is distinct from mere synchronization. Synchronization of an oscillator requires its period to match the external cycles, but for entrainment, the oscillator should acquire a unique and reproducible phase-relationship with the synchronizing cycle. Entrainment of circadian clocks is believed to occur via two possible modes proposed by the non-parametric and parametric models of entrainment (Pittendrigh and Daan, 1976; Beersma et al., 1999; Daan, 2000; Sharma and Daan, 2002; Sharma, 2003b; Sharma and Chandrashekaran, 2005). According to the non-parametric model, entrainment occurs through daily phase resetting of the circadian rhythm by the amount needed to correct the error in its period, while the parametric model proposes that entrainment occurs through

time dependent changes in the circadian period (Roenneberg et al., 2010b). Evidence in favor of the parametric model comes from studies where period changes have been reported as a result of exposure to light (Beersma et al., 1999; Daan, 2000; Sharma, 2003b). Currently the entrainment model which is widely accepted is the non-parametric or discrete model which suggests that entrainment of circadian clocks occurs due to time dependent phase shifts by light exposure (Daan, 2000; Dunlap et al., 2004). However, in reality the mechanism is likely to lie somewhere between the two models. In a study where features of both the models were incorporated to give a unified model of entrainment, the stability of phase of entrainment was maximal when circadian clocks entrained using both phase as well as period changes (Beersma et al., 1999). More recently a new model of entrainment has been proposed that utilizes the circadian integrated response characteristic of the clock. Unlike the previous models, this model makes no assumptions regarding phase and period responses, and proposes that entrainment of circadian clocks can be achieved by any kind of Zeitgeber by merely changing shape, asymmetry, and cycle length (Roenneberg et al., 2010a, b). Whatever be the mechanism of entrainment that circadian clocks may be using, adjustment to environmental cycles is the most important characteristic of circadian clocks which keeps organisms in-tune with its external environment.

In *D. melanogaster*, molecular mechanisms underlying entrainment to LD cycles is triggered when the light sensitive photopigment CRYPTOCHROME (CRY) binds to TIM in a light-dependent manner causing its degradation (Ceriani et al., 1999). Light causes conformational changes in CRY which makes it possible to bind TIM. After binding, CRY causes posttranslational modification in TIM allowing Skp1/Cullin/F-box

(SCF) E3 ubiquitin ligase complex JETLAG (JET) to target TIM leading to its degradation (Koh et al., 2006). In nature there are two allelic forms of *tim*, one which is light sensitive (23 amino acid shorter) version called *s-tim*, and the other *ls-tim* that encodes both short and long forms (Sandrelli et al., 2007; Tauber et al., 2007). The light sensitivity of *s-tim* is believed to be due to its ability to bind CRY more easily compared to *ls-tim* (Sandrelli et al., 2007). CRY is also degraded by JET in the presence of light; however, association of CRY with JET depends upon the presence of TIM as seen by the greater stability of CRY when more amount of TIM is present (Peschel et al., 2009). Following degradation of TIM, even CRY is degraded allowing fresh TIM to build up to start a new cycle.

In mammals multiple cell types form a network which is entrained by light via different types of photoreceptors (Herzog, 2007). Rods and cones - the classical photoreceptors detect light and are responsible for non image forming (NIF) vision including circadian photo-entrainment. Studies in the late nineties showed that rhythms in mice lacking rods and cones can also be phase shifted by light similar to the wild type animals, suggesting that mammalian circadian pacemakers use novel photoreceptors (s) (Freedman et al., 1999; Lucas et al., 1999). These photoreceptors were found to be intrinsically photo-sensitive, receive light information from rods and cones, and relay it to different brain centers (Baver et al., 2008). They express the photopigment melanopsin, which is mostly localized in the retinal ganglion cells (ipRGCs). In a separate study it was shown that absence of all three receptors (rods, cones and melanopsin) in triple knock-out animals abolished the non-visual responses to light (Hattar et al., 2003), suggesting that melanopsin was the novel circadian photoreceptor which took care of

photo-entrainment in absence of classical rod and cone photoreceptors. Melanopsin null (*Opn4*$^{-/-}$) mice display altered phase shift responses to light pulses (Panda et al., 2002). Mice lacking rods (*rd/rd*) show normal phase-shifts but fail to entrain to weak LD cycles (with light intensity < 1.0 lux). Similarly mice without rods and cones (*rdcl/rdcl*) show no change in their phase shift responses to bright white light (Semo et al., 2003). In coneless (*cl/cl*) mice which lacks almost all mid-wavelength (MW) cones (peak sensitivity > 500 nm) and majority of short wavelength (SW) cones (peak sensitivity < 500 nm), phase shift to mid wavelength light stimuli is not affected (Freedman et al., 1999). Interestingly, melanopsin deficient (*Opn*$^{4-/-}$) mice entrain normally to LD cycles but their response to light stimuli (< 480 nm) is significantly affected, which suggests their role in the photo-entrainment process (Panda et al., 2002, 2003). The thyroid hormone receptor knockout mouse, missing both *β* isoforms *β*1and *β*2 (*TRβ* $^{-/-}$), lacking MW responsive cones, show altered phase shift responses, phase-relationship, and altered rate of re-entrainment to phase shifted LD cycles (Dkhissi-Benyahya et al., 2007). These mice show reduced responses at 530 nm, and higher photon flux compared to 370 nm, 480 nm and low photon flux, suggesting a role of the MW cones in photo-entrainment. These studies suggest that there are two dedicated photopigments in the mouse circadian light input system, one responsible for sensing blue light and the other red light. Marked changes in the visible spectra occur during twilights, in terms of the ratio of blue versus red light, and presence of both of these pigments may be critical for survival and adaptation under field conditions (Panda, 2007). Therefore, absence of any one of them may compromise the animal's ability to entrain correctly and hence render them vulnerable to predators, although single photopigment is enough to entrain circadian

rhythms under laboratory LD conditions where generally white light is used as light source. Animals with targeted ablation of ipRGCs had more severely compromised circadian photo-entrainment and pupillary light reflex (PLR) than those with melanopsin deficiency, however, these manipulations had no deleterious effect on the image forming vision of animals (Guler et al., 2008; Hatori et al., 2008), which suggests that it is the cones and rods that transmit light signals to ipRGCs for photic-entrainment. Both photic and non-photic stimuli are known to reset the phase of circadian clocks in mammals utilizing neurotransmitters which act on SCN (Harrington, 1997). Thalamic intergeniculate leaflet (IGL) projections to the SCN are believed to be involved in the photic and non-photic phase shifts of circadian clocks. The IGL contain neurochemicals like neuropeptide Y, GABA, enkephalin, nitric oxide synthase, serotonin, histamine, and dopamine, which act though G protein coupled receptors (GPCRs), and non-GPCRs (for NMDA), located in the SCN (Harrington, 1997; Maywood and Mrosovsky, 2001). Dexras1/AGS (Activator of G protein signaling 1) is a member of family of G proteins which cycle with a peak during night. The *dexras*$^{-/-}$ animals display altered photic and non-photic phase shifts, suggesting that G-protein coupled receptor signaling pathways are involved in the phase resetting of circadian pacemaker in mice (Cheng et al., 2004). The above studies suggest that light for circadian photo-entrainment in mammals is perceived by a variety of photo-pigments and relayed to the SCN via ipRGCs (Baver et al., 2008).

Although LD cycles serve as the most important Zeitgeber for circadian clocks in a wide variety of organisms, there is evidence to suggest that non-photic environmental signals can also act as Zeitgeber and bring about circadian entrainment. For example,

entrainment of circadian clocks by temperature has been shown to occur in many ectotherms including fruit flies *D. melanogaster* (Matsumoto et al., 1998; Yoshii et al., 2005; Boothroyd et al., 2007; Busza et al., 2007). Temperature cycles entrain circadian rhythms of *Drosophila* even in conditions like constant light (LL) (Glaser and Stanewsky, 2005), where rhythms of wild type flies is abolished (Konopka et al., 1989). Synchronization of circadian clocks by temperature cycles occur at a relatively slower pace compared to those by LD cycles, which this can be speeded-up by removing the morning group of clock cells (Busza et al., 2007). It is believed that the circadian photopigment CRY plays an important role in entrainment of circadian clocks to temperature cycles, and temperature induced phase shifts are reduced substantially in the CRY hypomorphic mutants (cry^b) (Kaushik et al., 2007). Furthermore, the clock gene *per* occurs in two spliced variants, and it is found that *per* splicing is more predominant at cooler temperatures leading to phase advances in circadian rhythms, whereas hotter temperature reduces splicing yielding phase delays in circadian rhythms (Majercak et al., 1999). Apart from *cry*, another member of the photo-transduction pathway family $norpA^{P41}$ is also believed to play a key role in circadian entrainment to temperature cycles (Majercak et al., 1999,2004 ; Glaser and Stanewsky, 2005). Furthermore in *Drosophila*, circadian activity rhythm of *nocte* (no circadian temperature entrainment) mutants do not to entrain to temperature cycles, which makes it the only gene that has been implicated in temperature entrainment and is not known to also play a role in photo-entrainment (Glaser and Stanewsky, 2005). Entrainment of circadian rhythm to temperature has also been reported in Neurospora (Liu, 2003), diurnal palm squirrel (Rajaratnam and Redman, 1998) and isolated sections of SCN (Herzog and Huckfeldt, 2003). In plants circadian

core clock components such as PSEUDO RESPONSE REGULATOR9 (PRR9), PRR7, PRR5, and TIMING OF CAB2 EXPRESSION (TOC1) are responsible for temperature entrainment as evidenced by the failure of quadruple mutants (*prr9 prr7 prr5 toc1)* to display robust oscillations in the clock controlled genes (Yamashino et al., 2008), which suggests that these genes play important role in adapting to the temperature cycles. In cyanobacteria three Kai proteins and ATP have been shown to be sufficient to function as self-sustained circadian oscillator (Nakajima et al., 2005). Phosphorylation of KaiC protein can be entrained to temperature cycles (20-28 ^{o}C), and temperature steps of 30-45 ^{o}C are able to cause phase shifts in a time dependent manner (Yoshida et al., 2009). The above studies suggest that temperature cycles serve as an effective Zeitgeber for the circadian entrainment in a wide variety of organisms.

Organisms as diverse as bees, birds and mammals show food anticipatory activity (Mistlberger, 2009). Some food sources are available only at a specific time of the day and specific seasons of the year (Stephan, 2002), and can hence serve as important cue for entrainment under a given set of conditions. Rats fed one meal per day showed increase in activity 2-4 hours before their meal time which is referred to as food anticipatory activity (FAA). Such anticipatory behavior changes the phase of liver clock after only two days whereas the suprachiasmatic nucleus (SCN) retained the phase even after 19 days of food restriction resulting in the uncoupling of peripheral oscillator (liver) from the master oscillator (SCN) (Damiola et al., 2000). Food anticipatory behaviour is rescued in *Bmal1* null animals by expressing *Bmal1* in the dorsomedial nucleus region of the hypothalamus (DMH), which suggests that the food entrained oscillator of mammals is located in the DMH (Fuller et al., 2008). However, in a recent study it was found that

even *Bmal1* null mutants can anticipate food availability quite well and exhibit robust FAA (Mistlberger et al., 2008; Pendergast et al., 2009), indicating that the properties of the food entrainable oscillator is somewhat different from those of the light entrainable oscillator. The circadian rhythms of the house sparrow *Passer domesticus* can be entrained to restricted feeding (RF) cycles of 25 hr, and it free runs when the length of RF cycles is 23.5 hr (Hau and Gwinner, 1992). Shorter duration of food availability makes the RF cycles stronger with clearer anticipation to the time of feeding than longer food access duration where often no clear anticipation is seen, and in some cases even a negative phase-relationship is observed (Hau and Gwinner, 1996). The locomotor activity of house sparrows is synchronized to feeding cycles (Hau and Gwinner, 1992, 1996). Above studies suggest that food entrainable oscillator in house sparrow behaves differently from the mammalian food entrainable oscillator as there is anticipation in the latter, whereas in the former there is synchronization or even negative phase-relationship to the presence of food. In case of *D. melanogaster*, fat bodies perform a similar function as the mammalian liver, and were found have cyclic expression of *tim* levels which dampened in Clk^{jrk} mutants (Xu et al., 2008) suggesting presence of circadian oscillator. Expression of the dominant negative form of CLK in the fat bodies dampened cycling of *tim* transcript resulting in a disruption of the food entrainable clock, associated with increased intake of food during the night, which in turn makes them more sensitive to starvation (Xu et al., 2008). From the above studies it is clear that feeding rhythm in *Drosophila* is clock controlled, however, it remains to be seen what the contribution of circadian clock network is in regulating this rhythm, since feeding rhythm is only shifted by 8 hr, but not abolished when dominant negative form of CLK is expressed in the fat

bodies (Xu et al., 2008). Furthermore, it remains to be seen whether the food clocks of flies respond to restricted feeding or caloric restriction in the same way as mammals do.

Apart from temperature and food cues, there is another important means of adaptation to the surrounding environment which is facilitated by social interaction within and among members of any taxa, and such interactions have been shown to modulate the phase and period of circadian clocks which can bring about synchronization. The role of social interactions in synchronizing circadian rhythm of several species is clear when foraging ants, honey bees, birds, mammals leave and return to their dwelling sites in groups (Chandrashekaran, 1982). Social entrainment has been found across taxa, from primitive insects to highly evolved mammals, and it occurs in many different ways. For example, dominant individuals may entrain clocks of the subordinates as observed in case of the estrous females of golden hamsters (Handelmann et al., 1980), or one individual may pass time information to another as seen in mice (Viswanathan and Chandrashekaran, 1985), bats (Marimuthu et al., 1981; Marimuthu and Chandrashekaran, 1983a), and rats (Reppert and Schwartz, 1986).

Here, we review our current understanding of the role of social interactions on circadian clocks. We have categorized the discussion into two broad sections - social interactions among vertebrates and invertebrates.

1.2 *Effect of social cues on the circadian clocks of vertebrates:*

1.2.1 Fish: Circadian rhythms have been reported in the feeding behaviour of killifish *Fundulus heteroclitus* and goldfish *Carassius auratus* (Davis and Bardach, 1965; Aranda et al., 2001), and restricted feeding cycles were found to entrain circadian activity rhythm in goldfish *Carassius auratus*, where activity starts free running from the previous

feeding schedule (Aranda et al., 2001; Sunuma et al., 2008). In a study on a shoal of 12 fish trained to feed around mid day in a brightly lit area of the of the tank the trained fish show food anticipatory behavior by leaving the shady area and spending time in the illuminated area of the tank slightly ahead of feeding time (Reebs, 2000), while naive fish do not display such anticipation and spend most of their time in the shady part of the tank. However, naive fish along with the experienced ones in 7:5 or 9:3 or 11:1 ratios were able show food anticipatory activity which was stronger in cases were the proportion of experienced fish were more, suggesting social synchronization of circadian clocks (Reebs, 2000). The circadian period length of a day active species of Killifish *Fundulus heteroclitus* is affected by group size (Kavaliers, 1980). Group of five loosely aggregated units mostly behave as solitary individuals compared to a shoal of 25 fish, whose daily behaviours appear to be more synchronized, suggesting that coherence in circadian behaviours is achieved by social interactions among group members and extent of synchronization is probably a function of the probability of physical contact.

1.2.2 Birds: Many bird species are social and synchronize their foraging flight and return back to the roosting sites in great synchrony (Chandrashekaran, 1982). Such synchrony in birds may be due to auditory communication between individuals and therefore auditory signals has been suggested to serve as Zeitgeber for avian circadian timing system (Davidson and Menaker, 2003). In a study on the house sparrow *Passer domesticus*, three out of ten sparrows (both males and females) maintained under constant dim green light were found to entrain to sound recordings (70-80 decibels, db) of conspecifics when played daily for 4.5 hours. Eight of them showed either change in period or phase, while the remaining two were not affected by sound cues (Menaker and

Eskin, 1966). Similar studies on the Eurasian siskin (*Carduelis spinus*) and European serin (*Serinus serinus*) show strong male-female contact especially during the breeding season, while for the rest of the year they live in male or female-only groups (Gwinner, 1966). When three Siskin and one Serin female were subjected to daily 60-80 db song for 12 hr, all birds show entrainment of activity rhythm in spite of the fact that their circadian period was 23 hr. Furthermore, these birds were able to re-entrain to phase shifted sound cycle (Gwinner, 1966). However, in both these studies the control experiment with sound tracks from other species or white noise was not performed leaving the likelihood of non-specific sound cues in entraining circadian clocks. Such studies were subsequently performed (Reebs, 1989), which showed that white noise was equally effective in entraining circadian clocks of birds as the sound of conspecifics. This suggested that circadian clocks of birds respond to any kind of sound cue, and therefore it would be interesting to examine whether there is difference in the entrainment by white noise and non-specific song cycles. In case of Japanese quail *Coturnix japonica,* higher percentage of offspring raised by biological mothers show circadian and ultradian feeding rhythm as compared to those which have been artificially hatched (Formanek et al., 2009). Furthermore, higher percentage of brood raised by rhythmic mothers show circadian feeding activity as compared to those raised by arrhythmic mothers, suggesting social synchronization of circadian clocks by mothers. Therefore, bird circadian clocks seem to be sensitive to social cues.

1.2.3 *Mammals:*

1.2.3.1 ***Mice:*** The earliest studies demonstrating social entrainment were carried out on blind mice (*Mus musculus*) by Halberg and co-workers (Halberg et al., 1954) who

showed that blinded mice kept under LD cycles where able to entrain if co-housed with normal mice. Crowley and Bovet (1980) observed that deer mice *Peromyscus maniculatus* show social synchronization of the circadian locomotor activity rhythm when housed together under dim LL; circadian periodicity of the group was distinctly different from the periodicities when housed solitarily, suggesting a role of social interactions in synchronizing circadian clocks. Studies on the nocturnal field mice *Mus booduga* showed that activity rhythm of pups exposed to PA cycles of mother under LL and DD display entrainment, albeit with opposite phases, suggesting that mother can entrain circadian clocks of pups (Viswanathan and Chandrashekaran, 1985). In a separate study it was shown that pups considered mother's presence as subjective day and her absence as subjective night (Viswanathan, 1999). In C57BL/6J also, cyclic presence and absence (PA cycle) of mother is effective in synchronizing the clocks of offspring only until 23-26 days of age, and thereafter entrainment is lost, suggesting that PA cycles are effective only during the early stages of development when pathways responsible for photo-entrainment is not fully developed (Viswanathan, 1989, 1999). Some species of mice have greater propensity to respond to social cues than others. For example, the B6 (C57BL/6J) strain of mice are found to be more responsive to social cues than the BALB (BALB/c) strain (Panksepp et al., 2008). Furthermore, B6 mice display daily rhythmicity in socially relevant behaviours such as sniffing and direct contact with ano-genital area, and social grooming (Panksepp et al., 2007, 2008). However, in mice social cues are found to be ineffective under LL (15-25 lux) conditions (Viswanathan and Chandrashekaran, 1987), suggesting that functional clocks are necessary for social

synchronization. The above studies indicate that social cues can serve as Zeitgeber for mice circadian clocks.

1.2.3.2 ***Hamsters:*** Social synchronization has also been reported in golden hamsters *Mesocricetus auratus*; circadian clocks of host animals was synchronized to that of the visitors when they were co-housed for half an hour every day (Mrosovsky, 1988). Furthermore, re-entrainment to 8 hr phase advanced LD cycles was faster when males were presented with females for three hours on the first day of new LD cycle (Honrado and Mrosovsky, 1989). However, when out-of-phase males and females were kept together in a box separated by a mesh to avoid socio-sexual interactions, presence of females had no effect on the circadian rhythm of the males, although there was an effect of estrous cycle on activity levels of males (Davis et al., 1987), suggesting that the circadian clocks of hamsters are tightly linked to reproduction related behaviours. In hamsters, estrous cycle of the dominant female is believed to delay or speed-up estrous in other females in the group (Handelmann et al., 1980). Such synchrony has often been regarded as being merely a chance event (Schank, 2000). It has been argued that the stress of being confined in groups and/or being handled could have caused such synchronization (Schank, 2000). This view is supported by the studies that show that hamster females in groups having synchronous estrous cycle to start with become asynchronous after 2-3 weeks under isolation (Gattermann et al., 2002), suggesting that findings of Handelmann et al. (1980) study was masked by pseudo entrainment (Schank, 2000). Golden hamsters *Mesocricetus auratus* which were blinded by bilateral enucleation continued to free run in the presence of other males and females which were co-housed with them for three hours daily (Aschoff and von Goetz, 1988; Refinetti et al.,

1992). Similarly no phase shifts were observed when hamsters were subjected to social pulses at a time point which usually induces phase advances in the activity rhythm (Refinetti et al., 1992). In the diurnal rodent *Octodon degus*, females co-housed in DD but separated by a mesh from visitor females who were entrained to one hour of light exposure daily, exhibited partial entrainment resulting in the change of circadian periodicity, whereas control group of females subjected to daily disturbance free ran under similar conditions (Goel and Lee, 1997a). Olfaction is believed to play a major role in such synchronization, where olfactory cues cause expression of immediate early genes such as *c-fos* in tissues which innervate SCN (Amir et al., 1999). Furthermore, free running rhythms in DD can be entrained by 1 hr and 3 hr pulses of species-specific (Jechura et al., 2006) olfactory cues (Goel and Lee, 1997b; Governale and Lee, 2001). Ovariectomised individuals do not speed-up the rate of re-entrainment to phase shifted LD cycles, and hence are less responsive to intact donors, however, implantation of hormones such as estradiol and progesterone increased the rate of re-entrainment, suggesting that these hormones are critical for odor production as well as odor responses (Jechura et al., 2006). Thus role of social cues in modulating circadian rhythms of hamsters is not completely understood with some evidence for and also against it and clearer understanding awaits further studies.

1.2.3.3 ***Rats:*** As in case of mice social entrainment of pups by the mother has also been reported in rats. Pups blinded by enucleation on the first day of their birth and transferred to foster mother who were entrained to inverted LD cycles with respect to their biological mother (Sasaki et al., 1984), were in-phase with other offspring raised by the foster mother suggesting synchronization of circadian clocks by social cues. Another example

of maternal influence on the circadian rhythm of pups is seen in terms of pineal N-acetyl transferase (NAT) rhythm in 10 day old pups (Reppert et al., 1984). When pups were transferred to foster mothers which were 180^{o} out-of-phase to biological mother, average (litter) profiles of NAT in pups becomes arrhythmic, while individual profiles show variable post-natal regulation by the mother suggesting that NAT oscillations are in transition stage between the two mothers (biological and foster), and arrhythmicity in NAT profiles was due to greater spread in the phases among individuals. This finding was further confirmed in a study on NAT profile of pups born to SCN-lesioned mothers and raised by SCN intact mothers (Reppert and Schwartz, 1986). NAT profiles of pups born to SCN-lesioned mothers were more scattered than those of pups born to intact mothers. Furthermore, periodic exposure of intact mother to phase shifted LD cycles entrained the water intake and corticosterone rhythms of enucleated pups to the new regime (Shimoda et al., 1986). Maternal influence is most effective only during the first few days of birth (Reppert et al., 1984), and fetus from SCN intact mothers show rhythmicity in glucose utilization while those from SCN lesioned mothers do not (Reppert and Schwartz, 1986). Although there is a general consensus on maternal effect on circadian clocks in rats, similar studies related to the effect of socio-sexual or aggressive interactions on circadian clocks have thus far never been reported. In a separate study it was shown that male rats subjected to daily 1 hr social interaction with visiting estrus females showed no phase shift in circadian activity rhythm (Meerlo and Daan, 1998). Furthermore, submissive males do not show any phase shift when subjected to cyclic aggression by being co-housed with dominant males (in the cage of the dominant male) for one hr daily. Similarly co-housing of two blinded rats does not

cause any phase synchrony between their circadian rhythms (Richter, 1970). The failure of social cues in entraining circadian clocks in rats is not due to their inability to sense olfactory signals, as it has been shown that cyclic cues in the form of cedar wood oil entrained the *c-fos* expression rhythm in the SCN of rats (Amir et al., 1999). While cedar oil presented briefly either during subjective night or subjective day did not cause measurable phase shift in the free running rhythm or in the expression level of *c-fos*, when presented along with a light pulse, the treatment elicited much larger phase shifts associated with greater expression of *c-fos* in the SCN than light alone, suggesting that circadian clocks of rats are sensitive to olfactory cues. Interestingly, cedar wood oil alone was able to alter the expression of *c-fos* in other parts of the body which innervate SCN, and therefore it was hypothesized that these peripheral tissues may be influencing the SCN to bring about exaggerated phase shifts. Therefore in rats cyclic maternal social cues are believed to be important for survival of the young ones as during a critical phase of their lives it is able to entrain their circadian clocks. This ability to respond to social cues is either reduced or lost completely at later stages.

1.2.3.4 ***Bats:*** Circadian clocks of bats like many other organisms are sensitive to social cues, which brings about synchrony in the onset of flight activity (Chandrashekaran, 1982). Microchiropteran bat *Hipposideros speoris* live in colonies consisting of 400-500 individuals in deep caves in absolute darkness and almost the entire colony flies out of the cave in the evening within a span of 10-15 min (Marimuthu et al., 1981). Some members of the colony, kept captive in a cage, inside the cave were also found to start activity as the same time as the free living individuals of the colony. However, activity rhythm of individuals of same species obtained from a nearby cave kept captive without

conspecifics free run (Marimuthu et al., 1981), and was not affected by sound of crows, mynas, and frogs (Chandrashekaran, 1982), suggesting that social synchronization of circadian clocks is possible only when the partners involved in social interactions are from the same species. This was confirmed in another study where insectivorous microchiropteran bats *Taphozous nudiventris kachhensis*, kept captive inside a cave were found not to entrain by individuals of the species *Hipposideros speoris* and free run with period less than 24 hr (Marimuthu and Chandrashekaran, 1983a). This suggests that synchronization of circadian clocks in bats occur due to social interactions between members of the colony either via flight activity, ultrasound, or due to some yet unknown chemical signals (Chandrashekaran, 1982). Social synchronization of captive bats was abolished in the presence of constant light intensity (10-20 lux), and the individuals start free running with circadian period greater than 24 hr (Marimuthu and Chandrashekaran, 1983b), suggesting that LL has an inhibitory effect on rhythmic social interaction in bats, and that social signals are effective in DD only.

1.2.3.5 ***Other mammals:*** That the domestic cat *Felis catus L.* can be entrained by social cues was demonstrated in a study in which cats were divided into two sets, one set consisting of six cats were grouped in a colony and the other set of four cats were maintained solitarily (Randall et al., 1990). The locomotor activity behaviour of the cats living solitarily was monitored under DD, and was found to free run. It was possible to entrain the rhythm of solitary individual cats to the soundtracks of animals living in a colony when played daily for 8 hours.

Further evidence of social synchronization in mammals came from North American beavers *Castor Canadensis*, which are nocturnal during summer and remain,

confined in groups inside burrows during ice and snow covered winter (Davidson and Menaker, 2003). They display 27 hr coherent rhythms during winter conditions while they are in burrows only to return to 24 hr rhythmicity during summer, suggesting social synchronization among group members (Bovet and Oertli, 1974). In case of Australian sugar glider *Petaurus breviceps*, rhythms of co-housed males and females are not synchronized and are found to free-run with different periodicities (Kleinknecht, 2004). The phase of circadian rhythms of the day active common marmosets *Callithrix jacchus* was able to be reset by non-photic cues such as agitation of cages and sprinkling with water for one hour every day (Glass et al., 2001). However, there was no effect of social cues on the circadian clocks (Erkert and Grober, 1986).

1.2.3.6 ***Humans:*** As in the case of clocks in animals discussed thus far, light is the most effective Zeitgeber for entrainment of human circadian clock also (Czeisler and Dirk, 2001). However, it is quite natural to assume that social cues may play a much bigger role in entraining circadian clocks of humans. Social cues in humans has been a controversial topic (Wever, 1989; Czeisler, 1995); some human rhythms such as that of 17-hydroxy corticosteroids levels have been found to disappear in DD, and social cues act as an entraining agent for this rhythm (Aschoff, 1955). Humans living in groups when subjected to four days of entrainment to LD cycles followed by another 4 days of DD continued to show oscillations with 24 hr period quite like their entrained LD behaviour (Aschoff et al., 1971), suggesting social synchronization of circadian clocks. Social synchronization was also implicated in a separate study by Wever and co-workers (Wever, 1989). The sleep-wake cycles of 15 blind individuals under societal conditions showed synchronized 24 hr rhythmicity suggesting timekeeping by social cues (Klerman

et al., 1998). In blind individuals, who had no conscious light perception, negative electroretinogram, and abnormal visually evoked potential, synchrony of melatonin synthesis rhythm is not affected under forced desynchronization schedules (Klerman et al., 1998). Nine out of fifteen individuals showed entrainment of melatonin synthesis rhythm, while those of other six individuals free ran. Furthermore, unlike normal sighted individuals, the entrained individuals do not show phase shift in core body temperature minima due to light exposure (Duffy et al., 1996), suggesting that circadian clocks of human subjects can be entrained socially and that they differ in terms of their capacity to respond to social cues (Klerman et al., 1998). Co-housing of humans in groups of two resulted in synchronization of the subjects (Wever, 1979), however, these studies were performed in the presence of light and it is possible that self-selected light schedules of one subject entrained or masked the rhythms of others, which resulted in mutual synchronization of circadian rhythms. In another study melatonin and rectal body temperature rhythms of six healthy males kept together as a group having access to digital clocks free ran, suggesting that social synchronsation reported in previous studies was due to entrainment of circadian rhythms due to self-selected LD cycles and not because of social cues (Middleton et al., 1996; Middleton et al., 2002). Similar results were obtained in a study where circadian rhythms of a group of four human subjects were studied in Antartica (Kennaway and van Dorp, 1991). When six human subjects were exposed to LL for six weeks, rhythms in five of them free ran with period longer than 24 hr, and there was little or no influence on their sleep-wake schedule in spite of the subjects working and living together (Steel et al., 1995). In American submarines an 18 hour work schedule with 6 hr duty and 12 hr of rest entrains the rhythms of crew

members in spite of having access to 24 hr cues in the form of the knowledge of local time (due to the accessibility of watches) and social contacts through persons who are exposed to 24 hr LD cycles (Kelly et al., 1999). Blind people are ideal subjects for studying role of social cues as Zeitgeber and therefore rigorous and systematic studies could be carried out on them. Bilaterally enucleated human subjects exhibit free running circadian rhythms even in presence of non-photic cues including social cues (Skene et al., 1999a). In recent studies melatonin rhythm of blind individuals with no light perception (n = 30) living with family or partners was monitored for a period of 3-5 weeks (Skene et al., 1999a;b). While most individuals had abnormal melatonin rhythm (23 out of 30), it was more prevalent in uni-and bi-nucleated subjects. Ninety-one percent of subjects with no eyes, 71% with one eye, and 17% with both eyes showed free running rhythm with period of 24.13 - 24.79 hr. Furthermore, melatonin and cortisol rhythms free run in subjects with no conscious vision (Skene et al., 1999b). Light/dark cycles of period greater or lesser than 24 hr are used to desynchronize circadian rhythms from other environmental cycles (Kleitman, 1939, Czeisler et al., 1999). Two out of four human subjects who underwent a self imposed 26 hr (13:13 hr) sleep-wake cycle showed entrainment while the other two did not (Eastman, 1987). The ability to entrain was substantially improved when 26 hr self-imposed cycle was coupled with exposure to either evening or morning light (Eastman and Miescke, 1990), suggesting that LD cycles along with social cues entrain circadian rhythms in humans. Thus contrary to what one may believe about how heavily humans are influenced by other members of the society, it may be concluded that social synchronization of circadian timing system in humans is rather poor, and may act only to help fine-tune rhythms entrained by LD cycles.

1.3 *Effect of social cues on the circadian clocks of invertebrates:*

1.3.1 *Drosophila* and other insects

Since social cues have been reported to synchronize circadian clocks of many vertebrate species in spite of the fact that many live solitarily, it is expected to be even more relevant in invertebrates where many species are known to live as aggregates or colonies, and some have even evolved partial or complete polyethism. For example, in the cockroach species *Leucophaea maderae*, circadian clocks are found to regulate social interaction, however, circadian clocks *per se* are not entrained by cyclic social cues (Knadler and Page, 2009). In fruit flies *D. melanogaster*, rhythmicity in many physiological and behavioural processes such as activity/rest, copulation and sleep rhythms are affected by social cues (Levine et al., 2002; Fujii et al., 2007; Ganguly-Fitzgerald et al., 2006). It has been observed that flies kept in groups show considerably higher phase coherence in their circadian rhythms than those kept solitarily (Levine et al., 2002), suggesting that social cues synchronize circadian clocks in *Drosophila*. Phase coherence of wild type flies is substantially reduced when they were co-housed with arrhythmic per^{01} flies in 4:1 ratio (Levine et al., 2002). The extent of effect of social interactions depends on the clock phase and composition of the social group because it was found that per^{L} hosts co-housed with per^{S} visitors in 4:1 or 2:1 ratios influenced phase of the host, while per^{S} hosts visited by per^{L} visitors do not have any significant impact on the phase of per^{S} hosts (Levine et al., 2002).

Given that social cues modulate circadian clocks in *Drosophila*, it is expected that they would also influence clock related phenotypes. For example, when wild type flies are maintained in heterogeneous groups (6 wild type females, 4 wild type males, and 2

per^{01} males), they show attenuated expression of clock genes in the oenocytes and brain which eventually results in higher percentage of mating (Krupp et al., 2008). There is almost 22% increase in mating frequency of wild type flies in heterogeneous groups which translates to roughly 1.7 fold more compared to those in homogeneous group (6 wild type females, 6 wild type males). Heterosexual couples in *D. melanogaster* exhibit rhythmicity in close proximity interactions, where they spend ~50% of the total time together compared to homosexual couples who spend ~25% of the time together (Fujii et al., 2007). Close proximity rhythm is controlled by males, as males entrained to 11 hr apart resulted in the shift of close proximity rhythm by a similar magnitude. Social interactions in *Drosophila* are olfaction mediated as phase synchrony of paralytic olfactory mutants such as *para*$^{Sbl\text{-}1}$ and *para*$^{Sbl\text{-}2}$ are not affected by the presence of arrhythmic *per*^{01} visitors. Peripheral clocks are believed to play a pivotal role in regulating such interactions (Levine et al., 2002; Krupp et al., 2008; Fujii et al., 2007), as rescue of oscillations in LN_v and LN_d clock neurons in arrhythmic *per*^{01} mutants do not affect the phase desynchrony in *per*^{01} mutants (Levine et al., 2002). It is speculated that monoenes and methyl branched compounds acts as signals during social communication in *Drosophila* (Kent et al., 2008; Krupp et al., 2008).

Social interactions are believed to have significant impact on sleep of individuals housed together. Sleep is a clock-controlled process and there is an increase in sleep rebound when two wild type flies are made to interact socially (or socially enriched) (Ganguly-Fitzgerald et al., 2006). The *rutabaga* (*rut*^{2080}) mutants, deficient in adenylyl cylase, failed to respond by enhancing sleep rebound due to social enrichment; however, restoring expression of adenylyl cylase in the PDF-positive clock neurons rescued sleep

rebound following social enrichment (Ganguly-Fitzgerald et al., 2006). Another fly mutant *blistered* (*bs*) also does not show sleep rebound, but rescue of *bs* in the LN_v neurons of *bs* flies rescued the phenotype thus suggesting that *bs* in LN_v neurons are necessary and sufficient for social interaction related sleep rebound in *Drosophila* (Ganguly-Fitzgerald et al., 2006). The levels of the epidermal growth factor receptor (*Egfr*) in *Drosophila* brain increases after social enrichment, and is found to interact with *bs* (Donlea et al., 2009). Expressing dominant negative form of *Egfr* in the PDF-positive neurons prevents increase in social enrichment mediated sleep, suggesting that social cues operate though *Egfr* signaling and that PDF-positive neurons play a critical role. Social enrichment leads to the increase of pre- and post-synaptic proteins which include Bruchpilot (BRP), Cysteine String Protein (CSP), Discs Large (DLG), Synapsin (Syn), and Syntaxin (Syx), and their levels decrease after sleep (Donlea et al., 2009; Gilestro et al., 2009), suggesting that sleep may be important in maintaining synaptic homeostasis (Gilestro et al., 2009). Thus social interactions directly modulate circadian clocks and clock related phenotypes in *Drosophila*.

1.3.2 Social insects: Eusociality is defined by three specific traits (a) caring for young ones (not their own offspring) by members of the colony, (b) division of labor, where sterile female workers, otherwise capable of breeding, participate in raising the brood of fertile ones, and (c) overlap of two or more generations (Wilson, 1971). Social insects have evolved division of labor where specific groups of individuals perform specific functions unique to them (Wilson, 1971; Hölldobler and Wilson, 1990). Adults in eusocial insect colony can be broadly classified into reproductives who contribute to the propagation of the society through sexual means, and non-sexuals who perform daily

duties of maintenance (Hölldobler and Wilson, 1990; Brian, 1978). Social insects over-winter either in the form of hibernating queens inside or away from the nest or as adults of colonies (Brian, 1978). Critical processes such as growth, development, mating, and formation of new colony occur at a favorable season, and therefore seasonality is intimately connected with social behaviour (Brian, 1978).

In ants which are social insects, duration of pre-adult development varies from species to species, it ranges from one to few months, and development is halted during winter (Hölldobler and Wilson, 1990). Some species of ants such as *Formica polyctena* speed up their development either by raising nest temperature using heat produced by their metabolic activities or by exposing the nest to sunlight (Hölldobler and Wilson, 1990). There is differential development of different castes depending on their body sizes and environmental conditions, apart from social structure of the colony and social interactions (Brian, 1978; Hölldobler and Wilson, 1990). These studies thus suggest that factors that influence development are likely to influence social interactions as well.

Ants are successful in colonizing different habitats and their ecological dominance is clear as they contribute to half of the terrestrial biomass along with termites (Wilson and Hölldobler 2005). It is therefore intriguing to find that circadian timing systems in ants and the role of social organization in managing their day-to-day repertoire has never been systematically and rigorously studied. The ability to keep time is of prime importance to social insects especially for the production of sexual castes, scheduling their nuptial flights, and for establishing new colonies (Tauber et al., 1986). In ants, time of mating is species-specific, and is influenced by many environmental factors such as temperature, light intensity, and availability of food (Talbot, 1945; Hölldobler and

Wilson, 1990). For example, among army ants *Neivamymex*, males of different species are found to undertake mating flights at different times of the year (Baldridge et al., 1980), suggesting a role for timing systems in regulating mating flights. Furthermore, in case of harvester ant *Veromessor andrei*, males leave their nests for mating flight just before dawn, while those of Argentine ant *Iridomyrmex humilis* fly out two hours before dusk (McCluskey, 1965; Hölldobler and Wilson, 1990), suggesting species-specific regulation of rhythmic process. Similarly, reproductives of *Camponotus clarithorax* fly out for mating during the first half of the day, while those of fire ant *Solenopsis saevissima* do so during the second half of the day (McCluskey, 1965). In African *Dorylus* ants, reproductives of *D. moestus* species fly out for mating in the morning hours*, D. burmeisteri* species in the evening hours, while other species do so at different times during night (Haddow et al., 1966). These studies suggest a possible role of circadian clock in timing mating in ants. Although, circadian activity rhythm has been studied in a few species of carpenter ants, our understanding of the circadian timing system involved in mating and its behavioural and physiological impact is quite primitive (McCluskey, 1965; North, 1987; Sharma et al., 2004a,b). In five species of ants *Paraponera clavata*, *Iridomyrmex humilis*, *Solenopsis saevissima*, *Veromessor andrei*, and *Camponotus clarithorax* males were active at different times of the day. However, in four out of five species activity levels were high during light to dark transition, suggesting that carpenter ants time their mating flights to maximize fitness by avoiding predators and preventing desiccation by day time heat (Levin et al., 2009; Whitcomb, 1973).

For ant queens of *Veromessor pergandei* and *Pogonomyrmex californicus* species, mating is followed by immediate, drastic, and long lasting changes in clock behaviour (McCluskey 1967; McCluskey 1992). A similar post mating change in activity rhythm has been reported in *Camponotus compresses* queens, where mating results in the loss of circadian rhythm (Sharma et al., 2004 a, b). Such loss of rhythmicity in the ant queens could be due to the fact that soon after mating ant queens start laying eggs. While activity rhythms are lost after mating it is quite likely that circadian rhythms in other behavioural and physiological processes persist. However, circadian activity rhythm in the mated queens was restored as soon as the egg laying phase was over. How mating changes circadian rhythms in case of ants is not known yet, however, it is likely to be due to changes in hydrocarbon profiles, which we know from previous studies to change within minutes of mating (Oppelt and Heinze, 2009). Furthermore, there are changes in the brain volume and different parts of the brain undergo apoptosis (Julian and Gronenberg, 2002).

In case of honeybees, the type of daily colony tasks and circadian phenotypes of the workers are correlated. For example, nurses who perform functions inside the colony are arrhythmic while relatively older workers which go out for foraging are rhythmic (Bloch and Robinson, 2001). The levels of *per* mRNA in honeybee is developmentally regulated, and is found to be independent of flight experience, light and colony conditions (Bloch et al., 2004). Forager bees can be trained to feed at an artificial feeding site at any time of the day. This trained feeding behaviour free runs under DD, is amenable to phase shifts by light, can be entrained to LD cycles of 20-26 hr, suggesting that trained feeding behaviour of honeybees is a circadian clock controlled process

(Moore, 2001). This notion is further supported by the finding that when foragers were transferred to another colony, they were found to visit the food source two times during the day, once at the time corresponding to their native colony, and the second time at the time corresponding to host colony (Medugorac and Lindauer, 1967), suggesting that social interactions can influence circadian clocks of honeybees. Flight activity of a honeybee colony monitored for many weeks was found to exhibit circadian rhythmicity with a single period, suggesting that individuals have synchronized activity-rest rhythm (Frisch and Aschoff, 1987; Frisch and Koeniger, 1994; Moore, 2001). Circadian flight activity of honeybee colony can be shifted by light, temperature and feeding cycles (Moore, 2001). Honeybee workers living in groups were found to have synchronized metabolic rhythms (Southwick and Moritz, 1987), which is further confirmed by the finding that period of isolated bees drift away from the period of the colony (Frisch and Koeniger, 1994). Furthermore, young bees co-housed with old bees tend to have earlier onset of activity compared to those kept with same age group individuals, and the advanced phase of activity rhythm persists even after separation, suggesting a role of social interactions (Bloch, 2010). In honeybees and ants, queens loose their circadian activity rhythm soon after mating and start behaving like young nurses (Johnson et al., 2010). Such social interactions may be due to physical contacts, pheromones, and change in body temperature due to physical contact.

Apart from honeybees, circadian rhythms of no other social insect has been rigorously and systematically studied thus far. Ants, which have been otherwise extensively studied for many behaviours (Hölldobler and Wilson, 1990), have rarely (McCluskey 1967; Sharma et al., 2004a-c) been a model for investigating consequence of

social interactions on circadian clocks even though they offer many interesting contrasts to bees in terms of social structure. The broad aim of my research was to study the effects of social interactions on circadian clocks of two related species of *Camponotus* ants, and fruit flies *D. melanogaster*. After observing ant colonies the first question that came to my mind was how members of the colony, many of whom spend most of their time in dark underground nests, become aware of time in their local environment. This is especially intriguing given the degree of precision with which they perform their activities particularly the reproductives coming out of the colony for mating flights. The results of my study examining how ants entrain circadian clocks of their colony mates living under constant conditions of the nest are described in the second chapter of my thesis. After observing that circadian clocks of ants can be entrained socially, I became interested in examining if sensitivity to social cues was typical of social insects alone and whether insects less studied for their social behaviour would show such sensitivity. To test this I chose fruit flies *D. melanogaster* and studied the effect of social cycles on circadian clocks, the results are described in the third chapter of my thesis. From the results of social entrainment described in chapter three, it was clear that circadian clocks of *D. melanogaster* can not be entrained socially; however, social cues alter the phase and period of circadian rhythms. I asked if social cues are able to cause phase synchrony among socially interacting flies. This is addressed in the chapter four of my thesis.

The males and females of *Camponotus* ants come out of their nests at species-specific time close to dusk, and perform in-flight mating after which males die and females land on the ground and start digging nests to start a new colony. I was interested in studying the impact of sexual interactions on the circadian clock of queens which

forms the subject matter of the fifth chapter. Recent studies in *Drosophila* suggest that socio-sexual interactions among males and females results in enhanced night time activity. I decided to extensively study this behaviour by recording locomotor activity and sleep in various strains of wild type and mutant flies. The purpose of this study was to identify the responsible clock neurons and specific olfactory receptor(s) responsible for the communication of social signals between the two sexes and the results are described in the sixth chapter. To examine whether such socio-sexual interactions operate in larger groups of individuals and if such interactions have any after-effect on circadian clocks, I performed experiments where males and females were made to interact in a group of 30 individuals, and the results are discussed in the seventh chapter of my thesis.

Organization of a social insect colony depends upon timely development of certain individuals and their capacity to influence rate of development by bringing essential resources to the colony (Brian, 1978). This suggests that pre-adult development in ants is plastic. I was interested in studying if environmental light/dark condition plays any role in modulating the rate of pre-adult development in ants. It is possible that different castes of ants develop as pre-adults at different rates, and ant colonies use photoperiods as indicators of time of the year. To study these I assayed pre-adult development time of two species of *Camponotus* ants under different light conditions. This forms the eighth chapter of my thesis.

Chapter 2

Timekeeping Through Social Contacts

2.1 Introduction

In insects, circadian clocks regulate a variety of rhythms including those in locomotor activity (Petersen et al., 1988), adult emergence (Pittendrigh, 1954), mating (Sakai and Ishida, 2001), egg laying (Howlader and Sharma, 2006), olfaction (Krishnan et al., 2005), and in processes as fundamental to life as metabolism (Nakahata et al., 2009), and release of haematopoietic stem cells (Mendez-Ferrer et al., 2008). These clocks help organisms keep track of local time by entraining to variety of time cues (Zeitgebers), in a way that period of the rhythm becomes indistinguishably close to 24 hr (Daan and Aschoff, 2001; Dunlap et al., 2004; Sharma and Chandrashekaran, 2005). Although light/dark (LD) cycles are considered to be the strongest time cue for circadian clocks of a wide range of organisms, and is known to act through fairly well understood pathways, at least in fruit flies and mice, there is evidence to suggest that non-photic temporal signals such as cycles of temperature, food, social interactions can also serve as Zeitgebers (Saunders, 2002).

Cyclic social interactions serve as Zeitgeber for circadian clocks in a many organisms including fruit flies, honeybees, birds mice, rats, and bats, and are among the key and perhaps the only source of temporal information for individuals that remain confined in timeless environments (Halberg et al., 1954; Menaker and Eskin, 1966; Bovet and Oertli, 1974; Crowley and Bovet, 1980; Handelmann et al., 1980; Marimuthu et al., 1981; Marimuthu and Chandrashekaran, 1983a; Viswanathan and Chandrashekaran, 1985; Reppert and Schwartz, 1986; Goel and Lee, 1997a; Levine et al., 2002). It is believed that these organisms use visual, physical, olfactory, and auditory stimuli to socially communicate temporal information for circadian timekeeping (Davidson and Menaker, 2003).

Social interaction is known to synchronize circadian clocks in fruit flies *Drosophila melanogaster*; flies kept in groups show greater among-individual phase coherence in circadian activity/rest rhythm than those maintained in solitude (Levine et al., 2002). Social synchronization in flies is primarily mediated via olfaction as phase coherence of olfactory mutants $para^{Sbl\text{-}1}$ and $para^{Sbl\text{-}2}$ remains unaltered following social interaction with arrhythmic per^{01} visitors (Levine et al., 2002). Social interactions may be governed by peripheral clocks possibly those located in the fly antennae, as rescue of molecular oscillation in core clock (lateral and dorsal) neurons in arrhythmic per^{01} mutant has no effect on the phase synchrony of socially interacting flies (Levine et al., 2002; Krupp et al., 2008). Olfactory signals have been shown to have a significant effect on circadian clocks as they alter the expression of core clock genes in oenocytes and brain of flies (Krupp et al., 2008). While these studies suggest that social cues can act as Zeitgeber for circadian clocks of *Drosophila*, our understanding of entrainment of circadian timing system via social cues still remains in its infancy.

Although synchronization of circadian rhythms through social interactions has been reported in a variety of organisms ranging from fruit flies to mammals, systematic and rigorous empirical studies in eusocial insects which depend on within-species social interactions to a much higher degree for the survival of the individuals and the colony are very rare. Honeybees are known to have the ability to convey temporal information by synchronizing each other's circadian clocks (Frisch and Aschoff, 1987; Frisch and Koeniger, 1994). The fact that flight activity of honeybee colony shows a single period, suggests that clocks of individuals are highly synchronized (Frisch and Aschoff, 1987; Moore, 2001). Social synchronization is also evidenced by the fact that circadian period

of solitary individuals from a honeybee colony drift away from that of the colony (Frisch and Koeniger, 1994). Forager bees trained in one hive and transferred to another in which training schedule was different are found to forage at both the timings, suggesting that foraging behaviour of honeybees is influenced by social cues (Medugorac and Lindauer, 1967). Interestingly, a single queen presented to a colony of ~150 workers was able to shift the phase of circadian oxygen consumption rhythm of the entire colony suggesting that honeybee queens have the ability to synchronize circadian rhythms of the colony (Moritz and Sakofski, 1991). Similarly, a group of workers synchronized their metabolic rhythm when they came in physical contact with each other (Southwick and Moritz, 1987). Thus, the above studies suggest that circadian clocks are amenable to changes in phase and period due to social interaction, however, to the best of our knowledge entrainment by cyclic social interactions has never been reported (Bloch, 2010).

In this chapter I report the results of our study aimed at probing if freely moving ant workers are capable of entraining the circadian clocks of individuals confined under constant dark conditions (DD) of the colony. For this, I maintained workers and queens of the day active ant species *Camponotus paria* under DD, and allowed them to interact socially for 12 hr daily (either in pairs or in groups), with workers kept in 12:12 hr light/dark (LD) cycles. The results suggest that circadian rhythms of these ants entrain to social cues; however, ants assume the duration of pair-wise interactions as subjective day, and multi-individual group interactions as subjective night.

2.2 Material and methods

Three ant colonies of *C. paria* were collected from the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore campus (12°43'28" N, 77°26'45" E) and maintained under 12:12 hr LD cycles in the laboratory with light intensity of 100 lux during the light phase and dim red light of wavelength greater than 650 nm during the dark phase. The humidity (70-80%) and temperature (24.5 ± 0.5 °C) was maintained constant by keeping the colonies in temperature controlled incubators. The colonies were kept in acrylic petri plates (diameter × height = 90 mm × 15 mm) and provided with *ad libitum* food comprising of Bhatkar diet (Bhatkar and Whitcomb, 1970) and 10% honey solution.

2.2.1 ***Photic entrainment of workers:*** To assess entrainment of circadian locomotor activity rhythm some workers (n = 16) were first exposed to 12:12 hr LD cycles (with lights-on between 04:00 and 16:00 hr) for 7 days and then allowed to free-run under DD for minimum 5 days. The phase of entrainment of individual ants to LD cycles was subsequently determined by drawing a regression line through the onsets of activity and extrapolating it back to the last day of LD cycle. Timings mentioned through out the text are in local time (hours).

2.2.2 ***Pair-wise interactions in a colony workers:*** To study entrainment of circadian rhythm of ants to social interaction cycles, some workers (n = 62) were arbitrarily picked in roughly equal numbers from each colony, pooled and divided into two groups, making sure that both individuals participating in pair-wise social interaction belong to the same colony to avoid inter-colony aggression. The individuals of one of the groups (n = 31) were marked with non-toxic white paint (guests), while those of the other group were left

unmarked (hosts). The worker ants were kept individually in acrylic petri plates (diameter × height = 90 mm × 15 mm) and provided with food *ad libitum*. The locomotor activity behaviour of host ants was monitored individually using indigenously fabricated activity recording system that uses two pairs of infra red beams to track movements of insects kept inside a petri plate (Sharma, 2003c). Both groups of ants were kept under 12:12 hr LD cycles for the first 7 days, after which hosts were introduced into DD, while guests remained under LD cycles. After 5 days, guests were paired during their night phase with hosts for 12 hr. This cyclic social interaction was repeated for a period of 10 successive days. Following this the locomotor activity behaviour of hosts was recorded individually under DD for a minimum of 12-13 days.

2.2.3 ***Multi-individual interactions in a colony:*** To further elucidate the role of social interactions as Zeitgeber we carried out another study on a freshly collected colony of *C. paria* consisting of a large number of workers ($n \sim 100$), virgin queens ($n = 17$), and males ($n = 15$). We reared the colony in a manner similar to that in the previous experiment, but this time ants were kept in specially designed plastic containers (Figure 3). The containers were filled with a layer of half inch sand sprinkled with water (to maintain humidity), with four openings on either side of the box, wide enough for ants to move in and out. Prior to starting of experiment the colony with unmarked 40-45 workers, 27 marked workers (with non-toxic white paint), 15 queens, and 12 males was introduced into DD. The colony was kept undisturbed in DD for 12 days and food was provided *ad libitum*. After 12 days, marked workers were allowed to move out of the colony at 23:00 hr (local time) through the narrow openings and were brought back to the colony at 11:00 hr. During this interval (between 23:00 hr and 11:00 hr) marked workers

were kept under light intensity of 100 lux in groups of 4-5 individuals, in petri-plates supplemented with food *ad libitum*, and other factors such as temperature and humidity similar to what they experienced while they were in the colony. Virgin males and queens were unable to come out of the colony due to their large body size, while unmarked workers which came out though the channels were carefully transferred back to the colony in an area where there were no ants. The marked workers continued to exit and revisit the colony for 12 hr daily for 10 successive days, after which locomotor activity behaviour of virgin queens (n = 11) and unmarked workers (n = 12) was recorded individually for a period of 14-15 days under DD. The phase of entrainment of individual ants was subsequently determined by drawing a regression line through the onsets of activity and extrapolating it back to the last day of social cycle.

2.2.4 ***Impact of endogenous clock of guest and time of interaction:*** We study the importance of phases of the two interacting partners in determining social synchronization we first maintained a set of workers (hosts, n = 18), individually in petri plates while continuously monitoring their activity (using the recording system described previously) under LD cycles (with lights-on between 04:00 and 16:00 hr). After 6 days, hosts were divided into two groups (S and O groups) and introduced into DD. Another set of marked workers (guests, n = 18), also maintained individually in petri plates, were divided into two groups; the first set was introduced into LD cycle with lights-on between 04:00 and 16:00 hr (LD1, n = 8), and the other set into oppositely phased LD cycles with lights-on between 16:00 and 04:00 hr (LD2, n = 10). After 6 days in DD, the individually housed S group of hosts received one member each from the guest group LD1 between 10:00 hr to 22:00 hr. This procedure was repeated for 8 successive days.

In a similar manner O group of hosts interacted with guests from LD2 between 22:00 hr to 10:00 hr for 8 days. Thus each type of host (S or O) interacted with guests which were oppositely phased and at opposite phases of the social interaction cycle. Thereafter the activity of individual hosts was monitored in DD for approximately 12 days. The phase of the rhythm was estimated by extrapolating the phase on to the last day of social interaction. This was done by drawing a regression line through the onsets of activity.

The locomotor activity data was analyzed with CLOCKLAB, Actimetrics. The phase values of a particular host group were used to generate replicate data set by bootstrapping with replacement. The phase data was subsequently analyzed using circular vector analysis (Batschelet, 1981) to find out if social interactions alter the phase of circadian rhythm. Phase coherence vectors were estimated on a scale of 0-1, 1 indicates perfect coherence and 0, no coherence. This data was then used to estimate the magnitude (r) and direction (a^0) of the phase coherence vector. The replicate r and a^o values were used for analysis of variance (ANOVA) using STATISTICA (StatSoft,. 1995). Post-hoc multiple comparisons were performed using Tukey's test. To test if phase of social interactions is synchronous we used Rayleigh's test for circular non-uniformity (Batschelet, 1981). Further, to analyze whether any given pair of phase distributions were different we used Watson-Williams test (Batschelet, 1981). For all tests, $p < 0.05$ was considered as statistical level of significance.

2.3 Results

2.3.1 ***LD cycles synchronize locomotor activity rhythm of worker ants:*** To first quantify the degree of entrainment of individual worker ants to LD cycles they were (n = 16) were exposed to LD cycles (with lights-on between 04:00 and 16:00 hr) for 7 days,

and then allowed to free-run in DD for a period of 5 days. The phase of entrainment was determined by drawing a regression line through the onset of activity on subsequent days in DD (Figure 1a). The magnitude (r) of phase coherence vector (which is an indicator of inter-individual synchrony of the rhythm of individual ants) is found to be 0.98, with a mean angle of + 0.31 hr relative to lights-on (04:00 hr local time). The phase distribution is non-uniform as per Rayleigh's test ($p < 0.0001$) (Figures 1a, c). The locomotor activity rhythm of about 100 % of ants entrain to 12:12 hr LD cycles indicating the effectiveness of LD cycles as Zeitgeber for this ant species.

2.3.2 ***Pair-wise cyclic social interaction synchronizes phase of locomotor activity rhythm of host worker ants:*** To study the effect of cyclic social interactions on the circadian locomotor activity rhythm, worker ants (hosts, $n = 31$) maintained under DD after entrainment under LD cycles were paired for 12 hr daily with guest worker ants maintained under LD cycles. The guests were introduced to hosts during their night phase. Based on the analysis of locomotor activity data about 75% of host worker ants entrain to social interaction cycles. The magnitude of phase coherence vector is 0.89, with mean a^o value of - 0.58 hr relative to onset of social cycles (Figures 1b, c). The phase distribution is non-uniform as determined by Rayleigh's test ($p < 0.0001$).

To compare the effect of LD cycles with that of social interaction cycles we performed an ANOVA on the r values. Significant effect of Zeitgeber type (LD/social) ($p < 0.001$) was detected. Post-hoc multiple comparisons revealed that r value of phase coherence vector is significantly greater when ants were subjected to LD cycles than to social interaction cycles ($p < 0.001$). Similarly, ANOVA on a^o values showed significant

Figure 1

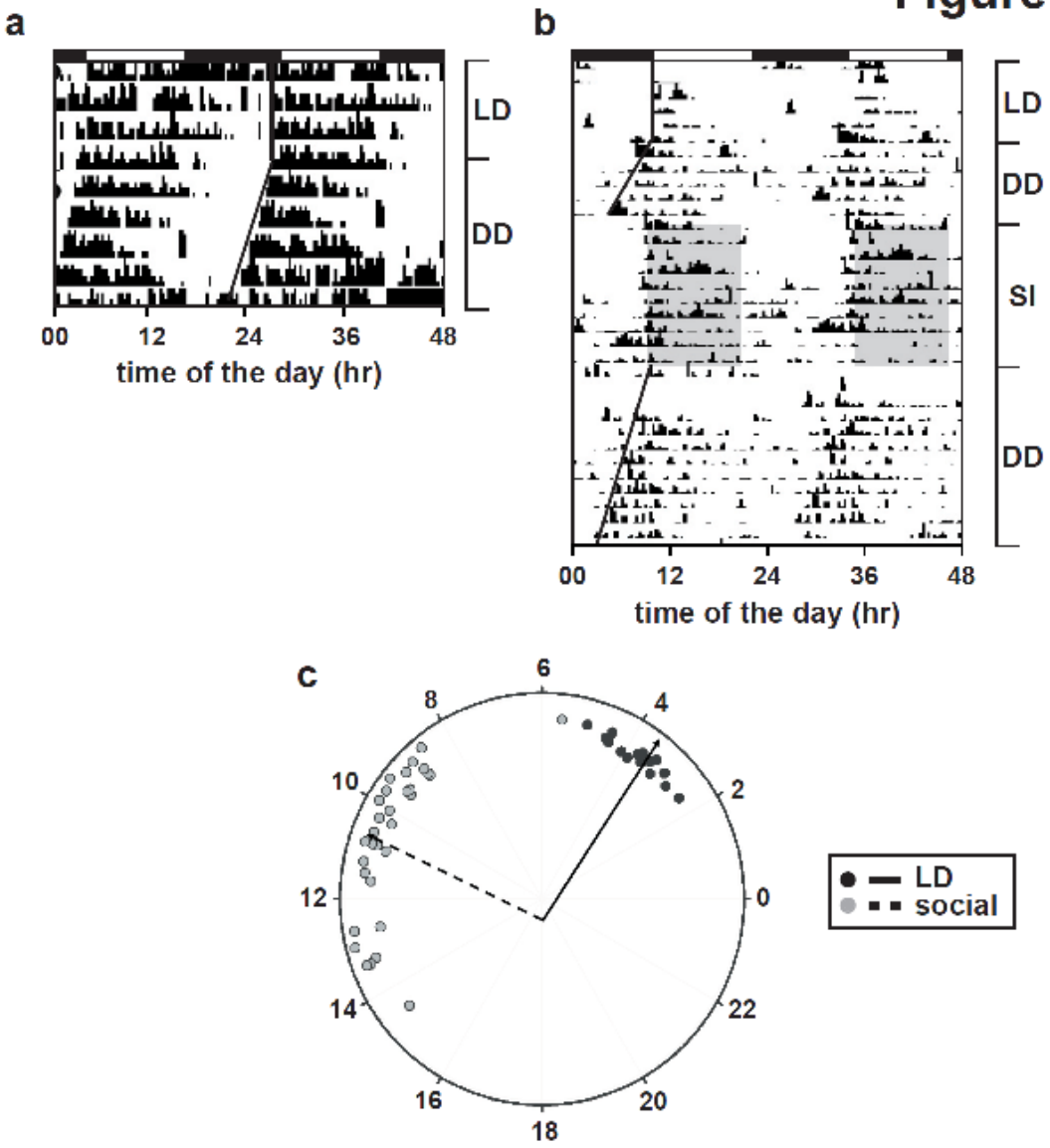


Figure 1 ***LD and social cycles synchronize locomotor activity rhythm of worker ants.*** (a) Actogram showing locomotor activity of a representative worker ant maintained under 12:12 hr light/dark (LD) for 7 days (only last 4 days are shown), and then introduced into constant darkness (DD). (b) Actogram showing locomotor activity behaviour of a representative host worker ants, maintained for 7 days under LD cycles (only last 4 days are shown), and then subjected to DD for 5 days before being exposed to social interaction (SI) cycles with guests for 12 hr for 10 successive days (rectangular grey shaded box). (c) Circular plot of phase of activity rhythm of host worker ants subjected to LD (black filled circles with solid arrow) and social cycles (grey circles with dashed arrow). The magnitude of phase coherence vector under LD cycles ($r = 0.98$) is significantly greater than that ($r = 0.89$) for social cycles ($p < 0.0001$). The direction of phase coherence vector, plotted with reference to onset of LD or SI cycles, is significantly different from that of workers subjected to social cycles ($p < 0.005$). The phase distribution of activity rhythm is non-uniform in both the cases ($p < 0.0001$), and the distributions are significantly different as per Watson-Williams test ($p < 0.0001$). However, phase distributions of ants do not differ when phase values are estimated with reference to Zeitgeber ($p > 0.05$). A total of 31 individuals (hosts) were used to study effect of social cycles, and 16 individuals for LD cycles.

effect of Zeitgeber type ($p < 0.0001$). Post-hoc multiple comparisons revealed that a^o value of phase coherence vector relative to a specific phase reference point in the social interaction cycles is -0.58 hr. The phase distributions of ants subjected to LD and social cycles are significantly different based on Watson-Williams test ($p < 0.0001$); however, phase distributions of ants entrained to LD and social cycles did not differ ($p > 0.05$) when phase values were estimated relative to the Zeitgeber. Thus social cycles are found to synchronize ant workers although less effectively compared to LD cycles.

2.3.3 ***Group-wise social interactions synchronizes the phase of locomotor activity rhythm of hosts:*** Since we found phase synchrony to occur as a result of pair-wise social interactions, we asked if such a phenomenon exists at the colony level where multi-individual interactions are more likely to take place. We marked 27 workers (guests) living under LD cycles with non-toxic paint, and allowed them to visit a host colony daily. The host colony was maintained under DD and comprised of unmarked workers, virgin queens and males. The visits were timed to occur in a daily fashion for exactly 12 hours and to coincide with the night phase of the guest ant workers. The guests were introduced into the host colony, at 11:00 hr and taken out at 23:00 hr. To evaluate the effect of such visits on the circadian clock of the hosts after 10 days of visit, virgin queens and unmarked workers (hosts) were removed from the colony, and their locomotor activity behaviour was recorded individually under DD. To assess the phase of their circadian clocks during cyclic social interaction we tracked the phase of their locomotor activity rhythm by drawing regression line through the onsets of activity and extrapolating it back to the last day of social interaction cycle (Figures 2a, b). The magnitude of phase coherence vector of virgin queens is 0.57, with a mean angle of +

0.49 hr, and the distribution of phase is found to be non-uniform as per Rayleigh's test ($p < 0.05$) (Figure 2c). The magnitude of phase coherence vector of workers is 0.74, with a mean angle of + 2.15 hr, and the distribution of phase is found to be non-uniform as per Rayleigh's test ($p < 0.001$) (Figure 2c).

ANOVA on the r values showed that the effect of caste is statistically not significant ($p = 0.44$), while ANOVA on a^o values showed significant effect of caste ($p < 0.005$). Post-hoc multiple comparisons revealed that workers start activity significantly earlier than virgin queens ($p < 0.005$). However, the phase distributions of workers and virgin queens are statistically not significantly different as per Watson-Williams test ($p > 0.25$). The magnitude of phase coherence vector of the colony as a whole is 0.65, with a mean direction of + 1.54 hr, and the phase distribution is not non-uniform as per Rayleigh's test ($p < 0.001$), which suggests that both virgin queens and workers are synchronized equally well, however, onset of activity of workers occurs one and half hours earlier than queens. We were unable to record the locomotor activity of males as most of them died within the first few cycles in isolation.

2.3.4 ***Timing of pair-wise interaction is critical in determining phase synchrony among hosts:*** One can think of at least two ways in which social synchronization may be achieved - if information about time is inferred by hosts based on the phase of the guest's clock, or hosts simply assume the duration of social interaction as day or night, irrespective of time in the guest's clock. To test between these two possibilities we used guest workers which were entrained in same LD regime as hosts, and allowed them to interact with host workers for 12 hr daily for a period of 8-9 days (Figure 4a). However, guests were allowed to enter the interaction arena with hosts only at mid-day and allowed

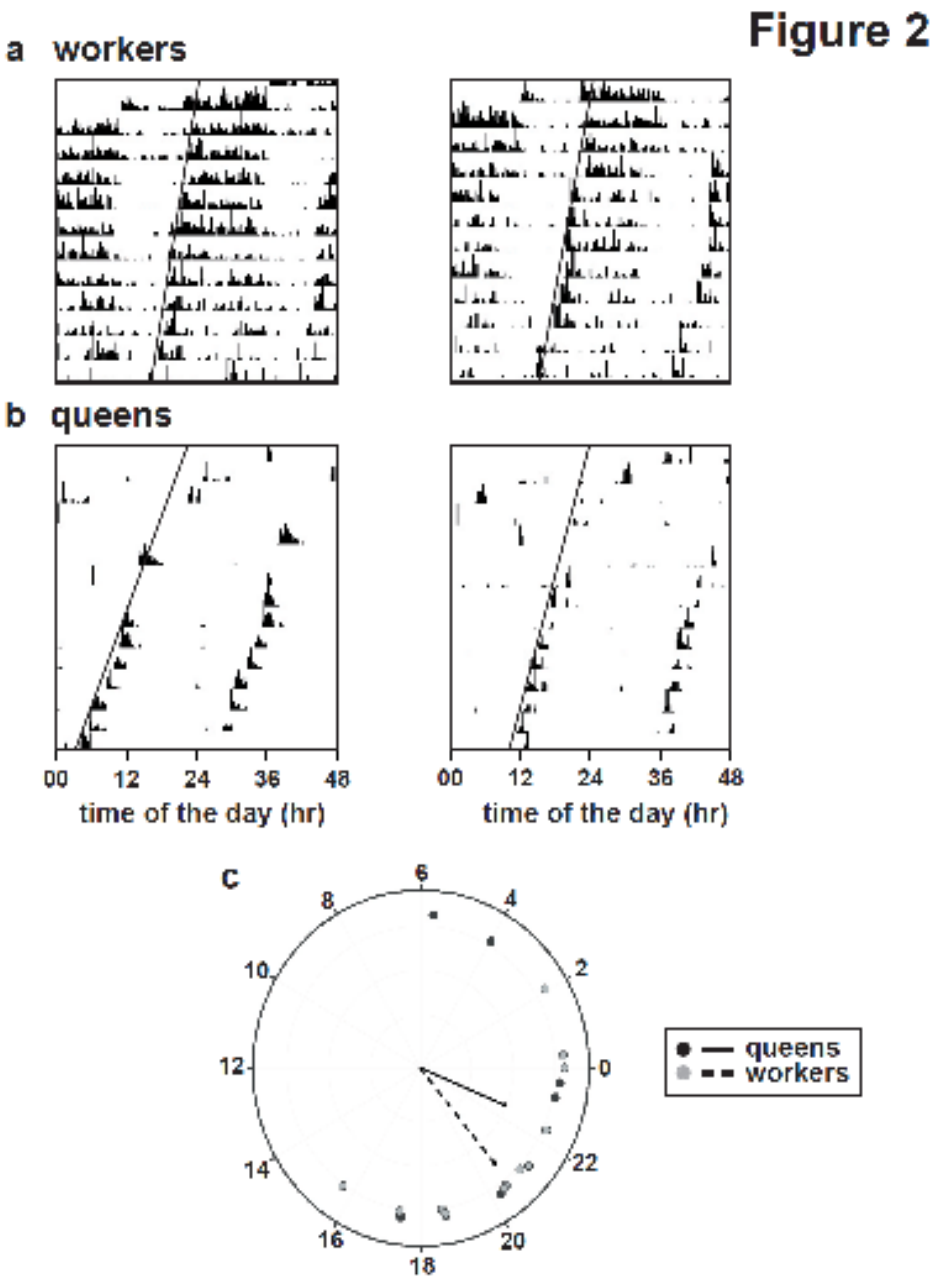


Figure 2 ***Group-wise social interactions synchronizes the phase of locomotor activity rhythm of hosts.*** (a) Actograms of two representative workers (out of $n = 12$), and (b) two virgin queens (out of $n = 11$) under constant darkness (DD), who had previously been subjected to 10 days of social interaction (SI) cycles with guest worker ants under colony conditions. Guests came from 12:12 hr LD regime (with lights-on between 23:00 hr and 11:00 hr). Guests interacted with colony members daily between 11:00 hr to 23:00 hr (night phase of the guest worker ants). See text and methods for details. (c) The phase coherence vectors of the host queens (black circles with solid line), and workers (grey circles with dashed line) are shown in a circular plot. The magnitude of phase coherence vector of queens ($r = 0.57$) and workers ($r = 0.74$) does not differ statistically ($p = 0.44$), however, its direction is significantly different ($p < 0.005$). Phase distributions of queens ($p < 0.05$) and workers are non-uniform ($p < 0.001$). The two distributions do not differ as per Watson-Williams test ($p = 0.25$). All other details same as in Figure 1.

to remain until mid-night (S group). The magnitude of the phase coherence vector of the S hosts is 0.71, with a mean angle of – 2.72 hr, and the phase distribution is found to be non-uniform as per Rayleigh's test ($p < 0.02$) (Figure 4c). In a separate experiment we used guest workers which were entrained to LD cycle out of phase by about 180^o relative to the prior LD cycle of host workers, and allowed them to interact with hosts daily for a period of 8-9 days (Figure 4b). However, guests were allowed to enter the host colony only at mid-night and remain until mid-day (O group). The magnitude of the phase coherence vector of O hosts is 0.72, with a mean angle of + 3.79 hr, and the phase distribution is found to be non-uniform as per Rayleigh's test ($p < 0.005$) (Figure 4c).

ANOVA showed that the effect of guest's phase on r values is statistically not significant ($p = 0.51$), while those on a^o valued showed significant effect of guest's phase ($p < 0.001$). The phase distribution of hosts receiving in-phase and out-of-phase guests is significantly different ($p < 0.001$) as per Watson-Williams test (Figure 4c).

2.4 Discussion

Our studies demonstrate that worker ants from colonies of the day active ant species *C. paria* are able to entrain the circadian locomotor activity rhythm of other workers and virgin queens maintained under DD. Cyclic social interactions result in the entrainment of circadian rhythms and social cues turned out to be an effective Zeitgeber in ants. Workers exposed to LD cycles are capable of entraining clocks of other workers and of a colony confined to DD. While circadian rhythms of both workers and queens entrained to cyclic social interaction with workers, worker ants assume a significantly advanced phase-relationship with the social cycles compared to virgin queens (Figure 2). Furthermore, our study suggests that phase of social interaction appears to be critical;

Figure 3

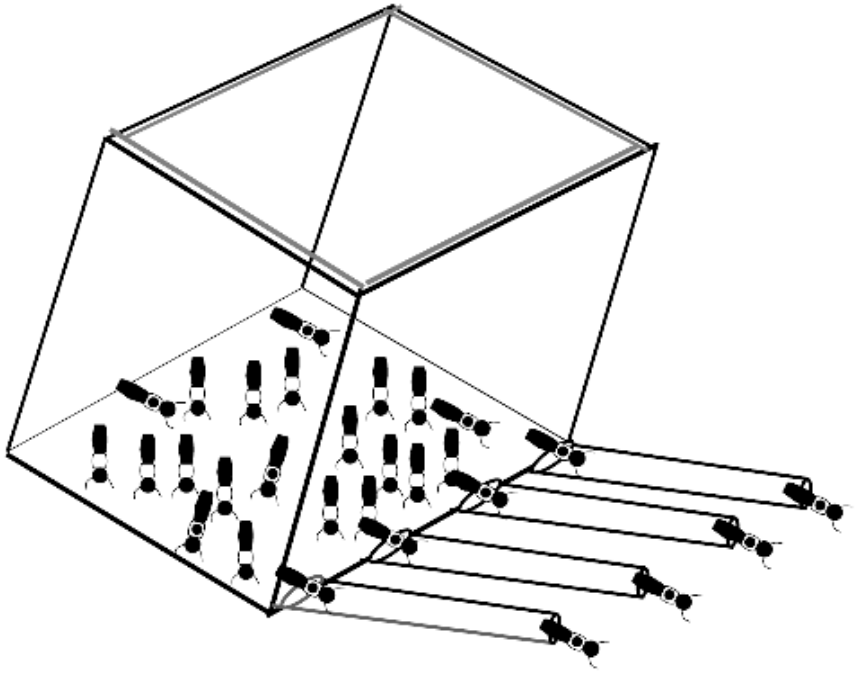
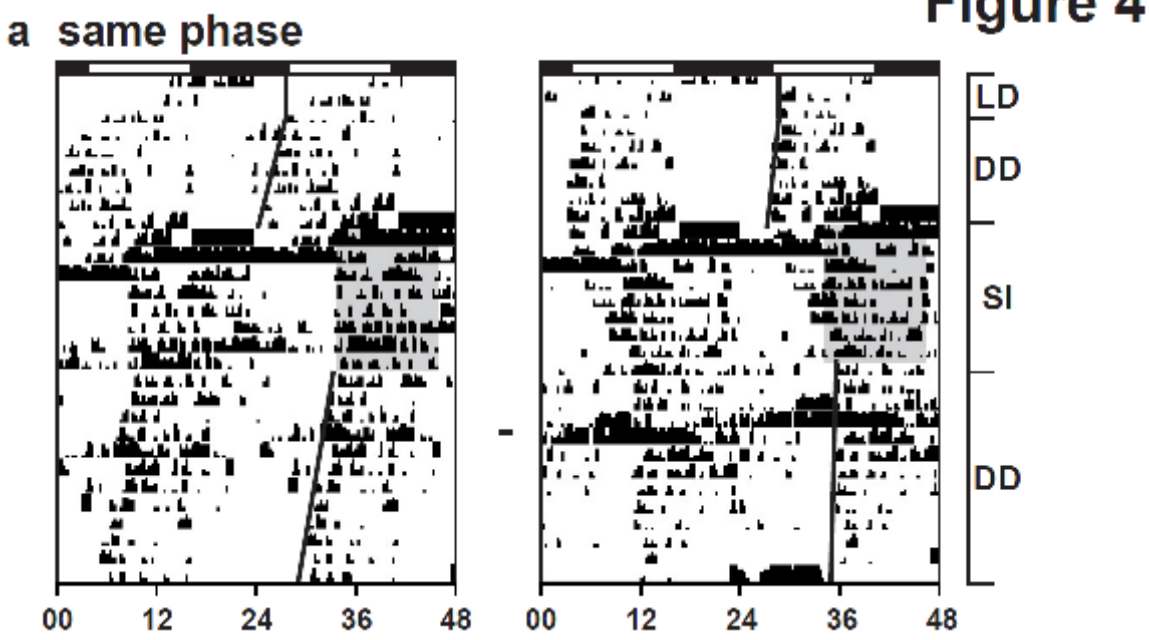


Figure 3 Diagram of colony setup for social interactions, dark spotted ants were allowed to move out of colony at 23:00 hr and returned to the colony at 11:00 hr.

Figure 4



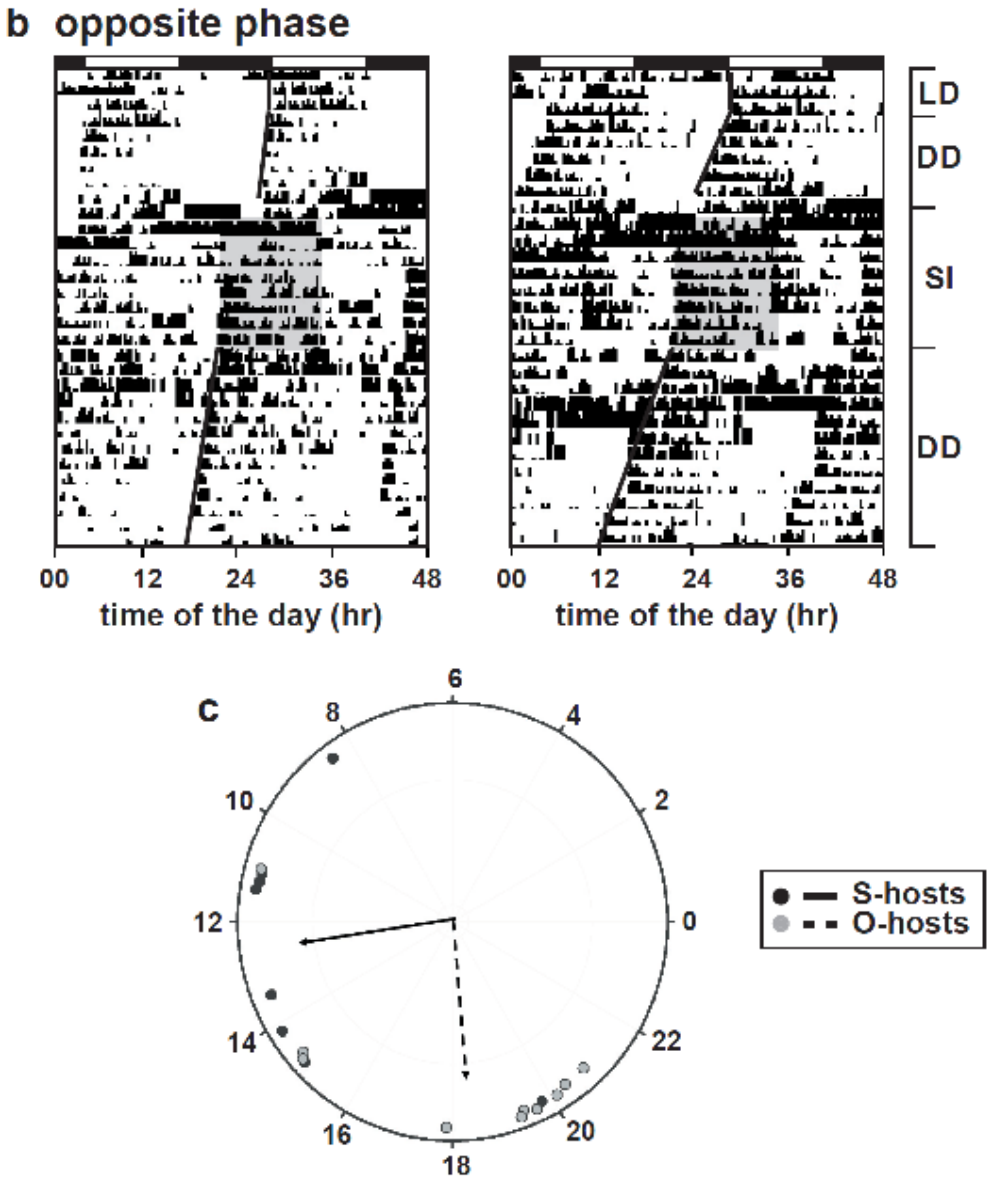


Figure 3 ***Timing of pair-wise interaction is critical in determining phase synchrony among hosts.*** Actograms showing locomotor activity behaviour of two representative host workers (out of $n = 18$), first kept under 12:12 hr light/dark (LD) cycles for 4 days, and then released into constant darkness (DD) for 5-6 days before being subjected to pair-wise social interaction (SI) cycles with guest worker ants who were maintained under LD cycles, either in (a) same or (b) opposite LD cycles as the hosts prior to being released into DD. In-phase guests were paired with hosts ($n = 8$) from 10:00 hr until 22:00 hr, and out-of-phase guests were paired with hosts (hosts, $n = 10$) from 22:00 hr until 10:00 hr. Circular plot of S and O group of hosts is shown in (c). Solid line with black circles indicate phase coherence vector and activity onset phase of hosts paired with in-phase guests, and dashed line with grey circles represent those of hosts paired with out-of-phase guests. The magnitude of phase coherence vector of S group ($r = 0.71$) and O group of hosts ($r = 0.72$) does not differ statistically ($p = 0.51$), however, their direction is significantly different ($p < 0.001$). The phase distribution of activity rhythm is non-uniform in both cases ($p < 0.005$), and the distributions are different as per Watson-Williams test ($p < 0.001$). All other details same as in Figure 1.

however, state of circadian rhythm of guests may also have some effect on the phase of entrainment. While entrainment of circadian clocks via social interaction has been reported in a wide range of organisms, many previous studies suggest little or no influence of social cues in communicating temporal information. For example, aggressive interactions between male rats or socio-sexual interaction between male and female rats do not have any impact on their circadian clocks (Meerlo and Daan, 1998). Similarly in cockroach *Leucophaea maderae*, cyclic social interactions fail to cause measurable effect on circadian clocks (Knadler and Page, 2009). In eusocial ants, division of labor forms the structural and functional bases for social organization (Hölldobler and Wilson, 1990), and therefore they are likely to respond to social cues more readily than insects that are not known to be social such as *Drosophila* (Chapter 3), cockroaches, and rats (Meerlo and Daan, 1998; Knadler and Page, 2009). Social insects such as honeybees and ants have evolved division of labor, wherein a particular caste or group brings in food for the colony (foragers), and other groups take care of other requirements of the colony (soldiers and nurses) (Hölldobler and Wilson, 1990). In *Camponotus* ant colonies, reproductive castes are represented by virgin queens and males, and the non-reproductive caste by major, media and minor workers which normally live in dark underground nests (Sharma et al., 2004b). The knowledge of local time becomes more decisive when reproductive castes have to undertake mating flight. The virgin males and queens synchronize their mating flights with utmost accuracy and precision (Haddow et al., 1966). It is believed that worker ants time circadian clocks of the reproductive castes, who are mostly confined to constant conditions of the colony (Hölldobler and Wilson, 1990). Our previous studies have shown that major workers can

be classified into three groups, those that (i) readily entrain to LD cycles and free-run under DD, (ii) display masked activity/rest behaviour under LD, but are arrhythmic under DD, and (iii) are arrhythmic under both LD and DD (Sharma et al., 2004b). In the present study we show that host ants maintained under DD entrain to cyclic social interactions, when made to interact pair-wise for 12 hr daily with guests coming from LD cycles. These results thus suggest that foragers, who periodically shuttle in and out of colony, entrain circadian clocks of individuals that are confined to the colony. These results are consistent with early findings in several species of birds and mammals, where presence and absence cycles are found to entrain circadian clocks of other individuals maintained under DD (Gwinner, 1966; Menaker and Eskin, 1966; Marimuthu et al., 1981; Viswanathan and Chandrashekaran, 1985; Goel and Lee, 1997a). Social cues in eusocial insects are meaningful only if it is effective at the colony level, and are capable of bringing about synchrony among members of different castes. We show that guest workers when made to interact with a host colony of workers and virgin males and queens, quite effectively synchronize circadian clocks of hosts. The circadian activity rhythm of both workers and queens is synchronized equally stably, suggesting that there is no difference in the level to which these two castes are synchronized. However, phase-relationships between activity rhythm and social interaction cycles differ between the workers and queens of the host colony, suggesting that during cyclic social interactions workers start activity significantly earlier than queens (Figure 2). Interestingly, phase-relationship of virgin queens (~0.49 hr before "dusk") is found to be close to the timing of their mating flights under natural conditions (~0.75 hr before "dusk") (Chapter 5). This suggests that virgin queens and workers residing in seemingly timeless environment

of the colony do keep track of local time by socially interacting with foragers who bring food to the colony. The results observed at the colony level are also consistent with the scenario where workers were made to interact one-on-one. At the level of the colony, presence of foragers is considered by members of the colony as subjective night, whereas when the foragers interacted one-on-one, their presence is considered as subjective day. This is not surprising as the carpenter ant species *C. paria* is day active, and a forager would meet another forager only during the day, while individuals living in the colony get to meet the foragers as a group only during night suggesting that social interactions can result in niche switching depending upon the nature of social interaction.

Is temporal information passed on from guests to hosts, or it is determined by the phase of social interaction between them? If the time of interaction is critical, S hosts should have onset close 10:00 hr, and O hosts at 22:00 hr, and if guest's endogenous timing is important, S hosts should have activity onset close to 04:00 hr, and O hosts close to 16:00 hr. On the other hand, if timing of host is supreme both groups of hosts should have activity onset matching their free running rhythms. From our study it is clear that while phase of social interaction is critical for social synchronization of the hosts, state of circadian rhythms of the guests is also important (Figure 4). This conclusion is based on the fact that guests with similar or out-of phase rhythms, when made to interact with hosts whose subjective day coincided either with the early or late subjective day of guests resulted in entrainment with about 6 hr difference in phase of entrainment. Such difference in phase of entrainment in the S and O type of hosts can be explained in terms of the differences in endogenous timing of the guests and hosts.

While it is clear that social cues act as Zeitgeber for the circadian rhythms of carpenter ant species *C. paria*, we do not yet know what signals these ants use to communicate information on time and it could be mechanical, olfactory, or auditory. While many organisms including fruit flies *D. melanogaster* (Levine et al., 2002; Krupp et al., 2008), diurnal rodent *Octodon degus* (Amir et al., 1999) use olfactory means to communicate temporal information via social interaction, several others such as birds (Gwinner, 1966; Menaker and Eskin, 1966), beavers (Bovet and Oertli, 1974) and bats (Marimuthu et al., 1981) use auditory means. It is noteworthy that in our study, individuals confined to the colony under DD entrained to social cycles, in spite of been given *ad libitum* food. In natural colonies, forgers are responsible for bringing in food, and we know from previous studies that cyclic availability of food can serve as Zeitgeber for circadian clocks, leading to entrainment (Mistlberger, 2009). Therefore, we can hypothesize that food cues associated with social interactions may serve as Zeitgeber when potent cues such as LD cycles are absent. Social cues may be quite helpful in maintaining synchrony among individuals under demanding ecological conditions such as caves, depth of the oceans, and underground nests such as those where *Camponotus* ants reside.

In summary the results of my study suggests that in carpenter ants *C. paria*, cyclic social interactions among workers and queen, for 12 hr act as Zeitgeber for their circadian clocks. Interestingly, social cues are as effective as light/dark cycles in bringing about stable entrainment of circadian locomotor activity rhythm. Worker ants (guests) are able to entrain the circadian locomotor activity rhythm of other workers and queens (hosts) confined to the colony in DD, when they are allowed to interact daily for

12 hr. The guests in our study can be equated with foragers ants who go out of the colony in search of food on a daily basis, and convey information on local time to other members of the colony. Furthermore, we show that phase at which guest workers interact with the hosts is important in determining synchrony during social interactions. Social cues may help ant colonies in tuning their clocks according to the local conditions, and hence serve as a timekeeper for the virgin males and queens, for whom mating is a species-specific critical event.

Chapter 3

Role of Social Cycles in Circadian Entrainment of *Drosophila melanogaster*

3.1 Introduction

Circadian clocks generate oscillations with near 24 hr periodicity and keep track of local time by entraining to external time cues (Zeitgebers) in a way that period of the rhythms that they control becomes indistinguishably close to 24 hr (Daan and Aschoff, 2001; Dunlap et al., 2004). These clocks use a wide range of time cues which include cycles of light, temperature, food, and social interactions (Daan and Aschoff, 2001; Dunlap et al., 2004; Sharma and Chandrashekaran, 2005). They make use of well developed signal transduction systems, dedicated to the perception of external time cues which can convey temporal signals to circadian clocks in a way that they can be translated to effectively modulate the overall behaviour and physiology. Such mechanisms for the perception of light signals have been quite extensively studied in mice and fruit flies *Drosophila melanogaster* (Emery et al., 1998; Stanewsky et al., 1998; Emery et al., 2000; Helfrich-Förster et al., 2001).

Although light is the primary and perhaps the most reliable Zeitgeber for circadian clocks in a wide variety of organisms, which acts through fairly well understood pathways, there is sufficient evidence to suggest that non-photic cues such as temperature, food, and social cycles may also serve as Zeitgebers (Saunders, 2002; Dunlap et al., 2004). For example in cockroaches (*Leucophaea maderae* and *Periplaneta americana*), temperature pulses cause phase shifts in the locomotor activity rhythm, and cycles of temperature can entrain the rhythm (Roberts, 1962). Entrainment to temperature cycles has also been observed in mosquito *Culex pipens pallens* (Chiba et al., 1993), and *Calliphora vicina* (Saunders and Hong, 2000), and fruit flies *D. melanogaster* (Matsumoto et al., 1998; Glaser and Stanewsky, 2005; Yoshii et al., 2005; Boothroyd et

al., 2007; Busza et al., 2007; Miyasako et al., 2007). Unlike light, entrainment to temperature is slow, and can be improved by the genetic manipulation of light sensitive morning oscillators (Busza et al., 2007). Temperature cycles are capable of entraining circadian locomotor activity rhythm in *Drosophila* even under constant light (LL) conditions (Glaser and Stanewsky, 2005), wherein locomotor activity and adult emergence behaviours become arrhythmic (Konopka et al., 1989). In *Drosophila*, temperature signals are perceived by chordotonal organs, which then relay it to clock neurons through yet unknown pathways (Sehadova et al., 2009). Clock neurons responsible for temperature entrainment are the dorsal group of cells (Busza et al., 2007; Miyasako et al., 2007; Picot et al., 2009), and the lateral posterior neurons (LPNs) (Miyasako et al., 2007). Thus *Drosophila* seem to have dedicated group(s) of clock neurons for light and temperature entrainment which is likely to help in efficient processing of temporal information. Further, these neurons may integrate temporal information through the clock network for circadian responses.

Many organisms use the presence of other individuals from the same or other species as time cues to the extent that cyclic presence and absence (PA) entrains their free running rhythms. For example, in organisms such as mice, bats, and birds, PA cycles of conspecifics entrain circadian locomotor activity rhythm (Menaker and Eskin, 1966; Gwinner, 1966; Marimuthu et al., 1981; Viswanathan and Chandrashekaran, 1985). In the bat species *Hipposideros speoris*, PA cycles of free-living conspecifics entrain circadian activity rhythm of individuals maintained in captivity inside a cave (Marimuthu et al., 1981), while rhythms of a different species of an emballonurid bat (*Taphozous nudiventris kachhensis*) also kept captive in the same cave remain unaffected suggesting

that social communication of temporal information in bats is species-specific (Marimuthu and Chandrashekaran, 1983a). Similarly in mice, PA cycles of the mother entrain circadian locomotor activity rhythm of pups (Viswanathan and Chandrashekaran, 1985), for at least 23-26 days of post-natal development (Viswanathan, 1999). Furthermore, in the Syrian hamster *Phodopus sungoru*, foster mothers were shown to be able to synchronize the rhythms of pups which had previously been entrained 12 hr out of phase by their biological mothers (Duffield and Ebling, 1998), suggesting that PA cycles can re-entrain circadian rhythms in hamsters. In the European rabbits *Oryctolagus cuniculus*, nursing by mother just for few minutes every day was able to entrain the oscillation of clock gene expression in the hypothalamus (Caldelas et al., 2007). The above studies suggest that PA cycles serve as Zeitgeber for the circadian clocks of a variety of mammalian species. While the precise nature of social signals still remains elusive, some of the likely candidates for signal transduction are via vision, olfaction, sound, and physical interactions (Davidson and Menaker, 2003).

Social cues are found to entrain circadian clocks of many insect species including eusocial honeybees. In honeybees, foragers trained to feed at a particular time of the day in one hive and then transferred to another hive in which the training schedule was different were found to forage at both times, suggesting that there is an influence of social interactions on the time of foraging (Medugorac and Lindauer, 1967). Honeybee queens caused greater shift in the phase of circadian rhythm of workers compared to a control group who were presented with a single worker (Moritz and Sakofski, 1991), suggesting that honeybee queens can modulate circadian clocks of workers. In fruit flies *D. melanogaster*, synchronization of circadian clocks by social cues was demonstrated in an

elegant study by Levine and co-workers (Levine et al., 2002). Wild type flies maintained in groups showed considerable degree of phase coherence in their circadian locomotor activity rhythm, suggesting synchronizing effect of social interactions on circadian clocks. When wild type flies were co-housed with per^{0} flies in 4:1 ratio, they show relatively less phase coherence than when they were maintained in homogenous groups. It was therefore suggested that synchrony among individuals living in homogeneous groups is achieved because of strong positive interactions among the group members (Levine et al., 2002; Krupp et al., 2008). Olfaction plays a key role in such social interactions because the phase coherence of paralytic olfactory mutant hosts such as $para^{Sbl\text{-}1}$ and $para^{Sbl\text{-}2}$ is not affected by the presence of arrhythmic *period* null (per^{01}) visitors (Levine et al., 2002). Peripheral clocks located in the olfactory neurons (Krishnan et al., 1999) were also implicated in such social interactions. The expression of clock genes in the fly head and oenocytes of wild type flies is altered when wild type flies were maintained with per^{01} flies in 4:1 ratio (Krupp et al., 2008). Rescue of circadian oscillation in the lateral and dorsal neurons in otherwise arrhythmic per^{01} mutant flies have little or no effect on the phase coherence of the group, suggesting that peripheral oscillators regulate social synchrony among hosts (Levine et al., 2002). Social interactions among flies involve pheromones released from the oenocytes (Wigglesworth, 1970) located in the fly abdomen (Demerec, 1994) which have recently been shown to have functional circadian clocks with cycling clock genes (Krupp et al., 2008). While previous studies do suggest that social interactions influence the phase of circadian rhythms in *D. melanogaster*, whether social cycles of PA can serve as a Zeitgeber for the circadian clocks of *Drosophila* remains an open question.

Here we present the results of our study which examined the effect of PA cycles of conspecifics on the circadian locomotor activity rhythms in fruit flies *D. melanogaster*. Wild type Canton S (*CS*) virgin males were first segregated into two groups, both of which were maintained for 4 days under 12:12 hr light/dark (LD) cycles. Thereafter one of the groups was transferred to constant darkness (DD) and served as 'hosts' to flies from the second group which continued to be under LD cycles and was used as 'visitors' to the first group by virtue of being co-housed with the host flies daily for 12 hr. The visitors were allowed to join the hosts daily during the dark phase of their native LD cycles, a schedule which was continued for 6-7 days thus providing PA cycles of conspecifics. Our results suggest that circadian clocks of fruit flies *D. melanogaster* do not entrain to social cycles, although PA cycles cause significant change in the phase of circadian locomotor activity rhythm.

3.2 Materials and methods

Freshly emerged *Canton S* (*CS*) males were collected and maintained under 12:12 hr LD cycles in glass vials (95 mm × 10 mm) as same sex groups of 30 individuals per vial. The flies referred to as hosts were individually loaded in locomotor activity tubes and subjected to LD cycles and then transferred to DD (dim red light of wavelength > 650 nm) for the rest of the experiment (Figures 1, 3, 6). A second set of flies with small cut on their wings (CS^{wc}), for ease of identification, were maintained under 12:12 hr LD cycles (as individuals or groups) and used as visitors. In some experiments we have used white eyed flies (*w*) maintained under LD cycles as visitors to study the effect of cyclic PA cycles of visitors from a different strain. For the PA cycles, visitors kept under LD cycles were transferred individually into the activity vial of host fly for 12 hr, either for

the entire duration of night (night visitors) or day (day visitors) with reference to the local day-night cycle; however, visitors were made to spend their night phase with hosts and light phase away from the hosts in the order to maintain their own clocks synchronized to LD cycles (Figures 1, 3, 6). This was continued daily for 6-7 days (henceforth such host flies will be referred to as flies with cyclic social interactions or simply CSI flies). The control flies were handled twice daily, in a manner similar to the CSI flies but did not receive any visitor (henceforth will be referred to as flies with cyclic disturbance or CD flies). In some experiments we have used another set of control flies, who were left undisturbed (henceforth will be referred as undisturbed control flies or UD flies). After treatments, we continued to record the locomotor activity behaviour of hosts under DD conditions to estimate their circadian phase on the last day of the cyclic treatment. The locomotor activity of flies was recorded in Drosophila Activity Monitors (DAM) system from Trikinetics, USA in 5 mm glass tubes. The activity data was collected in 15 min bins and analyzed with the help of CLOCKLAB software from Actimetrics, USA.

3.2.1 ***Effect of PA cycles on the phase synchrony of hosts:*** *CS* flies (hosts) (n = 59) were maintained under 12:12 hr LD cycles (with lights-on between 04:00-16:00 hr, local time) for 4 days and then transferred to DD. After 8 days in DD, another set of CS flies with cut marks on their wings (visitors), maintained throughout under 12:12 hr LD cycles (with lights-on during 08:00 – 20:00 hr), were made to join the hosts for 12 hr starting 20:00 hr, after which they were placed back in LD incubator for the subsequent 12 hr light phase before starting the next PA cycle (Figure 1). This continued daily for 7 successive days. The CD flies (n = 31) were first maintained for 4 days under 12:12 hr LD cycles and then transferred to DD. After 8 days, CD flies were handled twice daily,

once at 08:00 hr and then at 20:00 hr, in a manner similar to the CSI flies. After 7 days, locomotor activity behaviour of both CSI and CD flies was recorded under DD for a minimum of 10 days.

3.2.2 ***Effect of PA cycles of hosts with different visiting hours:*** In this experiment, host flies were presented with two types of visitors entrained under LD cycles 12 hr apart. We maintained three groups of host flies under 12:12 hr LD cycles (with lights-on during 14:00-02:00 hr) for 4 days and then transferred them to DD for 6 days. The first groups (n = 28) received visitors maintained in LD cycles (with lights-on between 08:00-20:00 hr – Group 1 visitors), while the second group (n = 50) received visitors maintained under LD cycles (with lights-on either during 20:00-08:00 hr – Group 2 visitors). Visitors interacted with hosts for 12 hr daily for 7 successive days, i.e. during their respective night phases from 20:00-08:00 hr (Group 1) or 08:00-20:00 hr (Group 2) (Figure 3). The third group was treated as CD controls (n = 30), and handled similar to the two CSI group of hosts but were not presented with visitors. After treatment the locomotor activity behaviour of flies from all three host groups was recorded in DD for the next 10 days.

3.2.3 ***Effect of PA cycles of clockless visitors:*** Wild type *CS* host flies were maintained under 12:12 hr LD cycles (with lights-on during 14:00-02:00 hr) for 4 days and then transferred them to DD. After 6 days, the hosts were divided into three groups of which one group (n = 55) was presented daily with per^0 visitors and another group (n = 50) with *CS* visitors. Both types of visitors were maintained under LD cycles (with lights-on during 08:00-20:00 hr), and during 7 successive cycles of PA, were made to spend their entire night phase with hosts kept in DD. After 4 days, the third group of flies (n = 28) were handled in the morning and evening (CD) in a manner similar to the CSI flies

(similar to Figure 1). Following 7 days of treatment, locomotor activity behaviour of CSI and CD flies was recorded under DD for a minimum of 10 days. Hosts visited by the two types of night visitors (*CS* vs per^0) were compared with CD controls to estimate the effect of intact and clockless visitors on the phase synchrony of hosts.

3.2.4 ***Effect of PA cycles on highly phase desynchronized hosts:*** To create a set of flies with a high degree of phase desynchrony *CS* flies were first placed under six different LD cycles (with lights-on during 05:00-17:00 hr, 08:00-20:00 hr, 11:00-23:00 hr, 17:00-05:00 hr, 20:00-08:00 hr and 23:00-11:00 hr), after which equal number of flies from each regime were pooled to form composite groups of 50-60 flies per vial. The magnitude of phase coherence vector of this set was as low as 0.0004 (on a scale of 0-1). Flies from this highly phase de-synchronous set were divided into three groups. The first group (n = 38) was introduced into DD and after 6 days were made to interact between 08:00 hr and 20:00 hr with *w* males (entrained under LD cycles with lights-on during 20:00-08:00 hr), the second and third groups were used as CD (n = 42) and UD (n = 30) controls, respectively (Figure 6). Following 7 days of treatment, locomotor activity behaviour of CSI, CD and UD flies was recorded under DD for a minimum of 10 days.

To ascertain sex-related effects on circadian rhythm a similar experiment was carried out with female visitors. First individual host males from the highly phase desynchronized set were divided into four groups. The following treatment was given to three groups between 08:00 hr and 20:00 hr daily: first group (n = 26) was presented with *w* females, second group (n = 22) with *w* males, third group (n = 24) was handled twice a day at 08:00 hr and 20:00 hr (CD controls), and fourth group (n = 26) was left unperturbed (UD controls). The visitors, both males and females were maintained under

12:12 hr LD cycles with lights-on during 20:00-08:00 hr (Figure 6). Following 7 days of treatment, locomotor activity behaviour of CSI, CD and UD flies was recorded under DD for a minimum of 10 days.

3.2.5 ***Effect of PA cycles on free running rhythm of CS flies:*** We first maintained two groups of *CS* flies (n = 17 and n = 18) for 4 days under LD cycles (with lights-on between 08:00 hr and 20:00 hr), and then transferred them to DD. From day one onwards the first group was presented with visitors between 08:00 and 20:00 hr (*w* males maintained under LD cycle with lights-on during 20:00-08:00 hr) while the second group was disturbed twice daily in the morning (at 08:00 hr) and evening (at 22:00 hr). Following 4 days of treatment, locomotor activity behaviour of CSI, CD flies was recorded under DD for a minimum of 8 days.

3.2.6 ***Effect of PA cycles on flies with labile circadian clocks:*** Temperature sensitive *shibre*^{ts} gene was ectopically expressed in the Pigment Dispersing Factor (PDF)-positive clock neurons (*pdfGAL4/UASshibre*^{ts} henceforth will be referred as *shibire* flies). Two groups of host male flies were entrained under LD cycle for 4 days and then transferred to DD. From the first day in DD onwards, one group (n = 21) was subjected to PA cycles of *w* males between 08:00-20:00 hr (entrained under 12:12 hr LD cycles with lights-on during 20:00-08:00 hr) and the other group (n = 18) to cyclic disturbance. Following 4 days of treatment, locomotor activity behaviour of CSI and CD flies was recorded under DD for a minimum of 8 days.

The locomotor activity data thus obtained was used to determine the phase of entrainment during treatments. A regression line was drawn through the daily offsets of activity following treatments, by extrapolating back to the last day of treatment. The

phase values thus obtained were subjected to circular vector analysis (Batschelet, 1981) to obtain the magnitude (on a scale 0 to 1), and direction (1-360^0 or 1-24 h) of the phase coherence vector. A magnitude (r) of 1 would mean all individuals in a given set have exactly the same phase, while a magnitude of 0 would mean that individuals in the group are highly phase desynchronized. The direction (a^0) of the phase coherence vector would assume values between 1^o to 360^o or 1 to 24 h (15^o equals to one hour). In every experiment, phase data was subjected to bootstrapping where re-sampling of data was done with replacement to generate replicate data sets. The r and a^o values thus obtained were used as replicates for analysis of variance (ANOVA), where r and a^o values were treated as fixed factors. ANOVA was followed by post-hoc multiple comparisons using Tukey's test. In addition, we subjected phase distributions of experimental and control treatments to Watson-Williams test to see if the phase distribution of CSI flies was significantly different from CD and/or UD controls. All statistical analysis was implemented using STATISTICA^{TM} (StatSoft, 1995) and circular statistics methods (Batschelet, 1981).

3.3 Results

3.3.1 ***Phase synchrony of host males is reduced by PA cycles of conspecifics:*** To study the effect of PA cycles on circadian locomotor activity rhythm we entrained male *CS* flies to LD cycles with lights-on during 04:00-16:00 hr for 4 days and then transferred them to DD, where they were subjected to one-on-one social interaction via PA cycles of male visitors who were maintained under LD cycles. During this period, male visitors were co-housed with host flies for part of the LD cycles, experiencing the dark phase in the company of the host fly and during the light phase were solitary (see method, Figure 1).

The *r* value of phase coherence vector of CSI flies is significantly smaller than that of CD controls ($p < 0.0005$), and the a^o values of CSI flies are significantly different from CD controls ($p < 0.001$) (Figure 2a-c). However, phase distributions of CSI and CD flies do not differ statistically as per Watson-Williams test ($p > 0.25$) (Figure 2c). Thus we find that the host flies that experienced cyclic social interactions were less synchronous than those that were merely disturbed periodically. This suggests that PA cycles reduce the phase synchrony of hosts.

3.3.2 ***PA cycles of conspecifics with different visiting hours influence the extent of phase synchrony among hosts:*** Since it is possible that the level of synchrony achieved is dependent upon the phase of circadian clocks of host flies at the beginning of social interactions (visiting hours), we conducted another experiment where the hosts received visitors at one of the two phases which were 12 hr apart (Figures 3, 4). ANOVA on *r* data showed significant effect of visiting hours ($p < 0.0001$). Post-hoc multiple comparisons revealed that while *r* values of CSI flies presented with Group 1 ($p < 0.005$) or Group 2 visitors ($p < 0.0005$) is lower than CD controls, hosts who interacted with visitors from Group 1 visitors have lower phase coherence than those that interacted with visitors from Group 2 ($p < 0.0005$). ANOVA on a^o data also showed significant effect of visiting hours ($p < 0.0001$). Post-hoc comparisons revealed that a^o of hosts presented with visitors from either Group 1 ($p < 0.01$) or Group 2 ($p < 0.0005$) are significantly different from CD controls. Furthermore, a^o values of the two groups of hosts also differed significantly from each other ($p < 0.0005$, Figure 4d-f), demonstrating that visiting hours determine the outcome of social interaction on circadian clocks.

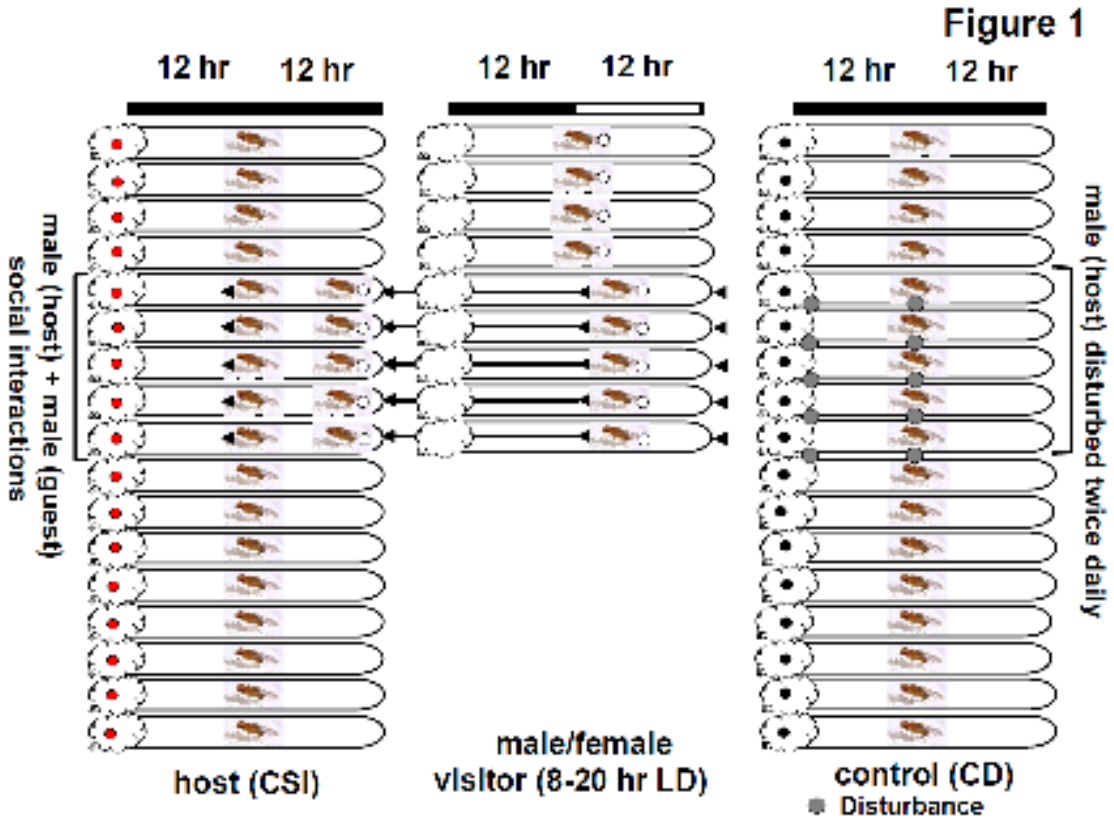


Figure 1 ***Effect of Presence and Absence (PA) cycles on circadian locomotor activity rhythm.***
The flies referred to as hosts were individually loaded in locomotor activity tubes and subjected to LD cycles and then transferred to DD (dim red light of wavelength > 650 nm) for the rest of the experiment (left panel). A second set of flies with small cut on their wings (CS^{wc}) [marked as small the white circle in the middle panel], for the ease of identification, were maintained under 12:12 hr LD cycles (as individuals or groups) and used as visitors. For the PA cycles, visitors kept under LD cycles were transferred individually into the activity vial of host for 12 hr; however, visitors always spent their night phase with hosts and day phase away from the hosts in the order to maintain their own clocks synchroned to LD cycle. This was continued daily for 6-7 days (henceforth the host flies will be referred to as flies with cyclic social interactions or simply CSI flies). The control flies were handled twice daily, in a manner similar to the CSI flies but did not receive any visitor (henceforth will be referred to as flies with cyclic disturbance or CD flies) [right panel]. After treatment both CSI and CD flies continued to remain in DD for the next 10 days.

Figure 2

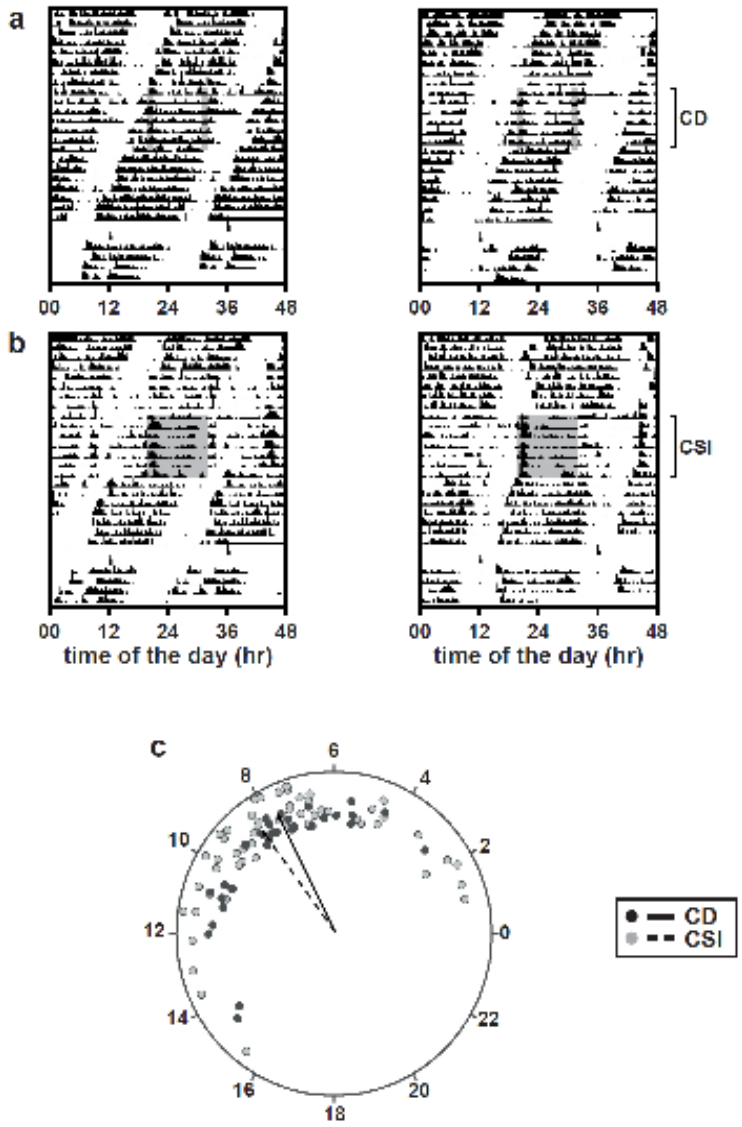


Figure 2 ***Phase synchrony of males is not altered by cyclic social interaction with conspecifics.*** Actograms of two representative host flies each from group of flies who were subjected to mechanical disturbance twice daily, at 20:00 hr and 08:00 hr (local time) (CD controls) (a), or to daily 12 hr social interaction with CS visitors between 20:00 hr to 08:00 hr (local time) (CSI flies) (b). The cyclic treatments were continued for 7 successive days, after which both CSI and CD flies were subjected to constant darkness (DD). The rectangular grey shade in (a) indicates timings of mechanical disturbance, while 12 h rectangular grey shades in (b) indicates timing of presence of visitor flies. Except for the rectangular dark grey areas, at all other times host flies were left undisturbed and solitary under DD. In the circular plot (c), grey circles and broken black line are phases and phase coherence vector of CSI flies subjected to presence and absence (PA) cycles, while black circles and black unbroken line phases and phase coherence vector of CD controls. The phase coherence vector of CSI flies is significantly different in magnitude ($p < 0.0005$) and direction ($p < 0.001$) than those of CD flies, however, the two phase distributions do not differ ($p > 0.25$) as per Watson-Williams test. Due to technical faults, activity recording was interrupted for ~1 day towards the end of the recording in DD as seen by the blank spaces towards the bottom of the actograms.

However, Watson-Williams test revealed that phase distribution of two groups of hosts does not differ statistically ($p > 0.20$) (Figure 4f).

3.3.3 ***Phase synchrony of hosts receiving clockless visitors is similar to those of controls physically disturbed twice a day:*** Having seen that the phase of circadian clock of the hosts has a significant effect on the social interaction mediated decrease in its phase synchrony, we asked whether robustness of the circadian clock of visitors may modify the outcome of social interactions on the circadian clock of host flies. ANOVA on *r* data showed significant effect of visitor's strain ($p < 0.0001$). Post-hoc multiple comparisons revealed that *r* value of CSI flies that received arrhythmic per^0 visitors does not differ statistically from CD controls ($p = 0.42$, Figure 5), however, *r* value of CSI flies receiving per^0 visitors is significantly greater than those that receive rhythmic *CS* visitors ($p < 0.01$, Figure 5d). ANOVA on the a^0 data showed significant effect of visitor's strain ($p < 0.0001$). Post-hoc comparisons revealed that a^o of CSI flies that received either type of visitors (per^0 or *CS*) is significantly different from CD controls ($p < 0.0001$), while that of CSI flies that received per^0 or *CS* visitors does not differ from each other ($p = 0.82$). The phase distribution of CSI flies that received *CS* visitors does not differ statistically from those that received per^0 visitors Watson-Williams test, $p > 0.25$ Figure 5d). Thus even arrhythmic visitors were able to evoke the same level of phase synchrony among hosts as cyclic mechanical disturbance (Figure 5c). Wild type conspecifics continued to show significantly lower ability to synchronize host's circadian clocks.

Figure 3

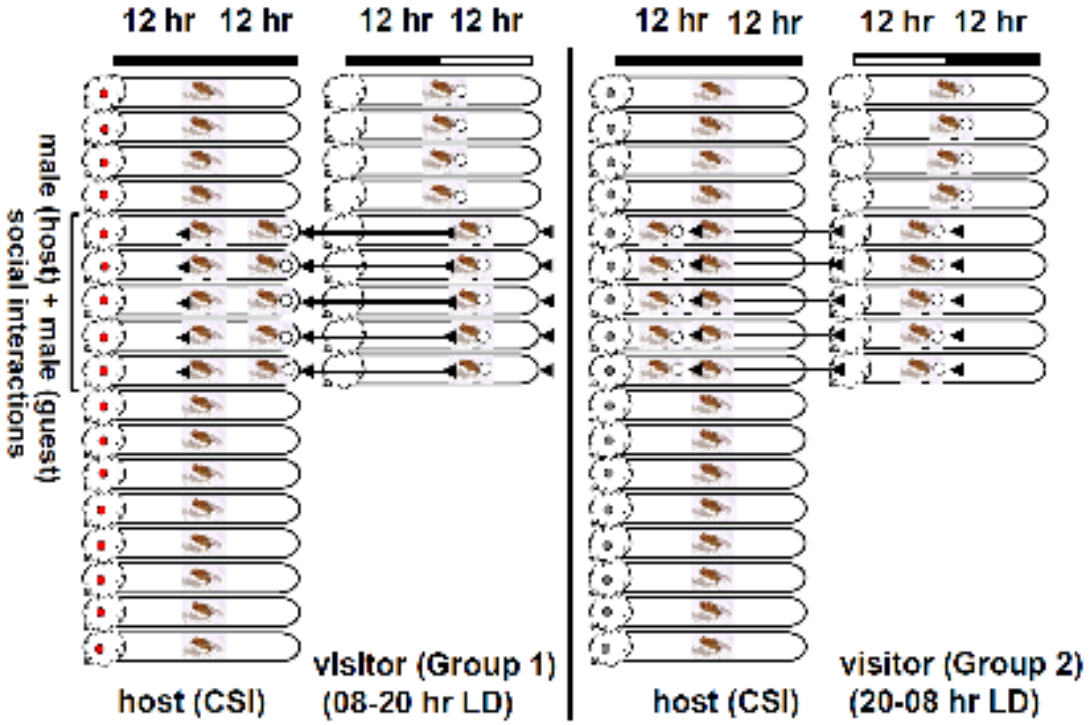


Figure 3 ***Effect of Presence and Absence (PA) cycles of guests with different visiting hours.***
Host flies were presented with two types of visitors entrained 12 hr apart. We maintained three groups of flies under 12:12 hr LD cycles (with lights-on during 14:00 – 02:00 hr) for 4 days and then transferred them to DD for 6 days. The first groups received visitors maintained under LD cycles (with lights-on either during 20:00 – 08:00 hr), while the second group received visitors maintained in oppositely phased LD cycles (with lights-on between 08:00-20:00 hr). Visitors interacted with hosts for 7 successive days, i.e. during their respective dark phases from 20:00-08:00 hr (Group 1) or 08:00-20:00 hr (Group 2). In addition to these two groups, we have CD controls as mentioned in Figure 1. After treatment flies from all three groups continue to remain in DD for the next 10 days.

Figure 4

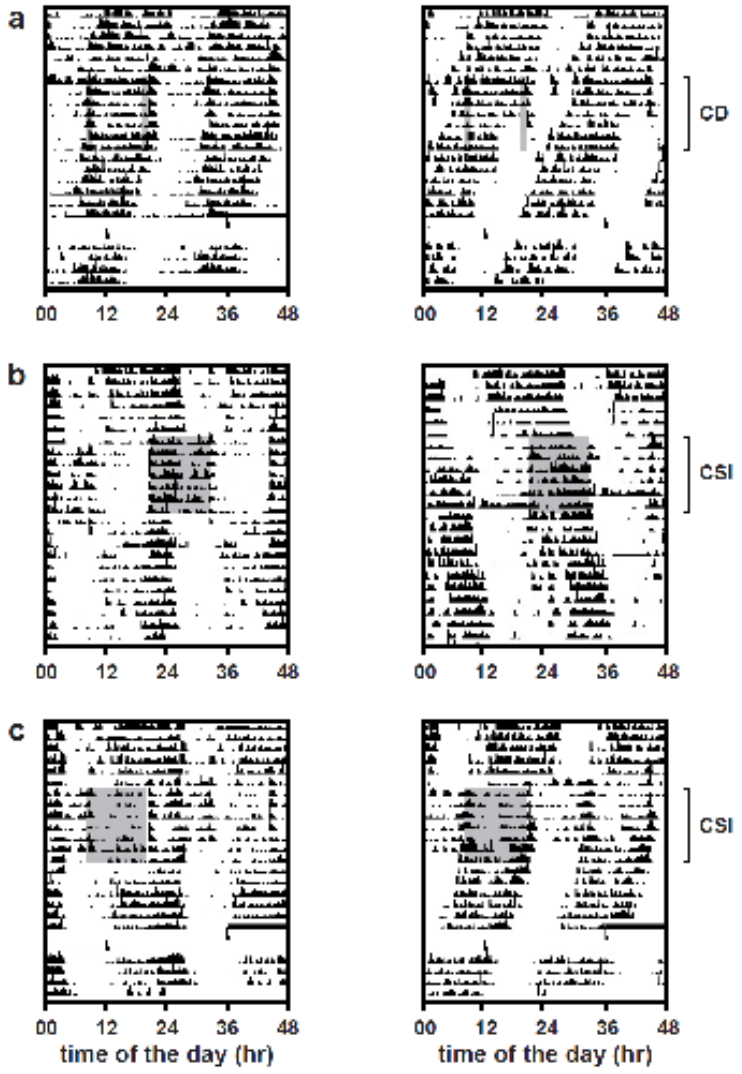


Figure 4 ***Extent of phase synchrony among hosts depends upon visiting hours of guests.*** Actograms of two representative control flies (CD controls) each from group of flies who were either disturbed twice daily at 08:00 hr or 20:00 hr (local time) (a), or presented daily with *CS* visitors for 12 hr (CSI flies) either between 20:00 and 08:00 hr (Group 1) (b) or between 08:00 hr and 20:00 hr (Group 2) (c). The cyclic treatments were continued for 7 successive days, after which both CSI and CD flies were subjected to constant darkness (DD). The rectangular grey shades in (a) indicate timings of mechanical disturbance; whereas 12 hr rectangular grey shades in (b and c) indicate cyclic presence of visitor flies. Except for the rectangular dark grey areas, host flies were left undisturbed and solitary under DD. All other details same as in Figure 2.

Figure 4

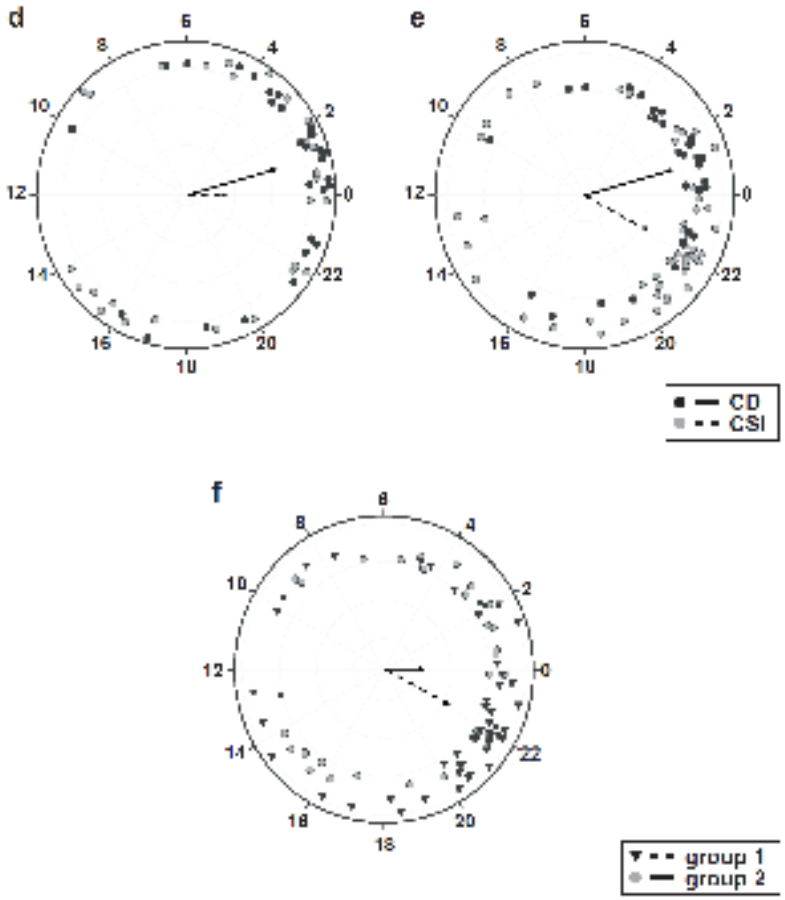


Figure 4 ***Phase distribution of hosts receiving guests at different visiting hours.*** In the circular plots (d-e), grey circles and broken black line show phase and phase coherence vector of CSI flies subjected to presence and absence (PA) cycles of visitors, while black circles and unbroken black line phases and phase coherence vector of CD controls. The magnitude ($p < 0.0005$) and direction ($p < 0.0005$) of phase coherence vector of hosts receiving Group 1 visitors (broken black line) are significantly different than those with Group 2 visitors (unbroken black line) (d-f). The phase coherence vector of hosts receiving Group 1 visitors is significantly different in magnitude ($p < 0.005$) and direction ($p < 0.01$) than those of CD controls. The phase coherence vector of hosts receiving Group 2 visitors is significantly different in magnitude ($p < 0.0005$) and direction ($p < 0.005$) than those of CD controls. However, phase distributions of CSI and CD, and CSI flies with Group 1 and Group 2 visitors do not differ statistically ($p > 0.20$) as per Watson-Williams test. All other details same as in Figure 2.

Figure 5

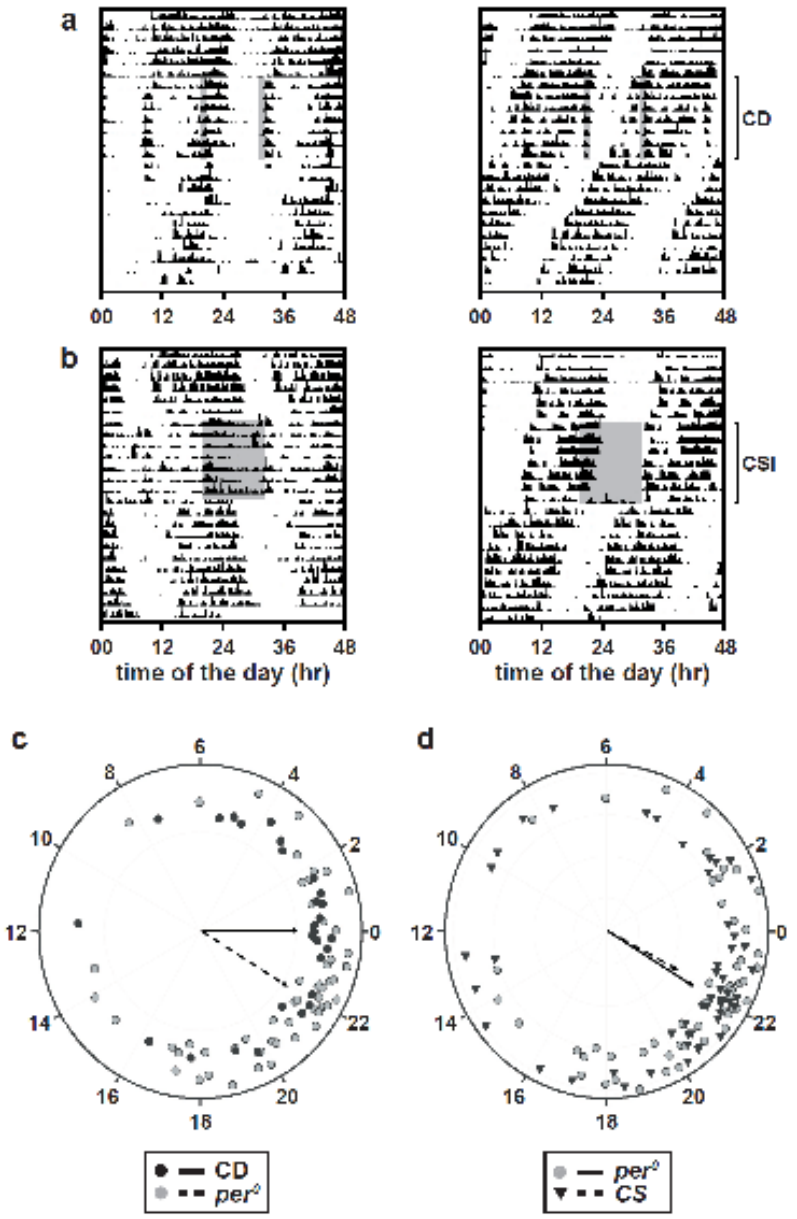


Figure 5 ***Effect of presence and absence (PA) cycles of visitors with and without functional circadian clocks on the phase synchrony of hosts.*** Actograms of two representative host flies (CD controls) each from group of flies who were either disturbed twice daily at 08:00 hr or 20:00 hr (local time) (a), or presented daily per^0 (b) visitors for 12 hr (CSI flies) between 20:00 and 08:00 hr. The phase coherence vector of hosts receiving per^0 visitors does not differ in magnitude ($p = 0.42$) but differ in direction ($p < 0.0005$) compared to those of CD controls (c). Furthermore, phase coherence vector of hosts receiving CS visitors is significantly different in magnitude ($p < 0.001$), but not in direction than those of hosts with per^0 visitors ($p = 0.82$) (d). The phase distributions of hosts exposed to PA cycles of CS and per^0 visitors do not differ statistically ($p > 0.25$) as per Watson-Williams test. All other details same as in Figure 2.

3.3.4 ***PA cycles of male or female visitors fail to synchronise phase of highly desynchronized hosts:*** Our studies thus far indicate that circadian clocks of the hosts determine the outcome of social interactions, and clockless per^0 visitors were equally effective in maintaining phase synchrony as CD controls. Therefore we tested whether the fact that hosts used thus far always had well synchronized clocks to start with may have an overriding effect on the phase synchrony even after cyclic social interactions. Hence we created a set of highly phase desynchronized hosts by pooling together flies from six different LD cycles (see methods, Figure 6). They were then subjected to 12:12 hr PA cycles of male visitors during the night phase of visitor's LD cycles. Two groups of hosts were used as controls in this experiment, one of them was disturbed twice daily (in the morning and evening) as described in previous experiments (CD controls) and a second control group was left undisturbed (UD controls). ANOVA on the *r* data showed a significant effect of treatment (CSI/CD/UD) ($p < 0.05$). Yet, post-hoc comparisons revealed that *r* values of the phase coherence vector of CSI flies do not differ statistically from those of CD and UD controls ($p > 0.05$) but there is significant difference between that of CD and UD controls ($p < 0.01$). The effect of treatments on a^o is statistically significant ($p < 0.01$). Post-hoc comparisons revealed that a^o of CSI flies does not differ from CD controls ($p = 0.13$) but is significantly different from UD controls ($p < 0.005$). The Watson-Williams test revealed that phase distributions of CSI and CD controls ($p > 0.20$), CSI and UD controls ($p = 1.00$), or CD and UD controls ($p > 0.20$) do not differ statistically (Figure 7a). Thus we see that cyclic social interactions with visitors also failed to synchronize host rhythms when hosts had highly desynchronous phases to begin with. Next we asked whether visitors of the opposite sex may be better able to cause

phase synchrony among hosts. To test this we subjected highly phase desynchronized flies (as described previously) to PA cycles of female visitors using a similar protocol as described above (Figure 6). Comparison of *r* and a^o values of the phase coherence vector revealed that phase and phase synchrony of CSI flies that received male or female visitors do not differ from CD controls ($p > 0.05$). The Watson-Williams test revealed that phase distributions of hosts receiving male or female visitors and of CD controls do not differ statistically ($p > 0.50$) (Figure 7b). Thus even PA cycles of female visitors were unable to cause phase synchrony among highly phase desynchronized male hosts.

3.3.5 ***PA cycles modulate circadian clocks of hosts with labile period:*** Next we asked whether a highly labile circadian clock can be entrained by social cycles. To do so we used flies in which the *shibre*^{ts} gene is ectopically expressed in the PDF-positive clock neurons (*pdfGAL4/UASshibre*^{ts}). The transgenic flies have circadian clocks with extremely labile circadian period which is known to change as a function of environmental temperature (Kilman et al., 2009). Two groups of host *shibire* male flies were entrained under LD cycles for 4 days and then transferred to DD. Starting from the first day in DD, one group (CSI, $n = 21$) was subjected to PA cycles by the introduction of *w* males between 08:00-20:00 hr (local time), which was their day phase based on the previously exposed LD cycles. These guests were entrained under 12:12 hr LD (with lights-on during 20:00-08:00 hr). The second host group was treated as controls and subjected to cyclic disturbance (CD, $n = 18$). Following 4 days of treatment, locomotor activity behaviour of CSI and CD flies was recorded under DD for a minimum of 10 days. Another experiment involved visits of *w* visitors to wild type CS hosts (CSI, $n = 17$) and CD flies as control ($n = 18$). ANOVA on *r* data showed significant effect of host

Figure 6

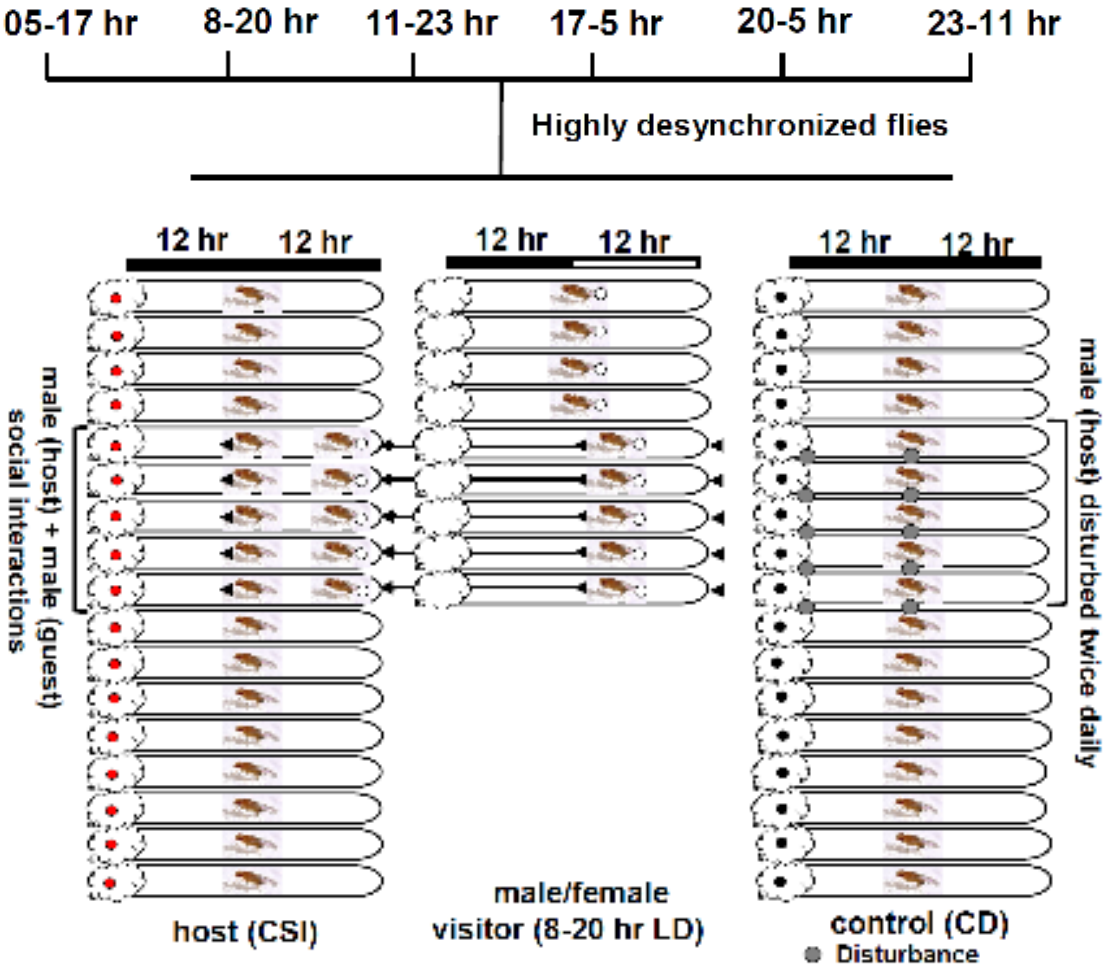


Figure 6 ***Effect of Presence and Absence (PA) cycles of visitors on hosts with highly desynchronized circadian rhythm.*** To create a set of flies with a high degree of phase desynchrony *CS* males were first placed in six different LD cycles (with lights-on during 05:00-17:00 hr, 08:00-20:00 hr, 11:00-23:00 hr, 17:00-05:00 hr, 20:00-08:00 hr and 23:00-11:00 hr), after which equal number of flies from each regime were pooled as groups. The magnitude of phase coherence vector of this set was as low as 0.0004 (on a scale of 0-1). Flies were divided into two host groups, one subjected to PA cycles of 12:12 hr (left) of visitors (entrained under LD12:12 hr cycle with lights on 20:00-8:00 hr) and flies of other group (right) was disturbed twice daily in the morning and evening (CD). After treatment both host groups continue to remain in DD for the next 10 days.

Figure 7

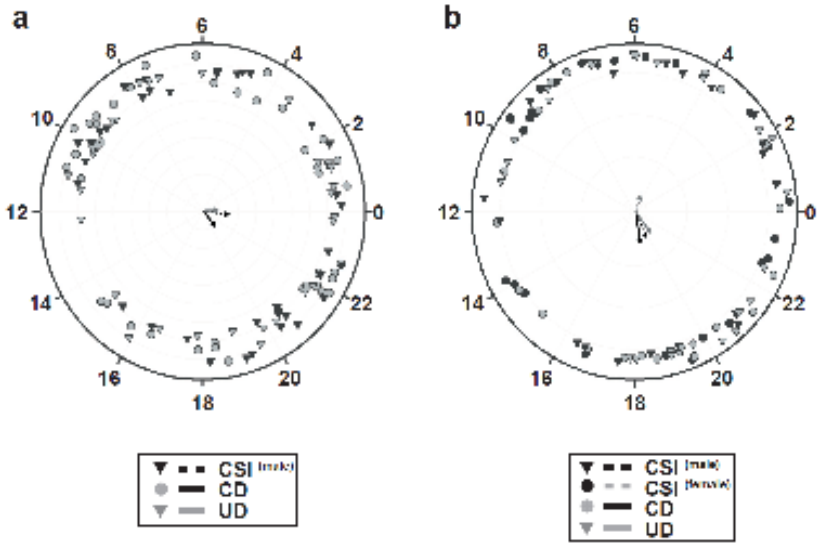


Figure 7 ***Effect of presence and absence (PA) cycles on the phase synchrony of highly phase desynchronized hosts.*** To create maximum degree of phase desynchrony among host flies, *CS* flies were subjected to six different LD cycles and then pooled to form three host groups. First group of flies were subjected daily to 12 hr presence and absence (PA) cycles, the second group was subjected to daily cyclic disturbance in the morning and evening (CD controls), and the third group were left undisturbed (UD controls). (a) Phase and phase coherence vector of CSI flies exposed to PA cycles is shown as black triangles and black broken line, CD flies as grey circles and unbroken line, and of UD flies as grey triangles and grey unbroken line. The magnitude of the phase coherence vector of CSI flies does not differ from CD or UD controls ($p > 0.05$). The direction of the phase coherence vector of CSI flies does not differ from CD ($p = 0.13$) but is significantly different from UD controls ($p < 0.005$). In (b) phase and phase coherence vector of CSI flies exposed to PA cycles of males are shown as black triangle and broken black line, and to PA cycles of females shown as black circles and broken grey line. The phase and phase coherence vector of CD/UD controls are shown as grey circles/triangles and black and grey unbroken lines. The magnitude and direction of phase coherence vector of CSI flies do not differ from CD and UD controls ($p > 0.05$).

strain (*shibire*/*CS*) ($p < 0.005$), and treatment (CSI/CD) ($p < 0.01$), however, effect of host strain × treatment interaction is statistically not significant ($p = 0.60$). Post-hoc comparisons revealed that *r* values of two CSI flies (that received *shibire* or *CS* visitors) do not differ from those of CD controls ($p > 0.05$). ANOVA on a^0 data showed significant effect of host strain ($p < 0.0001$), treatment ($p < 0.001$), and host strain × treatment interaction ($p < 0.005$). Post-hoc comparisons revealed that a^0 of socially interacting *shibire* flies is significantly lesser than CD controls ($p < 0.001$), while that of *CS* flies does not differ from CD controls ($p = 0.98$) (Figure 8).

3.3.6 ***cry^0 hosts with day visitors:*** Having seen that hosts with extremely labile circadian clocks can be phase shifted by PA cycles we asked whether PA cycles can influence circadian clocks of hosts whose internal synchrony is disrupted due to improper circadian photoreception such as those which occur in mutants of circadian photoreceptor CRYPTOCHROME. To study the effect of social interactions on light dependent splitting of activity rhythm of cry^0 flies, we maintained cry^{02} and cry^{03} males under 12:12 hr LD cycle (with lights-on between 20:00 hr and 08:00 hr) for 3 days, and then transferred them to LL. These mutants are rhythmic in LL in contrast to wild type flies (Dolozelova et al., 2003). Under LL they were made to interact daily for 12 hr with CS visitors coming from LD cycles (with lights-on between 08:00-20:00 hr) such that they now experience the day phase while interacting with hosts. Visual inspection of activity data reveals that there is no effect of visitors on the light-dependent splitting of cry^0 hosts, and splitting in CSI flies is as prevalent as in CD flies (Figure 9).

Figure 8

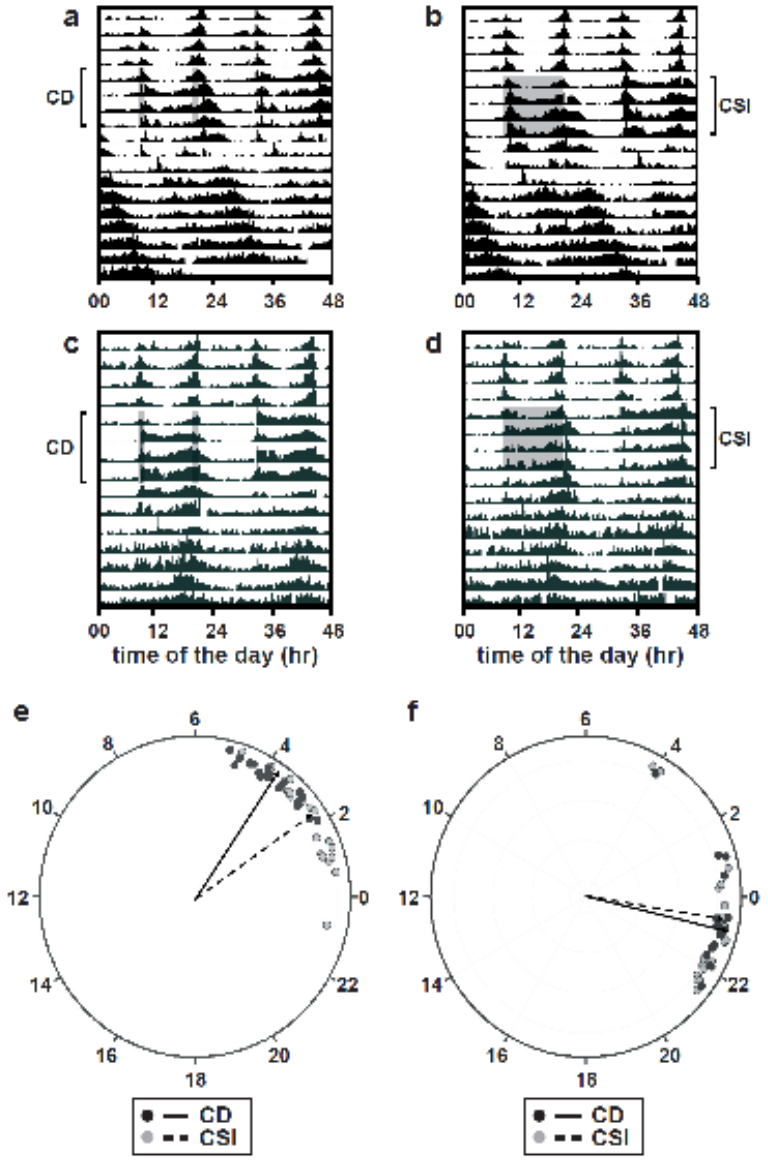


Figure 8 ***Effect of presence and absence (PA) cycles on the phase synchrony of phase synchronized hosts with labile circadian clocks.*** Average actograms of host CS flies (CD controls) who were either disturbed twice daily at 08:00 hr or 20:00 hr (local time) (a), or presented daily *white eyed* (*w*) (b) visitors for 12 hr (CSI flies) between 08:00 and 20:00 hr. Average actograms of host *shibire* flies (CD controls) from group of flies who were either disturbed twice daily at 08:00 hr or 20:00 hr (local time) (c), or presented daily *white eyed* (*w*) (d) visitors for 12 hr (CSI flies) between 08:00 and 20:00 hr. In CS (f) flies, presence and absence (PA) cycles of 4 days does not have any significant effect on the magnitude and direction of phase coherence vector ($p > 0.05$). In *shibire* flies (e) PA cycles of 4 days have significant effect on the direction of phase coherence vector ($p < 0.001$). Solid black circles and line indicate phase and phase coherence vector of CD controls, and grey circles and broken black line those of CSI flies.

Figure 9

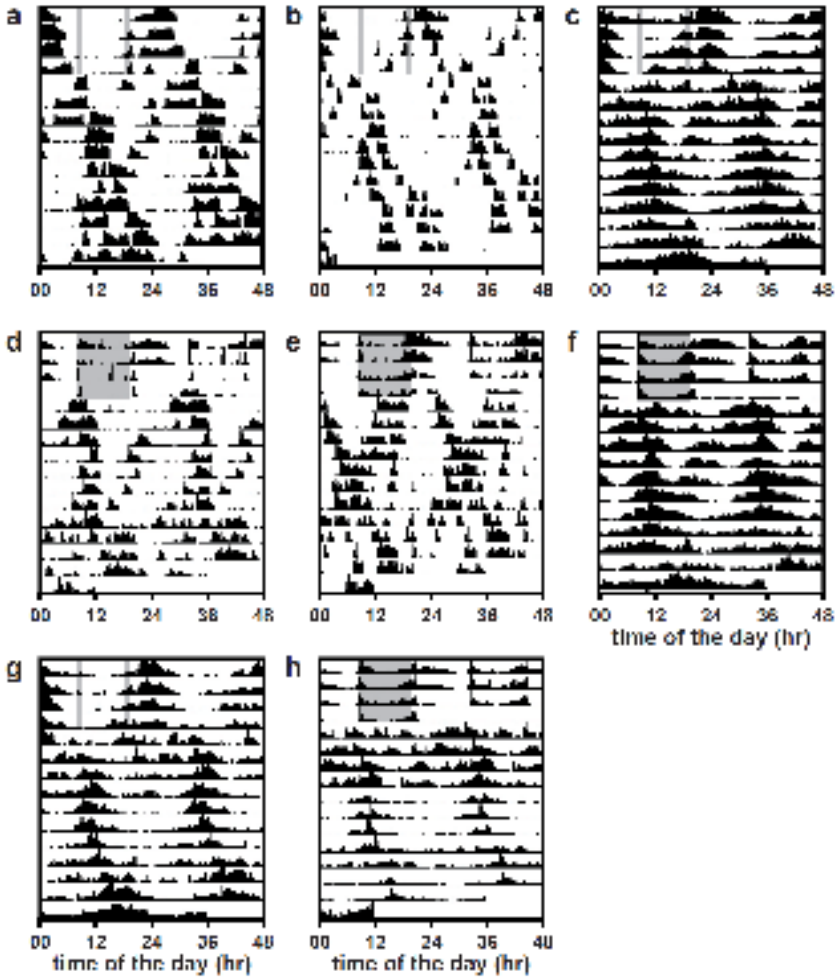


Figure 9 ***Social cycles have no impact on light-dependent splitting of Cry^{0} flies.*** Average actogram of host flies (*cry^{02}* - c; *cry^{03}* - g,) and one representative fly (*cry^{02}* - a; *cry^{03}*- b) of controls subjected to disturbance in the morning and evening. Average actogram of host flies (*cry^{02}* - f; *cry^{03}* - h,) and one representative fly (*cry^{02}* - d; *cry^{03}*- e) which were subjected to PA cycles of CS males for 4 days between 08:00 hr and 20:00 hr. PA cycles do not alter light-dependent splitting behaviour of these flies. All other details same as in Figure 2.

3.4 Discussion

In this report we show that the presence and absence (PA) cycles of 12:12 hr does not cause entrainment of circadian locomotor activity rhythm in fruit flies *D. melanogaster*. The phase of circadian rhythm of experimental flies remains by and large unaffected by PA cycles irrespective of the visitor's sex or strain. However, phase synchrony of hosts that received night visitors (Group 1) is significantly greater than those that received day visitors (Group 2). Relatively poor phase synchrony among host flies is primarily due to social interactions because CD controls who were handled similar to experimental CSI flies but were not presented with visitors showed significantly greater phase synchrony than flies exposed to PA cycles. Furthermore, phase synchrony among hosts is significantly greater when they received arrhythmic, clockless (per^0) visitors than when they received rhythmic wild type (*CS* or *w*) visitors, suggesting that social interactions visitors with functional clocks reduces phase synchrony of host flies. Ineffectiveness of PA cycles in synchronizing circadian locomotor activity rhythm of hosts is apparent when hosts with highly heterogeneous phases were made to interact with rhythmic visitors. While the phase of host's circadian rhythm does undergo change due to PA cycles, this is purely due to physical disturbance and not due to social interaction with visitors because magnitude and direction of phase coherence vector of CSI flies do not differ from CD controls, while those of CSI and CD flies differ significantly from UD controls. Furthermore, PA cycles where host males were made to interact with female visitors are equally ineffective in bringing about phase synchrony among hosts. Moreover, flies with labile circadian period (*pdfGAL4/UASshibire*) show some influence

of cyclic presence of visitors, suggesting that social cues may serve as a weak Zeitgeber for *Drosophila* circadian timing system.

Social cues have been shown to entrain circadian rhythms of a wide variety of organisms including mice, rats, bats, and birds (Halberg et al., 1954; Crowley and Bovet, 1980; Handelmann et al., 1980; Viswanathan and Chandrashekaran, 1985; Goel and Lee, 1997; Reppert and Schwartz, 1986). Social cues in the form of song cycles were reported to entrain circadian locomotor activity rhythm of birds (Gwinner, 1966; Menaker and Eskin, 1966), however, it was subsequently discovered that in some cases entrainment was due to physical disturbance and not due to social cues, e. g., in birds, cyclic white noise is also found to be equally effective in bringing about entrainment as the songs of fellow birds (Reebs, 1989). Among fish shoals, leaders who are aware of a foraging area act like informers and lead fellow fish to the foraging area in a time dependent manner by interacting with them. Synchrony within the group is directly related to the percentage of experienced fish in the group (Reebs, 2000), suggesting that social entrainment is responsible for phase synchrony of foraging behaviour. In fruit flies *D. melanogaster*, social interactions have been shown to influence the phase and period of circadian rhythm when flies are kept together in groups for several days (Levine et al., 2002). But until now the question remained as to whether social cues can entrain circadian rhythms of *Drosophila*. The results of our present study suggest that social cues may have very little influence on the circadian clocks of *Drosophila* (Figure 2). In fact daily social interaction with rhythmic visitors reduces phase synchrony of hosts to a relatively lower level than controls who were disturbed twice a day, once in the morning and then in the evening. Pair-wise social interaction with visitors during the day time (Group 2) does not

improve phase synchrony of circadian rhythm in hosts when compared to CD controls. However, between the day and night visitors, hosts that received Group 1 visitors have greater phase synchrony than those that received Group 2 visitors (Figures 4). This suggests that visiting hours play a critical role in determining the phase of synchrony in the hosts. This is consistent with the finding of a previous study where it was shown that "late visitors" do not have any effect on the circadian rhythm of "early hosts", while "early visitors" are able to alter the phase of "late hosts" significantly (Levine et al., 2002).

Apart from visiting hours, another factor which is likely to influence the outcome of cyclic social interactions is phase of the host's circadian clocks. In rats, fetuses born to suprachiasmatic nucleus (SCN)-lesioned mothers display desynchronized glucose utilization and disrupted N-acetyltransferase rhythm than those born to intact mothers (Reppert and Schwartz, 1986). Similarly in Syrian hamsters, pups born to SCN-lesioned mothers are found to have greater phase desynchrony than those born to intact mothers (Davis and Gorski, 1988), suggesting a role for circadian clocks of mother in socially entraining circadian clocks of pups. In a separate study it was shown that foster mothers were able to entrain circadian clocks of pups born to mothers that were 12 hr out of phase, although some pups continued to retain the phase of their biological mother suggesting circadian entrainment by social cues (Duffield and Ebling, 1998). We found that daily PA cycles of loss of function per^0 mutant visitors evoked similar response in hosts as cyclic physical disturbance, while PA cycles of visitors with intact clocks resulted in greater phase desynchrony among the hosts than CD controls (Figure 5) probably because of mismatch between the phase of host and visitor's rhythms. The per^0

visitors do not influence phase of the hosts because they have no phase identity of their own and they do not have rhythmicity in olfactory ability (Krishnan et al., 1999; Krupp et al., 2008). In a previous study, presence of per^0 flies amidst a group of wild type flies was found to result in decreased phase synchrony among the group members (Levine et al., 2002), and it was argued that this could be due lack of effective communication between CS flies in presence of per^0 visitors. Further, social interaction with visitors from different time zones has profound effect on the circadian phase of hosts (Levine et al., 2002), suggesting that reduced phase synchrony among the hosts in our study may be due to differences in the phases of host and visitor's clocks. Our study offers a different interpretation of the results. We propose that better phase synchrony among host flies interacting with per^0 visitors may be due to lack of effective communication between hosts and loss of function mutant visitors, while decrease in phase synchrony due to visitors with intact clocks may be because of the mismatch between the phase of circadian rhythms of visitors and hosts.

We further show that phase coherence in socially interacting flies is often comparable to CD control flies who experienced cyclic disturbance (Figures 2), suggesting that PA cycles can cause phase synchrony, however, observation of individual actograms suggests that there is no appreciable change in the phase of the rhythm following social interactions indicating that phase synchrony among host flies is not newly achieved. Given that cyclic physical disturbance is as effective as PA cycles, results of our study suggests that social cues at least in the form of PA cycles may not act as Zeitgeber for the *Drosophila* circadian timing system. To examine if such phase coherence is due to social interactions or merely an after-effect of living under periodic

LD cycles or living in groups, we first created maximum phase desynchrony in flies by keeping them in six different cycles and then pooling them together (Figure 7). We find that daily PA cycles are even less effective in bringing about synchrony in phase of highly phase desynchronized flies ($r = 0.0004$) suggesting that phase coherence observed in previous studies may be a carry over effect from previous entrainment regimes and not due to social cues (Figure 7a).

Previous studies have shown that male-female interactions have significant effect on the activity rhythm of *Drosophila* (Fujii et al., 2007; Fujii and Amrein, 2010; Hamasaka et al., 2010). Our study tested whether socio-sexual interactions can bring about phase synchrony and modulate phase of highly desynchronized flies by exposing phase desynchronized hosts to 12:12 hr PA cycles of females. The outcome remained unchanged, social interaction with females was unable to synchronize circadian clocks of male hosts (Figure 7b). These results thus suggest that social cues in the form of PA cycles do not serve as Zeitgeber in fruit flies *D. melanogaster*. If at all they do, it is a much weaker time cue compared to LD, temperature, and food availability cycles. Furthermore, social cues were ineffective in altering LL-mediated splitting of cry^0 flies. The phases of the two split components of activity in cry^0 flies subjected to PA cycles were similar to CD controls, suggesting that there is no effect of social cycle on the circadian rhythm of hosts (Figure 9).

To examine if social cues can help in retaining the phase of already set rhythm (from previous entrainment schedule) we subjected CS males to PA cycles of 12:12 hr. We found that phase of activity rhythm in DD drift away from the phase previously set by entrainment to LD cycles, suggesting that PA cycles are unable to retain the phase of

entrainment. To further test this we used *shibire* males with mean circadian period of 25.61 ± 0.15 h at 25 ^{o}C. The results revealed that phase synchrony of CSI and CD flies is similar, however, phase shifts after 4 days of free run in DD is significantly lesser in CSI flies than CD controls suggesting that social cycles have significant impact on circadian clocks provided flies have a labile circadian timing system (Figure 8). Our study suggests that PA cycles are unable to entrain circadian locomotor activity rhythm of *D. melanogaster*, however, consistent effect of social interaction is observed in terms of phase synchrony among socially interacting flies, confirming the findings of previous studies that circadian clocks are responsive to social cues (Levine et al., 2002), and are likely to fine-tune circadian rhythms at the group level.

My study suggests that fruit flies *Drosophila melanogaster* can not be entrained by the cyclic presence and absence (PA) of males or females visitors. However, there is consistent modulation of phase of the host's rhythm which indicates that *Drosophila* is sensitive to social cues. Synchrony among hosts depends on the time at which they interact with the visitors. While *Drosophila* clocks are sensitive to social cycles in a time dependent manner, it is also able to distinguish between clockless and intact visitors. Furthermore, social cycles are able to synchronize the host's clocks better when the host's circadian system is labile. These findings are especially relevant in case of host flies which were highly phase synchronized to start with. The visitors are however able to influence the phase of the host's rhythm that has been already set by LD cycles, though it is unable to synchronize clocks of highly desynchronized hosts. These results indicate that daily visits by male or female visitors are ineffective in causing phase synchrony suggesting that social cues in the form of PA cycles are very weak Zeitgeber for fruit flies

D. melanogaster, and may only be useful in fine-tuning of circadian clocks that are already synchronized by LD cycles.

Chapter 4

Social Interactions Modulate Period and Phase of *Drosophila melanogaster*

4.1 Introduction

Many organisms live in the face of threats from their competitors and predators. In order to manage such challenges many organisms have evolved the ability to perform their critical activities at an optimal time of the day, when risk associated with the behavior is low and chances of finding food and mates are high. To achieve this they use endogenous circadian clocks capable of measuring time on a 24 hr scale, and keeping track of time in the local environment using a variety of time cues such as cycles of light, temperature, and social interactions (Dunlap et al., 2004; Hardin, 2005; Wijnen and Young, 2006; Allada and Chung, 2010). In the course of evolution, further complexities seems to have been incorporated in the clock system wherein peripheral oscillators disturbed throughout the body have also assumed the ability to regulate rhythmic functions (Giebultowicz, 2000; Hall, 2003). Many peripheral oscillators such as those located in oenocytes (Krupp et al., 2008), malpighian tubules (Giebultowicz and Hege, 1997), gut (Giebultowicz, 2000), antennae (Krishnan et al., 1999), prothoracic gland (Myers et al., 2003), fat bodies (Xu et al., 2008) have been reported to function in *Drosophila*. Some of these oscillators function autonomously (Tanoue et al., 2004; Krupp et al., 2008), others such as those in prothoracic gland and fat bodies require inputs from the central brain pacemakers (Myers et al., 2003; Xu et al., 2008).

Although it is well recognized that many organisms use cycles of light, temperature, food, and social interactions to keep track of time in their local environments (Dunlap et al., 2004; Hardin, 2005; Allada and Chung, 2010), the cellular and molecular pathways by which light is able to synchronize circadian clocks is the best understood. In fruit flies *Drosophila melanogaster*, light is perceived by compound eyes,

ocelli, Höfbauer-Buchner eyelets, and by cell autonomous light sensor CRYPTOCHROME (CRY) (Höfbauer and Buchner, 1989; Emery et al., 1998; Yasuyama and Meinertzhagen, 1999; Emery et al., 2000; Helfrich-Forster et al., 2001). Temperature has also been reported to serve as time cue for the circadian clocks in many organisms including *D. melanogaster* (Saunders, 2002). In *Drosophila* adults, temperature signals are sensed by dorsal neurons (DNs) and lateral posterior neurons (LPNs) (Busza et al., 2007; Miyasako et al., 2007), and in larvae by the DN2 neurons (Picot et al., 2009). Temperature is perceived by the chordotonal organs and relayed to the clock neurons through yet unknown pathways (Sehadova et al., 2009). Apart from light and temperature, social cues also serve to convey timing information to many organisms including *Drosophila* (Levine et al., 2002; Krupp et al., 2008).

Several studies in mammals showed that mother's clock sets timing in offspring early during development (Viswanathan and Chandrashekaran, 1985; Reppert and Schwartz, 1986; Honma et al., 1987; Duffield and Ebling, 1998; Viswanathan, 1999). Social interactions speed up the rate of resynchronization to phase shifted light/dark (LD) cycles. For example, in the day active rodent *Octodon degus*, resynchronization of circadian rhythms in females following a phase advance in LD cycles by 6 hr is faster when they are paired with other females or males (Goel and Lee, 1997). Similarly, when hamster males are subjected to phase advanced LD cycle of 8 hr, their rhythms entrain faster when they are presented with females in estrous, however, mating with such females have any negative impact on the rate of re-entrainment (Honrado and Mrosovsky, 1989). Mice with variable circadian periods show synchronized behaviour as long as they are maintained together (Crowley and Bovet, 1980). Members of a family

of free living beavers *Castor Canadensis* display synchronized circadian period of ~27 hr when confined to caves in winters, only to return to a 24 hr entrained period in summer (Bovet and Oertli, 1974), suggesting that social interactions are responsible for maintaining synchrony among rhythmic behaviours during winter conditions. However, there are also reports of lack of social synchronization of circadian clocks in a few organisms. For example, in a study on hamsters it was found that locomotor activity rhythm of enucleated hamsters kept under LD cycles along with some normal sighted individuals free-run while that of sighted hamsters entrain to LD cycle, suggesting lack of social synchronization (Refinetti et al., 1992). Similarly in the sugar glider *Petaurus breviceps*, rhythms in individuals maintained in pairs free-run with different circadian periods (Kleinknecht, 2004), suggesting lack of social synchronization. Similarly in rat males, neither aggression by males nor sexual interactions with females could cause large enough effect on circadian clocks (Meerlo and Daan, 1998). Based on these studies it is clear that the role of social cues in circadian timekeeping is far from being resolved.

In eusocial honeybees, synchronization among colony members is achieved by social interactions (Frisch and Koeniger, 1994). In *D. melanogaster* too social interactions have significant effect on the period and phase of circadian clocks (Levine et al., 2002; Krupp et al., 2008). It is believed that social interactions maintain synchrony among individuals living in groups. This is evidenced by the finding that presence of arrhythmic loss of function *period* (per^0) mutants in a group of rhythmic wild type individuals decreases the over all phase synchrony of the group (Levine et al., 2002). In a similar and more recent study it was shown that presence of per^0 flies in 4:1 ratio in a

group of CS individuals attenuates the transcript levels of core clock genes in the fly head and oenocytes of wild type flies (Krupp et al., 2008)

In this chapter I report the results of my study aimed at understanding the effect of social interaction on circadian clocks of fruit flies *D. melanogaster*. For this, I estimated the percentage contribution (in terms of periodicity of free running rhythm) of each interacting partner on the cumulative rhythmic behaviour of pairs of socially interacting individuals under constant darkness (DD). I also studied the effect of multi-individual interactions on the rhythmic behaviour of group and estimated phase synchrony among individuals of different strains maintained in both homogenous and heterogeneous groups. Although it is known that social interactions improves phase synchrony among socially interacting individuals (Levine et al., 2002), we asked if such social interactions have the ability to synchronize highly phase desynchronized flies (created by pooling flies from several out-of-phase LD cycles). The results suggest that social interactions alter circadian period and phase of locomotor activity rhythm of flies and cause greater phase and period synchrony among the socially interacting individuals.

4.2 Materials and Methods

The general scheme followed in all experiments (with deviations as explained for specific experiment) was as follows: flies developed as pre-adults under 12:12 hr LD cycles, and freshly emerged flies were collected and maintained in glass vials (95 mm × 10 mm - length × diameter) as same sex groups of 30-40 flies per vial. Flies were kept for the first 4 days under LD cycles and then transferred into DD where they were maintained either in pairs, or in groups depending on the specific experiment.

4.2.1 ***Effect of pair-wise social interaction on circadian locomotor activity rhythm:*** To study the effect of one-on-one social interaction on circadian locomotor activity rhythm we paired two individuals from the same or different strains in activity tubes, and recorded locomotor activity behaviour of the pairs. From the activity data we estimated their free running period (τ) using CLOCKLAB, Actimetrics, USA. We paired per^S (τ = 18.77 ± 0.31 hr), per^L (τ = 28.96 ± 0.77 hr), and *CS* (τ = 23.58 ± 0.57 hr) males in the following combinations - $per^S + per^L$, per^S + *CS*, and per^L + *CS*. Further, in order to generate flies with smaller period differences we crossed per^S, per^L and CS flies, and paired their female offspring (because the *per* locus is on X chromosome and female offspring will have two X chromosomes). The mean τ of female offspring from per^L × per^S (per^{LS}), per^L × *CS* (per^{LC}), and per^S × CS (per^{SC}) crosses were 22.96 ± 0.21 hr, 25.30 ± 0.61 hr, and 21.49 ± 0.50 hr, respectively.

The locomotor activity of any given pair of flies comprising two different strains would display circadian period of both strains or of either strain, or arrhythmicity (Figure 1). We therefore estimated number and hence the percentage of pairs that showed short period, long period, both short and long periods, or arrhythmic activity. It is possible that different periodicities may arise merely due to mixing of two sets of time series data with intrinsically different periods without any involvement of interaction between individuals. To address this possibility we separately recorded locomotor activity behaviour of individual flies in DD for 10-12 days for each of the strains tested. For each strain the time series data obtained from individual flies was tagged numerically (1-32). For each type of empirical one-on-one interactions between a given pair of strains, we artificially summed time series data by making pairs of time series from two strains

having the same tag. For example, time series data of per^S strain was merged with time series data of per^L strain. The summed time series data was used as control (Control-T). The percentage contributions of different patterns (short period, long period, both short and long periods, or arrhythmicity) were estimated as described for empirically obtained time series data and the relative distributions were compared for each set of empirically and mathematically summed data. To observe whether one-on-one (pair-wise) social interaction has an effect on the circadian period of interacting flies we compared period values of the rhythmic components between empirical and control-T data using analysis of variance (ANOVA), where data type (empirical or control-T) and strain were treated as fixed factors. Post-hoc multiple comparisons were done using Tukey's test. All statistical analysis was carried out on STATISTICA™ for Windows Release 5.0 B (StatSoft, 1995).

Flies to be used were kept for the first 4 days under LD cycles and then transferred into DD where they were maintained either individually, or in groups depending on the specific experiment.

4.2.2 ***Effect of group-wise social interaction on circadian locomotor activity rhythm:*** To study if different strains of flies synchronize each other's circadian clocks when maintained in groups, we took flies from three strains - per^S, *CS*, and per^L, and their female progeny (per^{LS}, per^{LC} and per^{SC}) and maintained them under LD cycles. After 4 days we transferred the flies to DD and mixed flies of two different strains in equal proportions to form heterogeneous groups ($per^S + per^L$, $per^S + CS$, and $per^L + CS$, per^{LS} + CS, per^{LC} + CS and per^{SC} + CS) while homogeneous groups of flies were maintained in groups of similar size as controls. After 12 days, flies were separated, and their

locomotor activity behavior was recorded individually in DD. The phase of locomotor activity rhythm of flies, on the day of separation was tracked by extrapolating back the offset of locomotor activity rhythm. The phase data thus obtained was bootstrapped with replacement to obtain replicate data sets, which was then used to generate the magnitude (r) and direction (a^0) of phase coherence vectors. The r and a^0 values of mixed and control groups were analyzed using ANOVA.

4.2.3 ***Effect of social interaction on the circadian locomotor activity rhythm of shibire flies:*** The key contributors to phase synchrony in socially interacting flies are circadian period and the fly's ability to consolidate activity. In order to address this we took flies with *shibire*^{ts} expressed in the Pigment Dispersing Factor (PDF)-positive clock neurons (*pdfGAL4/UASshibire*^{ts} henceforth will be referred as *shibire* flies), whose circadian period is known to depend on the ambient temperature, and studied the effect of pair-wise social interaction between *shibire* flies and flies from two control strains (*CS* and *UASshibire* - henceforth will be referred as *UAS* flies). We estimated percentage contribution of four different phenotypes (short period, long period, both, and arrhythmic) in the activity data of interacting pairs, and compared with those obtained from the control-T data. To study if pair-wise interaction has any effect on the circadian period of interacting flies, we compared period of contributing strains in empirical and control-T data using analysis of variance (ANOVA), with data type (empirical or control-T) and strain as fixed factor.

To study if *shibire* flies are able to form synchronous group in combination with *CS* or *UAS*, we took these flies, and mixed them in equal proportion with *shibire* flies to form heterogeneous groups of 50-60 flies per vial. The *shibire* or *UAS* flies, maintained

as same strain groups of 50-60 individuals per vial, served as controls. These groups were transferred to DD for 12 days to enable social interaction following which their locomotor activity behavior was monitored individually in DD. The phase of locomotor activity rhythm on the last day of social interaction was assessed by extrapolating back the offset of locomotor activity rhythm. The phase values thus obtained was bootstrapped with replacement to obtain replicate data sets. The replicate phase values were then used to obtain r and a^0 values of phase coherence vectors.

4.2.4 ***Effect of long term group-wise social interaction on circadian locomotor activity rhythm:*** To study the effect of long term social interaction in groups on the circadian locomotor activity rhythm, we maintained *CS* flies in group of 50-60 flies first under LD for 4 days and then in DD for 21 or 35 days. Another set of CS flies were maintained as solitary individuals first under LD for 4 days and then in DD for 21 or 35 days (Figure 4). Following this, locomotor activity behavior of flies maintained in groups or solitary was monitored individually under DD. The phase of activity rhythm was estimated using regression lines drawn through the offsets of locomotor activity. The lines were extrapolated back to the last day of social interaction to obtain the phase of entrainment. The phase values thus obtained were bootstrapped with replacement to obtain replicate data sets for the estimation of phase coherence vectors using circular vector (Batschelet, 1981). These replicate set of phase values were then used to obtain replicate magnitude (r) and direction (a^0) values of the phase coherence vectors.

4.2.5 ***Effect of social interaction on the circadian locomotor activity rhythm of highly desynchronized flies:*** We wanted to study if social interactions are capable of bringing about phase synchrony among flies with high degree of phase desynchrony. For this, we

performed two separate experiments. In the first experiment we pooled flies kept in two LD cycles (10:00-22:00 hr and 20:00-08:00 hr) 10 hr out of phase, and in the second experiment we kept flies in six LD cycles (05:00-17:00 hr, 08:00-20:00 hr, 11:00-23:00 hr, 17:00-05:00 hr, 20:00-8:00 hr and 23:00-11:00 hr). In both the experiments, we first maintained flies under various LD cycles for a period of 4 days and then pooled them in equal proportion from each LD cycle, and transferred them to DD. Pooled flies from two opposite LD cycles were divided into two sets, and transferred into DD, where one set was maintained for 21 days in groups of 50-60 flies in vial, while flies from the other set were kept solitary. Pooled flies from six different LD cycles were divided into six sets and transferred to DD; three sets were placed in groups of 50-60 flies in vial, while the remaining three sets were kept solitary (Figure 5). The locomotor activity behavior of one set each of mixed and solitary flies was recorded after 2, 4 or 10 days to assess the extent of phase synchrony among individuals as a function of number of days of social interaction.

4.2.6 ***Role of olfaction in social interaction:*** Olfaction plays a crucial role in the social interaction of *Drosophila* (Levine et al., 2002; Krupp et al., 2008). Therefore, to study the role of olfaction in social interaction we took flies with loss of function mutation in *Or83b*, a receptor which is widely expressed in the fly olfactory circuit of the fly (Larsson et al., 2004). The $Or83b^{0}$ flies were maintained in six different LD cycles, in a manner described above, pooled, and divided into two sets and transferred to DD, each set having equal contribution from all six LD cycles. One set of flies was maintained in groups of 50-60 flies per vial, while the other set was kept solitary. After 21 days, locomotor activity behaviour of flies from both the sets was recorded individually under

DD. To reconfirm our results, we backcrossed w^{1118} and $Or83b^{0}$ flies to CS for six generations, and then repeated the above experiments, however, flies were maintained in DD for 12 days either in groups of 50-60 flies per vial or solitary.

4.3 Results

Pair-wise social interaction alter circadian period of per^{L} flies:

4.3.1 ***Percentage contributions of the interacting strains:*** The contributions from different strains of flies estimated empirically match closely with control-T data for all pair, except $per^{S} + per^{L}$. Based on the empirical data ~70% $per^{S} + per^{L}$ pairs display periods similar to per^{S}, ~4% similar to both, while in the control-T data the percentages are ~25% and ~61% respectively (Figure 2a). These results suggest that pair-wise social interaction does not alter the distribution of different patterns (short period, long period, both short and long periods, or arrhythmicity), except when per^{S} and per^{L} flies are made to interact with each other.

4.3.2 ***Circadian period of contributing strain:*** After estimating the percentage contributions of different strains of flies when socially interacting, our immediate goal was to find if flies can alter each other's circadian period. For this we took per^{S}, per^{L}, and CS males, and paired them as $per^{S} + per^{L}$, $per^{S} + CS$, and $per^{L} + CS$. In addition, we took per^{SC}, per^{LC}, per^{LS}, and CS females, and paired them as per^{LC} + CS, per^{SC} + CS, and per^{LS} + CS. ANOVA on the circadian period data showed a significant effect of pairing ($p < 0.0001$), strain ($p < 0.0001$), pairing × strain ($p < 0.0001$), pairing × data type ($p < 0.005$), pairing × strain × data type interactions ($p < 0.05$), however, effect of data type

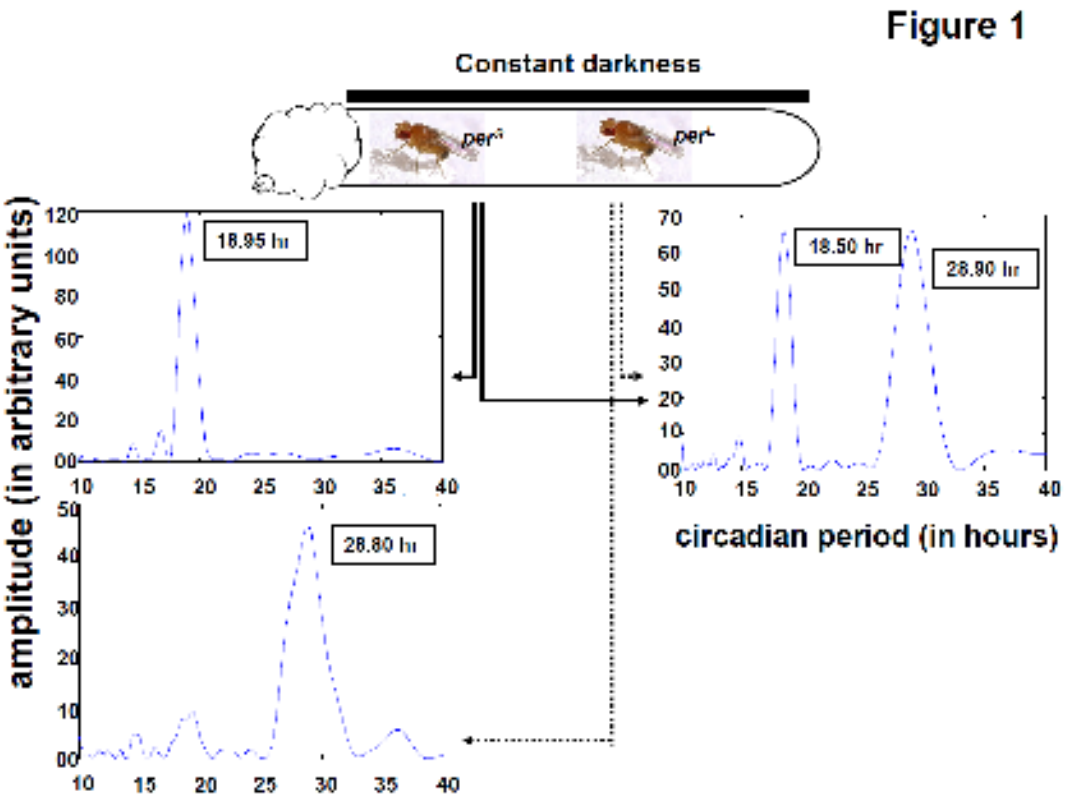


Figure 1 ***Effect of pair-wise social interaction on circadian locomotor activity rhythm.*** Flies from the same or different strains were paired in activity tubes, and their locomotor activity behaviour recorded. From the activity data we estimated free running period (τ) of activity rhythm of the pairs. The locomotor activity of any given pair of flies comprising two different strains display circadian period of both strains (per^S and per^L) or of either strain (per^S or per^L), or arrhythmicity.

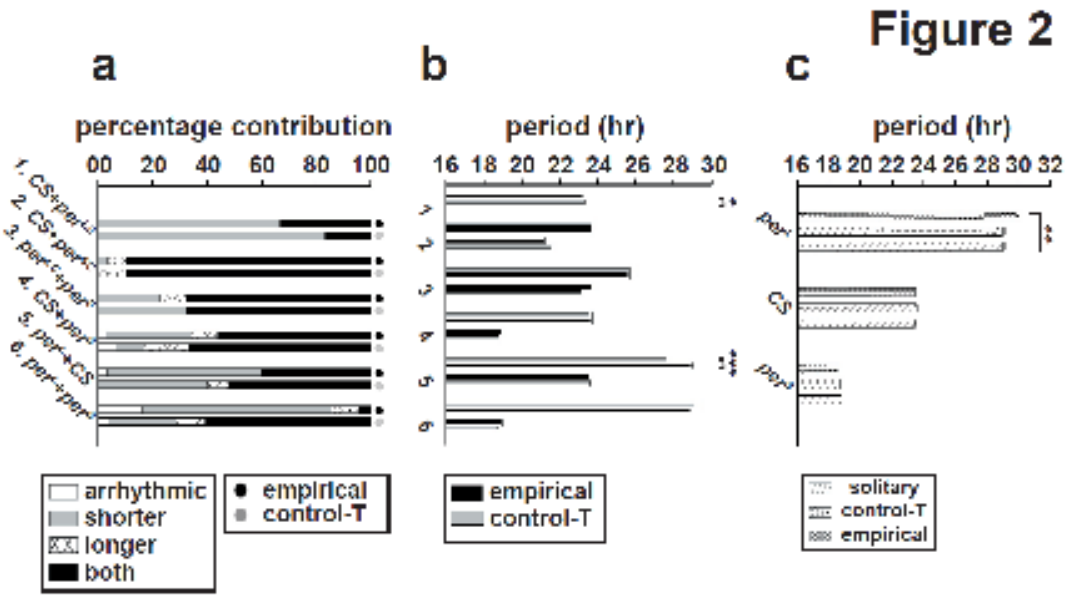


Figure 2 ***Effect of pair-wise social interaction on circadian locomotor activity rhythm*****.** (a) Percentage contributions of participating strains when allowed to interact in 1:1 context and controls obtained theoretically by mixing time series data of interacting individuals. The percentage contribution of arrhythmic pairs is shown as white shaded bar, while presence of both periodicities of the interacting pair is given by black shaded bar, when only one period is detected, and the value of the period is that of the partner with the lower/shorter period it is indicated by a grey shaded bar, while a hatched bar indicates that the period of the partner with the higher/longer period is detected as a result of the theoretical or empirical mixing. (b) Effect of pair-wise social interaction on circadian period. Except in per^{L}+ CS ($p < 0.0005$) and per^{LS} + CS ($p < 0.05$) pairs, interaction between no other strains caused significant change in circadian period ($p > 0.05$) than what can be seen by theoretical mixing of time series. Period values of two flies of same genotype per^{L} were significantly larger than its own simulated controls-S ($p < 0.005$) where there is no difference in period of other two genotypes (*CS* and per^{S}) (d). Grey circles on the top of figure a. represent control-T and dark cycles represent empirical data.

($p = 0.91$), and strain × data type interaction is statistically not significant ($p = 0.38$). Post-hoc multiple comparisons using Tukey's test revealed that except for per^L flies in $per^L + CS$ combination, which show shorter circadian period compared to controls ($p < 0.0005$), pair-wise social interaction does not have any effect on the circadian period of interacting partners ($p > 0.05$). We were unable to assign distinct period values for the $per^{LS} + CS$ pairs because circadian periods of both strains were indistinguishably close to each other, and therefore the data from this combination was excluded from the composite ANOVA, and was analyzed separately. ANOVA on the circadian period when per^{LS} and *CS* flies were paired together suggests that empirically obtained circadian period is significantly smaller than that from control-T data ($p < 0.05$) (Figure 2b).

To study if pair-wise social interaction among flies of same strain alters circadian period of their locomotor activity rhythm, we took per^S, per^L and *CS* flies, and paired them with flies of the same strain, and recorded their locomotor activity behaviour under DD, and compared circadian period of the pairs with those obtained from the control-T data. The circadian period of per^L pairs is significantly longer than that obtained from control-T data ($p < 0.005$), however, empirically obtained period values of CS or per^S pairs does not differ statistically from their respective controls ($p > 0.05$) (Figure 2c).

4.3.3 ***Social interaction among flies from different strains with varying period decreases phase synchrony of the group:*** Different strains of flies (*CS*, per^S, per^L, per^{LC}, per^{SC}, and per^{LS}) were maintained under LD cycles as same sex group of 50-60 flies per vial. After 4 days, they were divided into two sets, and transferred into DD. One set (control, n ~60) was kept as a group of 50-60 flies of same strain and the other set (experimental, n ~60) was mixed in equal proportion with flies from a different strain

(25-30 each strain). Flies were kept as experimental and control groups for a period of 12 days, following which their locomotor activity was recorded individually and the phase of locomotor activity rhythm determined. The phase values thus obtained were used to generate replicate data sets by bootstrapping with replacement. Replicate data were used to assess synchrony among flies in experimental and control groups. ANOVA on the *r* values showed significant effect of group ($p < 0.0001$). Post-hoc multiple comparisons using Tukey's test revealed that mixed groups $per^S + per^L$, per^S + CS, per^L + CS, per^{LC} + *CS*, per^{SC} + *CS*, per^{LS} + *CS* of flies have significantly lesser phase synchrony than homogeneous controls ($p < 0.0005$) (Figure 2d), suggesting that phase reduced phase synchrony among the interacting flies, in a heterogeneous group, is due to period differences among the interacting partners.

4.3.4 ***Pair-wise social interaction alter circadian period of shibire flies:*** We used another approach for social interactions among individuals with different circadian clock properties by driving the expression of temperature sensitive *shibire* in the ventral lateral neurons (LN_v) using the *pdfGAL4* driver (*shibire)*. Pair-wise social interaction of *shibire* flies ($\tau = 25.33 \pm 0.38$ hr), with either *UAS* ($\tau = 24.35 \pm 0.61$ hr), or CS ($\tau = 23.56 \pm 0.59$ hr) flies results in distribution of circadian phenotypes similar to controls. In *shibire* + *CS* pairs, contribution of long period in the control-T data is only ~21%, while in the empirical data it increased to ~67%. Similarly, contribution of both short and long periods in control-T data is as high as ~55%, while it is only ~8% in the empirical data (Figure 3a).

ANOVA on the circadian period data showed that there is significant effect of strain ($p < 0.0005$), data type ($p < 0.05$), strain × data type interaction ($p < 0.01$). Post-

Figure 2

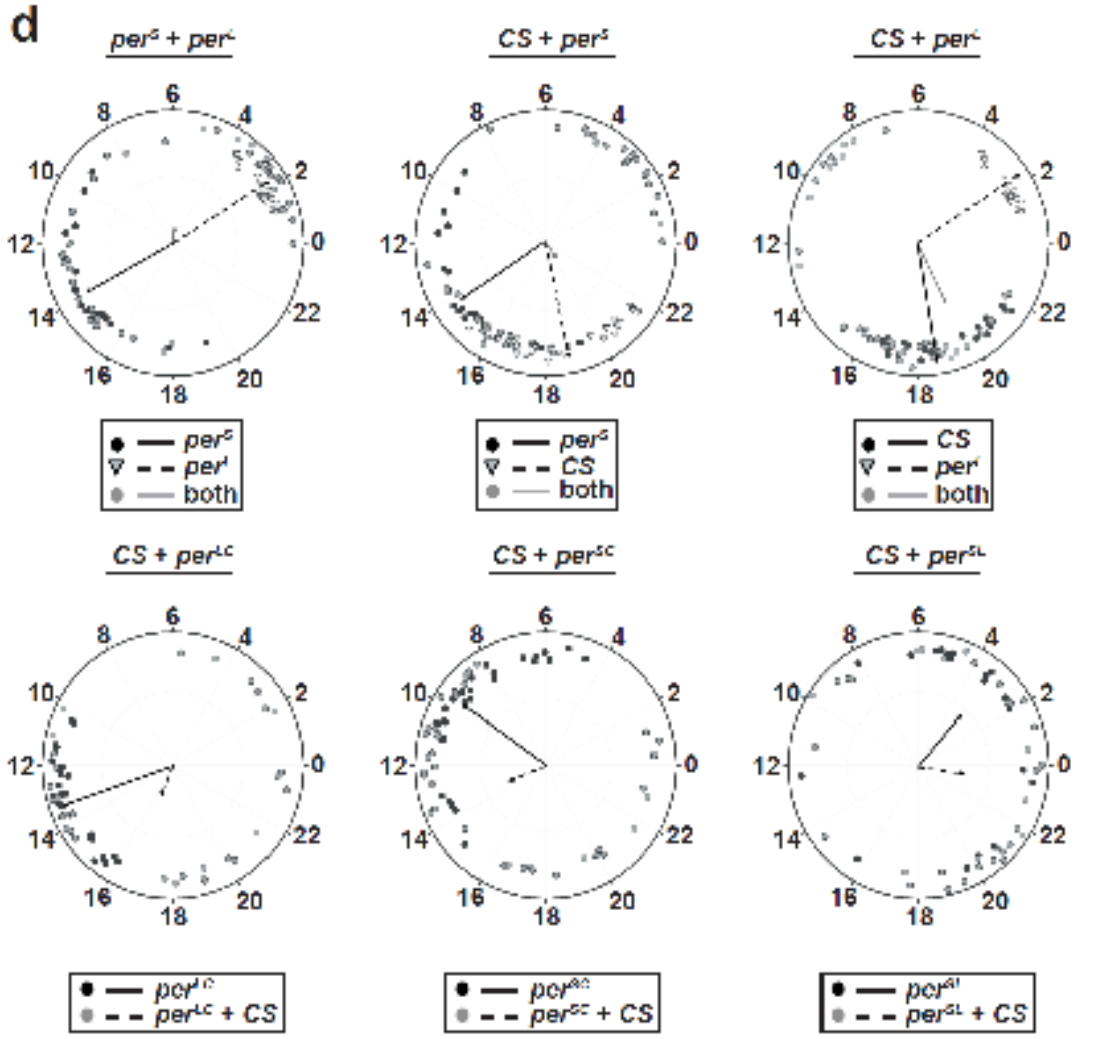


Figure 2 ***Social interaction among flies from different strains with varying period decreases phase synchrony of the group.*** (d) Flies maintained in heterogeneous groups have lower phase synchrony compared to controls. Dark cycles and dark arrow represent offset of lower period flies, grey triangles and broken black arrow represent higher period and grey circles and solid grey arrow represent offset of two strains of flies together. Similarly in lower panel, solid black circles and black line represents homogeneous control and solid grey cycles and broken black line represent heterogeneous group consisting of CS in combination in other strains.

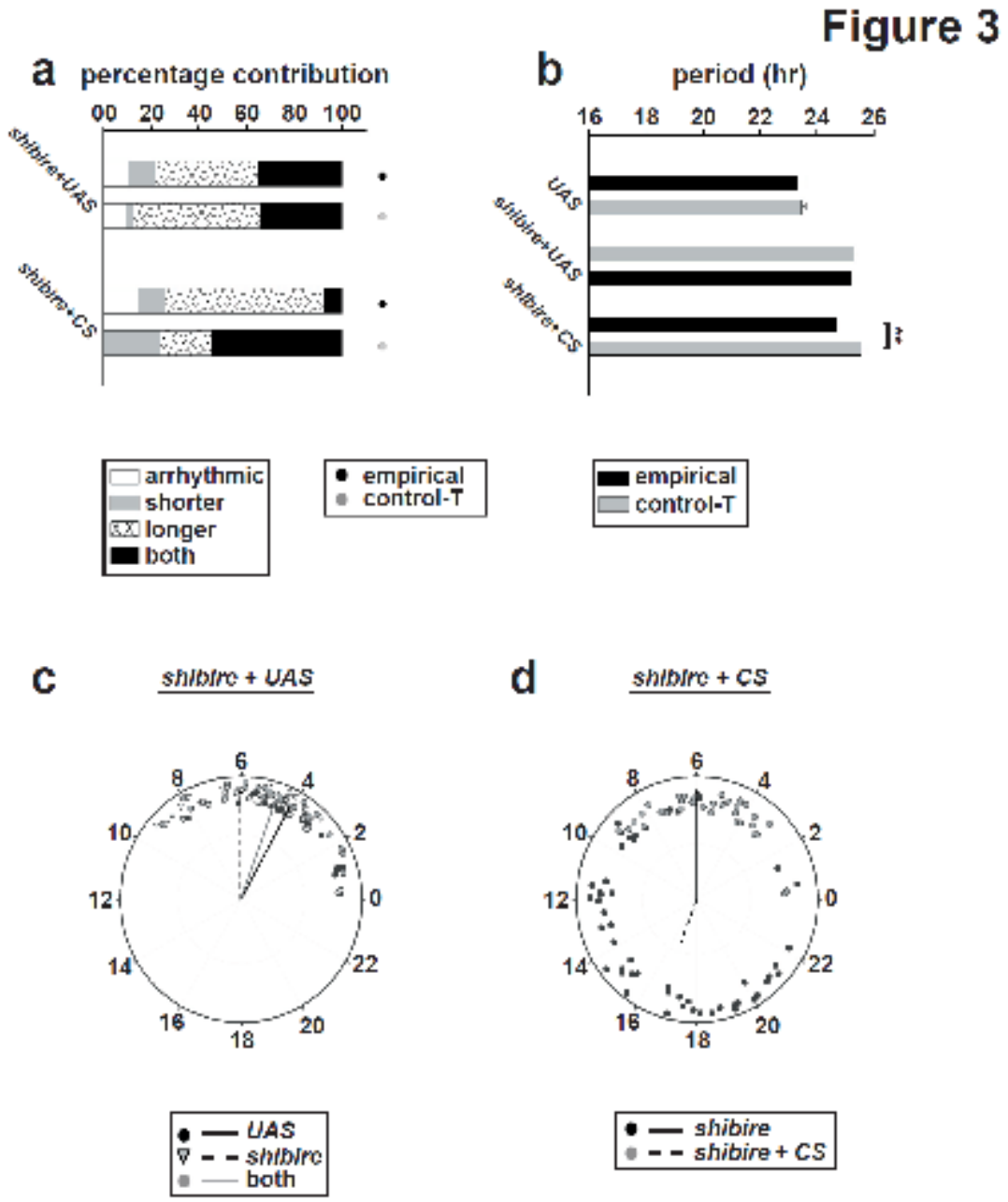


Figure 3 ***Pair-wise*** **and group level** ***social interaction in shibire flies.*** (a) Percentage contributions of *pdfshibire* in combination with *CS* or *UASshibire* when they were placed in 1:1 context and compared with controls obtained by mixing of data series of two parental controls. (b) Period of *pdfshibire* + *CS* were is significantly less than controls ($p < 0.001$) where as there are no differences when *pdfshibire* + *UASshibire* and *UASshibire* are compared control-T ($p = 0.99$). (c) There are no significant differences in phase synchrony when *pdfshibire* + *UASshibire* flies are compared with *UASshibire* or *pdfshibire* flies alone ($p > 0.05$).(c-left). In case of *pdfshibire* + *CS*, synchrony is less than homogeneous control ($pdfshibire^{ts}$) ($p < 0.0005$). Other details similar to Figure 2.

hoc comparisons revealed that circadian period of *shibire* and *UAS* flies in *shibire* + *UAS* combination does not differ statistically from controls ($p = 0.99$) for both short and long period components), while that of *shibire* in *shibire* + *CS* combination is significantly shorter than controls ($p < 0.005$) (Figure 3b). However, due to a small fraction of flies (~7%) with period close to CS in the empirical data, we could not compare its period between empirical and control-T data.

4.3.5 ***Group-wise social interaction enhances phase synchrony in shibire flies:*** To study if *shibire* flies can form synchronous group with CS or *UAS*, we maintained these flies in DD, for 12 days, in mixed (*shibire* + *UAS* or *shibire* + *CS*) groups, and homogenous (*shibire* or *UAS*) groups. The results suggest that phase synchrony of mixed *shibire* + *UAS* groups of flies is similar to homogeneous *shibire* or *UAS* controls ($p > 0.05$) (Figure 3c). However, phase synchrony of *shibire* + *CS* groups is significantly lower phase synchrony than homogenous *shibire* controls ($p < 0.0005$) (Figure 3d).

4.3.6 ***Long term group-wise social interaction enhances phase synchrony:*** Freshly emerged *CS* males were maintained under LD cycles for 4 days, then divided into two groups and transferred into DD. Flies from the first group were isolated and kept solitary, while those from the second group were maintained in groups of 50-60 individuals per vial. After 21 or 35 days, locomotor activity behaviour of flies maintained solitarily and in groups was recorded individually in DD, and their phase coherence compared. The results show that phase synchrony of flies maintained in groups for 21 days ($p < 0.0005$) or 35 days ($p < 0.001$) is significantly greater than their respective solitary controls (Figure 6a).

Figure 4

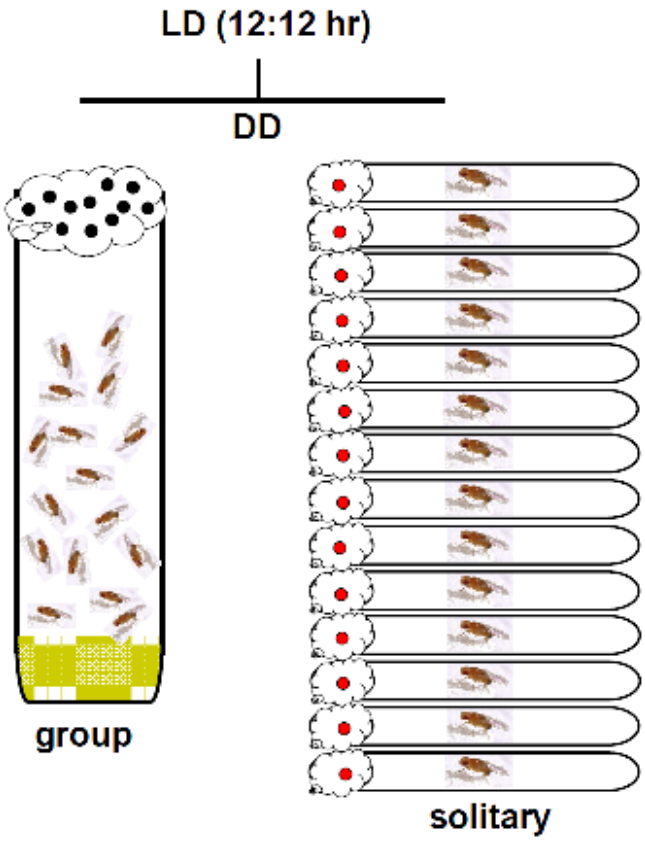


Figure 4 ***Effect of long term group-wise social interaction on circadian locomotor activity rhythm.*** To study the effect of long term social interaction in groups on the circadian locomotor activity rhythm, we maintained *CS* flies in groups of 50-60 flies per vial first under LD for 4 days and then in DD for 21 or 35 days. Another set of *CS* flies were maintained as solitary individuals first under LD for 4 days and then in DD for 21 or 35 days. Following this, locomotor activity behaviour of flies maintained either in groups or solitary was monitored individually under DD.

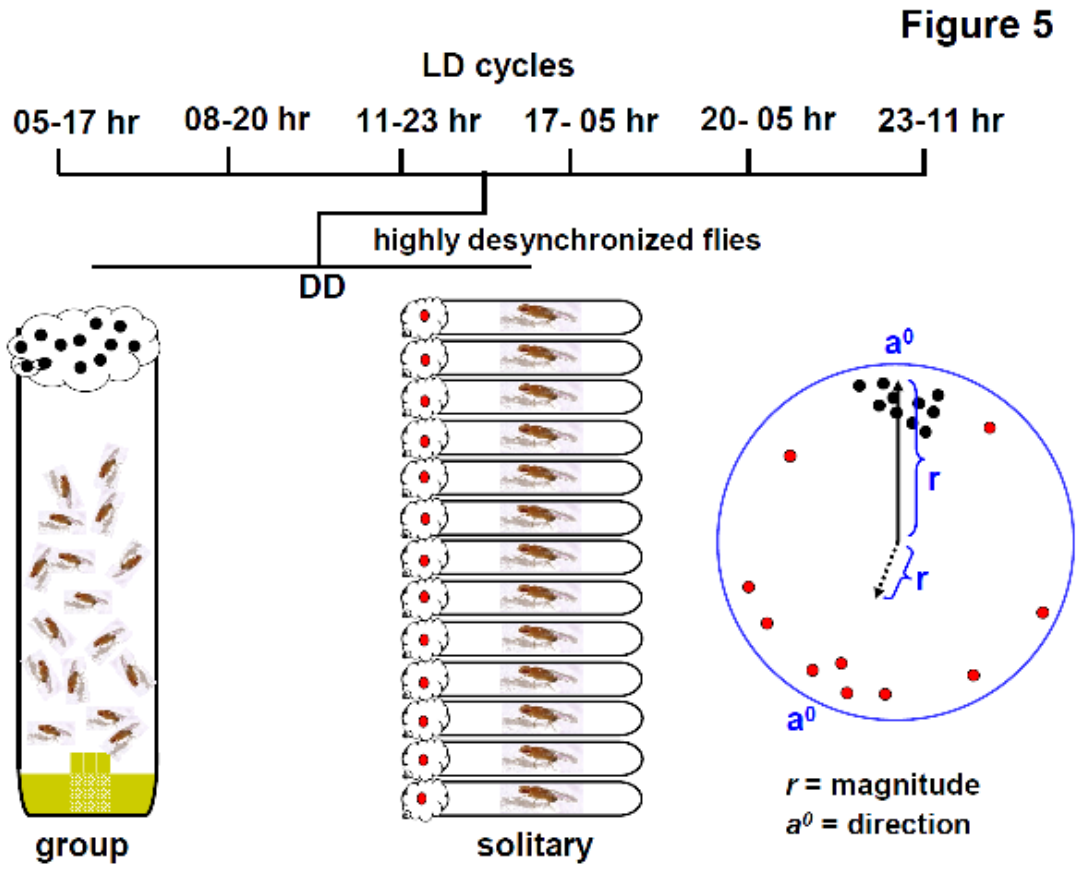


Figure 5 ***Effect of social interaction on the circadian locomotor activity rhythm of highly desynchronized flies.*** To create maximum phase desynchrony ($r = 0.0004$) among interacting flies, we maintained *CS* flies under six different LD cycles, and then pooled them together with equal contribution from each LD cycle to form six sets (only two sets are shown here). Three sets of flies were kept in groups of 50-60 individuals per vial (left), and the remaining three sets were kept solitary (right). Following this, locomotor activity behaviour of flies maintained either in groups or solitary was monitored individually under DD.

4.3.7 ***Group-wise social interaction enhances phase synchrony of highly phase desynchronized flies:*** Given that group-wise social interaction in CS flies resulted in enhanced phase synchrony, we wanted to study if flies with high degree of phase desynchrony can achieve synchrony via social interaction. For this, we first desynchronized the phase of locomotor activity rhythm of *CS* flies by keeping them under two oppositely phased LD cycles (with lights-on between 10:00-22:00 hr and 20:00-08:00 hr) for a period of 4 days, then divided them into two sets, and transferred to DD. Individuals from the first set were maintained solitary, while those from the second set were mixed, taking equal number of flies from both LD cycles, to form composite groups of 50-60 individuals per vial. After 21 days, locomotor activity behaviour of flies from both the sets was recorded individually under DD. Analysis revealed that magnitude of phase coherence vector of flies maintained as groups is significantly greater than those kept solitary ($p < 0.05$). However, direction of phase coherence vector of flies maintained in groups does not differ significantly from those kept solitarily ($p = 0.28$). Rayleigh's test showed that phase coherence of flies living solitary ($p = 1.00$) and in group ($p = 0.20$) does not differ significantly, however, the level of phase synchrony in flies living as groups is much better than those living solitary (Figure 6b).

To create maximum phase desynchrony ($r = 0.0004$) among interacting flies, we maintained *CS* flies now under six different LD cycles, and then pooled them together with equal contribution from each LD cycle to form six sets (Figure 5). Three sets of flies were kept in groups of 50-60 individual per vial, and the remaining three sets were kept solitary. To assess phase coherence, flies kept in groups under DD were separated after 2, 4 or 10 days for behavioural assay. After 2, 4, or 10 days locomotor activity

Figure 6

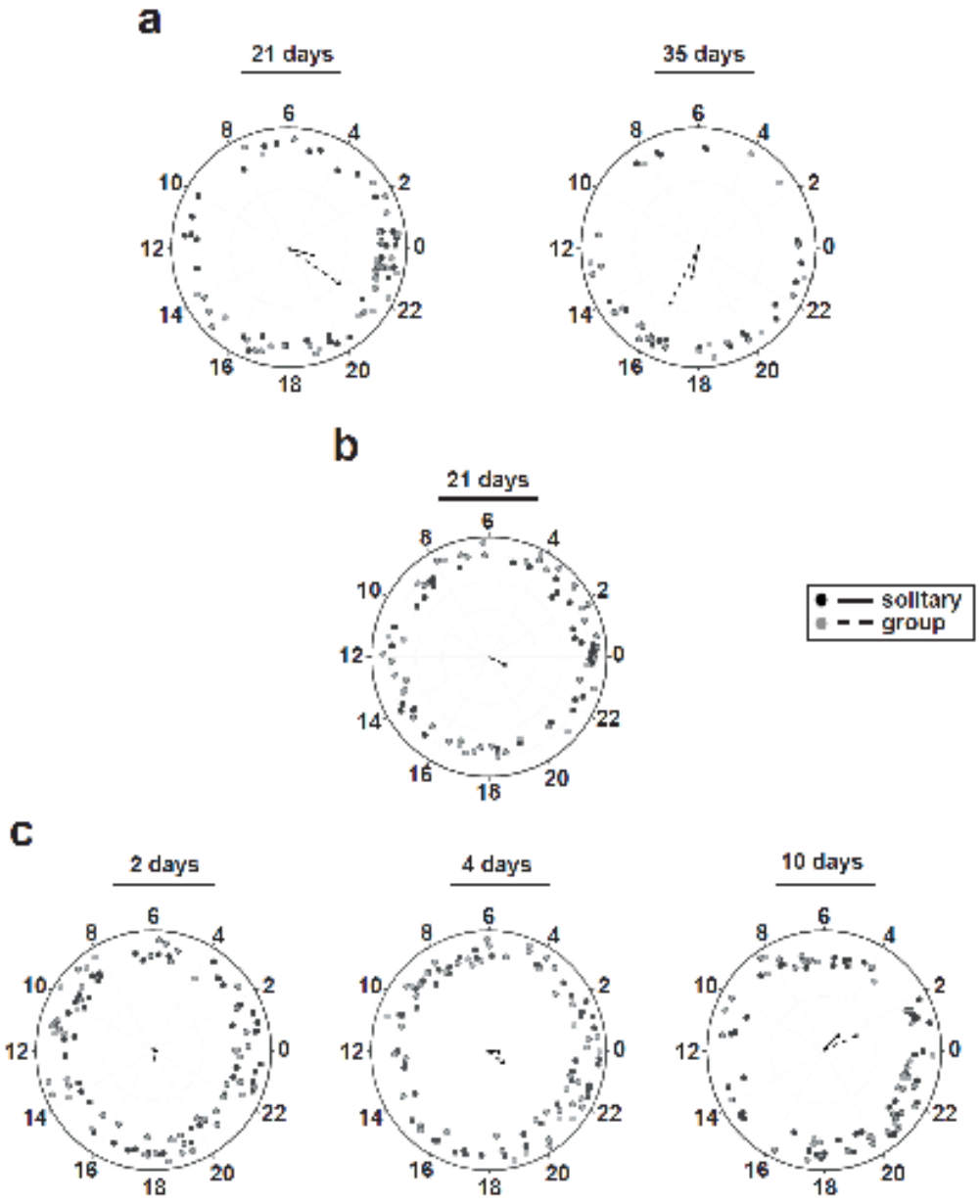


Figure 6 ***Interactions between flies kept as groups enhance phase synchrony of their individual circadian clocks.*** (a) The phases of activity rhythm of individual flies previously kept in groups for prolonged duration under constant darkness show greater synchrony when compared to isolates. After 21 days (left) in constant darkness the phases of grouped flies (grey dots) are more synchronous than their isolated controls (black dots, $p < 0.0005$). Similar difference is seen after 35 days (right) ($p < 0.001$). (b) Flies which were first entrained to two oppositely phase LD cycles (lights-on between 10 - 22 hr or between 20 – 8 hr) were allowed to interact in groups under DD, showed greater synchrony compared to their isolated controls ($p < 0.05$). (c) Flies first entrained to six different LD cycles were allowed to interact for either 2 days (2D), 4 days or 10 days in DD. Compared to controls synchrony of group maintained flies was greater for 2 days ($p = 0.84$), ($p = 0.59$) for 4 days and ($p = 0.08$) for 10 days. Black circles and arrow represent activity offset phase and degree of synchrony of isolates while grey circles and broken black arrow denotes the same for grouped individuals.

behaviour of experimental and control flies was monitored in DD individually. ANOVA showed that effect of social status (group/solitary) is statistically not significant (p = 0.07), while effect of number of days (2/4/10 days) ($p < 0.005$), and social status × number of days interaction is statistically significant ($p < 0.05$). Post-hoc multiple comparisons revealed that effect of group living for 2 ($p = 0.84$) or 4 days ($p = 0.59$) does not have any measurable effect on the phase synchrony of interacting individuals, however, after 10 days, differences between the phase synchrony of flies living in groups and solitary became greater only to reach a marginal level of statistical significance (p = 0.08). Rayleigh's test showed that phase coherence vector does not possess any direction in flies living solitarily ($p > 0.05$). However, in flies maintained as groups, although direction of phase coherence vector is uniform after 2 or 4 days of social interaction ($p >$ 0.05), after 10 days of social interaction it became significantly non-uniform ($p < 0.01$) (Figure 6c).

4.3.8 ***Flies lacking functional olfactory ability has lower phase synchrony:*** To study the role of olfaction in social interaction among flies maintained in groups, we took flies with loss of function mutation in a widely expressed olfactory receptor *Or83b* (*Or83b*^{0}), and placed them in six different LD cycles (with lights-on during 05:00-17:00 hr, 08:00-20:00 hr, 11:00-23:00 hr, 17:00-05:00 hr, 20:00-08:00 hr or 23:00-11:00 hr) for 4 days, and then pooled them in equal proportion from each LD cycles into two sets. While both sets were transferred simultaneously to DD, flies from the first set were kept solitary, and those from the second set in groups of 50-60 per vial. After 21 days, locomotor activity behaviour of flies was recorded individually in DD. Interestingly, phase coherence of flies living in groups does not differ statistically from those living

Figure 7

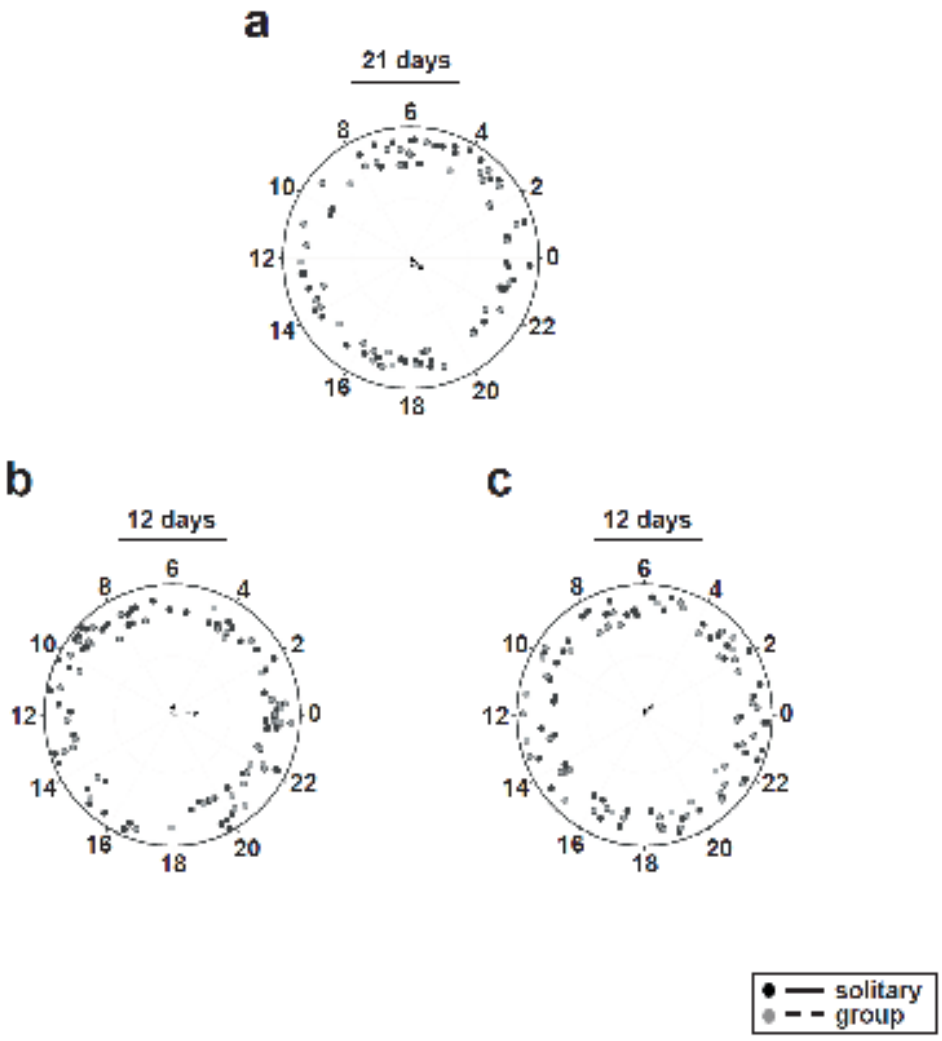


Figure 7 ***Social interaction between flies that allows greater phase synchrony requires olfactory communication.*** Flies that were desynchronized by entraining them to 6 different LD cycles were pooled together and kept in constant darkness to enable interactions. (a) No significant differences are seen *(p* = 0.35) between flies in group compared to isolates when *Or83bnull* flies are tested after 10days in DD. (b) *Or83bnull* and w^{1118} flies crossed to CS for 6 generations subjected to above protocol and tested after 12 days showed that synchrony of group living w^{1118} flies was significantly more ($p < 0.005$) than group living *Or83bnull* flies while there are no differences in isolated individuals of both the groups ($p = 0.97$) (b and c). Black circles and arrow represent offset phase and extent of synchrony of isolates while grey circles and broken black arrow denotes the same for grouped individuals.

solitarily ($p = 0.35$) (Figure 7a). We then backcrossed $Or83b^0$ and w^{1118} flies for six generations into *CS* background, and then repeated the above experiments with backcrossed $Or83b^0$ and w^{1118} flies. The phase synchrony of w^{1118} flies living in groups is significantly greater than $Or83b^0$ flies ($p < 0.005$), while that of flies living solitary does not differ ($p = 0.97$) (Figure 7b, c), suggesting that social synchronization in fruit flies requires functional olfactory ability.

4.4 Discussion

The results of our study suggest that flies fail to synchronize each other's circadian rhythms under DD when social interactions are limited to two individuals, as circadian period of interacting individuals does not differ from controls, except in the case when such interactions took place between per^L or per^{LS} flies and flies from any other strain. The strongest demonstration for synchronization of circadian clocks via social interactions is seen in the study when *CS* flies were made to interact in social groups of 50-60 individuals. Flies living in groups display enhanced phase synchrony than those living solitary. Social synchronization is further evidenced when highly phase desynchronized flies are made to interact socially in a group; flies displayed much improved phase synchrony compared to solitary controls. However, when flies were maintained in mixed groups of 50-60 flies per vial, only groups comprising of *shibire* flies showed phase synchrony similar to homogenous control groups, suggesting that flies with labile circadian clocks can get be synchronized by social cues even when their circadian periods are quite different. Furthermore, our study shows that flies with compromised olfactory ability ($Or83b^0$) fail to influence each others circadian rhythms

resulting in lack of phase synchrony, suggesting that social synchronization among flies require functional olfactory ability.

Circadian clock of fruit flies *D. melanogaster* is believed to be sensitive to social cues which modulate its period and phase (Levine et al., 2002). Apart from fruit flies, other organisms such as mice and beavers also show effect of social interactions on circadian period (Halberg et al., 1954; Bovet and Oertli, 1974; Crowley and Bovet, 1980). Our study shows that social interactions does not cause significant change in circadian period when made to interact one-on-one, except when per^S and per^L flies are paired together. About 70% of pairs show circadian period similar to per^S, as opposed to only ~25% in the controls. Furthermore, contribution from both the interacting partners is seen only in ~4% of the pairs in empirical data, compared to ~60% in controls (Figure 2a). In *shibire* + *UAS* pairs percentage contribution of the two interacting partners is similar to controls. However, in *shibire* + *CS* pairs, contribution from *shibire* in the pair is ~67% in empirical data as opposed to ~21% in controls, while contribution of both strains is ~7% in empirical data and ~55% in controls (Figure 3a). Flies failed to synchronize each other's circadian clocks in group-wise social interaction when their circadian periods were significantly different from each other (Figure 2d), suggesting that desynchrony among interacting flies may be due to phase of activity rhythm drifting away gradually. However in group, phase synchrony of *shibire* + *UAS* flies is similar to homogenous controls ($p > 0.05$) (Figure 3c), whereas that of *shibire* + *CS* flies is lower than controls ($p < 0.0005$) (Figure 3d). This asymmetry could be due to the fact that the difference in circadian periods of *shibire* and *UAS* flies is much smaller (~1 hr) than that between *shibire* and *CS* flies (~2 hr). These results suggest that social interaction may be

able to synchronize circadian clock only when periods of the interacting flies are labile and not too wide apart.

Social synchronization alters circadian period of interacting flies only in cases when per^L strain was involved either directly or indirectly (Figure 2b, c, 3b); circadian period of per^L and per^{LS} flies is significantly different than controls, however, no such effect is seen in pairs without per^L flies suggesting that per^L strains are sensitive to social cues. These results are in a way consistent with the findings of Crowley and Bovet (1980) where phase synchrony in mice was found to be achieved by period changes. Furthermore, in a previous study by Levine and coworkers (2002) it was shown that per^L host flies are responsive to per^S visitors whereas per^L visitors are not able to bring about significant change in the phase synchrony of per^S hosts, suggesting that it is the genetic makeup of per^L flies which makes them sensitive to social cues. Change in circadian period due to social interactions is quite evident when *shibire* flies are paired with CS flies. Circadian period of socially interacting *shibire* flies is shorter than controls. Such changes in period are probably due to the modulation of clock gene expression in socially interacting flies (Krupp et al., 2008). However, it would be interesting to know whether body temperature of flies is different when they are in group compared to when they are solitary, and whether such differences contribute to social synchronization in temperature sensitive *shibire* flies.

Previous studies have shown that in the event of absence of potential Zeitgebers, social cues can serve as time cues for *D. melanogaster* circadian clocks (Levine et al., 2002). This is seen in our study too, CS flies maintained together as groups for long enough period (21 or 35 days) in DD have significantly greater phase synchrony than

those kept solitary (Figure 6a), suggesting that social interaction enable synchronization of circadian clocks. Whether phase synchrony in flies is due to active manipulation of phase or due to accidental coincidence of similarity in their phase? We addressed this by allowing highly phase desynchronized flies to interact in social groups (Figure 5,6b, c). Flies with high degree of phase desynchrony show enhanced phase synchrony after as few as 10 days of group living (Figure 6c), suggesting that social cues act as Zeitgeber for fruit flies *D. melanogaster*.

From previous studies we known that olfaction plays a significant role in communication during social interaction (Levine et al., 2002; Fujii et al., 2007; Krupp et al., 2008). To critically examine this, we took flies with loss of function mutation for a widely expressed olfactory receptor *Or83b* (*Or83b*^{0}), which is known to disrupt response for many odors (Larsson et al., 2004), and allowed them to interact socially under DD for 21 days. Socially interacting *Or83b*^{0} flies have extremely poor phase synchrony (Figure 7a), confirming that social interaction in *Drosophila* is olfaction mediated (Levine et al., 2002; Fujii et al., 2007; Krupp et al., 2008). Furthermore, when *Or83b*^{0} and w^{1118} flies backcrossed to *CS* flies for six generations were used, phase synchrony of w^{1118} flies is significantly greater than *Or83b*^{0} flies ($p < 0.005$) (Figure 7b, c). These results thus suggest that social interactions play a significant role in enhancing phase synchrony among flies living in groups, and olfaction is a likely mode of communication between them.

My study suggests that pair-wise interaction among flies do not alter circadian locomotor activity rhythm in fruit flies *D. melanogaster*. Social interaction enhanced phase synchrony among individuals when circadian periods of interacting flies were

labile and the differences were not too large. Social communication among individuals living in groups is olfaction mediated. My study suggests that fruit flies *D. melanogaster* use phase changes to attain synchrony among socially interacting individuals. Social interactions may be of great advantage to organisms living in groups as it maximizes synchrony and hence the likelihood of most members taking part in community activity is increased which is critical for enhancing fitness of the society. However, our study suggests that social interaction alone may not be enough to entrain circadian clocks.

Chapter 5

Sex and Age Related Changes in Circadian Rhythms of *Camponotus* Ants

5.1 Introduction

It is believed that organisms have evolved the ability to display rhythmic behaviours as an adaptation to cyclic changes in their environment caused due to geophysical cycles (Dunlap et al., 2004). Such temporal adjustments are regulated by endogenous timekeepers that continue to tick under constant conditions (devoid of any time cues), and are buffered from subtle changes in environmental conditions (Dunlap et al., 2004). These clocks are responsible for timing daily rhythmicity in behaviour and physiology in a wide range of organisms, and at the molecular level constitute transcriptional-translational feedback loops involving mRNAs and proteins of core clock genes (Allada and Chung, 2010). In fruit flies *Drosophila melanogaster* circadian clocks regulate rhythmicity in a wide variety of behaviours such as adult emergence (Pittendrigh, 1954), locomotor activity (Petersen et al., 1988), olfaction (Krishnan et al., 2005), mating (Sakai and Ishida, 2001), and egg-laying (Howlader and Sharma, 2006).

The regulation of key life processes by circadian clocks (Alenghat et al., 2008), and its ubiquitous presence across several levels of complexity and organization suggests its adaptive importance (Saunders, 2002; Dunlap et al., 2004). Adaptive value of circadian clocks has been implicated in studies in the wild. In guillemots (*Uria lomvia*) (Daan and Tinbergen, 1979), European ground squirrels (DeCoursey et al., 1997), and free-living chipmunks *Tamias striatus* (DeCoursey et al., 2000), circadian clocks are believed to enhance survival. Importance of circadian clocks have also been suggested for organisms living under constant laboratory conditions, and in constant conditions inside the cave (Ouyang et al., 1998; Sharma, 2003a). Further, some studies suggest that circadian clocks play a vital role in enhancing the fitness of organisms and in providing

them with a competitive edge over others whose clocks fail to keep up with the environment (Ouyang et al., 1998).

In *Drosophila*, circadian clocks govern rhythmicity in courtship and mating (Hardeland, 1972; Ikeda, 1976; Sakai and Ishida, 2001). The *period* (*per*) gene, a core clock gene in the circadian molecular clockwork (Allada and Chung, 2010) is believed to be involved in the regulation of inter-pulse-intervals during courtship (Kyriacou and Hall, 1980), and in the regulation of mating rhythm (Wheeler et al., 1991; Sakai and Ishida, 2001; Tauber et al., 2003). In *Drosophila*, clock genes (*per* and *timeless*) have also been implicated in the regulation of release of sperms from testes to seminal vesicle (Beaver et al., 2002), duration of copulation (Beaver and Giebultowicz, 2004), and oocyte maturation (Beaver et al., 2003).

Many sympatric organisms temporally segregate their mating behaviour (Haddow et al., 1966; Tauber et al., 2003), probably to secure better mates and to avoid predators and competitors. For example, two sympatric species of *Drosophila* are found to mate at different times of the day; *D. pseudoobscura* mate close to dusk, while *D. melanogaster* mate ~3 hr before dusk (Tauber et al., 2003). In ants, mating is a once in a life time event, and a significant proportion of queens die within a few hours of leaving the nest, perhaps due to desiccation by heat, or due to predation (Levin et al., 2009), and therefore their mating flights should be precisely timed and effectively executed to ensure maximum efficiency (Hölldobler and Wilson, 1990). Time of mating in ants is species-specific, and is largely influenced by environmental factors such as temperature, light intensity, and availability of food (Talbot, 1945; Hölldobler and Wilson, 1990). For example, males of the harvester ant *Veromessor andrei* leave their nests for mating flight

just before dawn, while those of Argentine ant *Iridomyrmex humilis* fly out during the last two hours before dusk (Hölldobler and Wilson, 1990). Similarly, reproductives of fire ant *Solenopsis saevissima* mate during second half of the day, while those of *Camponotus clarithorax* during first half of the day (McCluskey, 1965). In African *Dorylus* ants, reproductives of *D. moestus* species fly out to mate at dawn*, D. burmeisteri* species at dusk, while others at different times during the night (Haddow et al., 1966). Although, circadian locomotor activity rhythm has been studied in a few species of carpenter ants, our understanding regarding the circadian timing system involved in mating and its behavioural and physiological impact is very preliminary (McCluskey, 1965; North, 1987; Sharma et al., 2004a-c).

In ant queens, mating is followed by immediate, drastic, and long lasting changes in behaviour (McCluskey, 1967). Within a very short span of time, often in the order of minutes, hydrocarbon profiles of queens change and they become less attractive to males for further mating (Oppelt and Heinze, 2009). Furthermore, they undergo changes in phototactic behaviour and in brain volume (Julian and Gronenberg, 2002). These changes are likely to enable ants to adapt to the new phase of their life during which they would start laying eggs to initiate a new colony (Hölldobler and Wilson, 1990; Julian and Gronenberg, 2002).

Apart from the mating related changes, many organisms undergo age dependent changes in their circadian clocks (Brock, 1991). Such changes in mammals are thought to be due to reduction in the volume and number of cells in the suprachiasmatic nucleus (Swaab et al., 1985; Brock, 1991), or due to decrease in the level of clock mRNAs of certain neuropeptide such as vasoactive intestinal polypeptide (Kawakami et al., 1997;

Krajnak et al., 1998). In peripheral tissues also, peak and overall levels of the gene expression reduces with increasing age (Malatesta et al., 2005). The amplitude of circadian rhythms is also affected with age, and older animals start displaying split activity rhythms (McAuley et al., 2002). Furthermore, in some species, older animals take longer to re-entrain to phase-shifted light/dark (LD) cycle, and their circadian period is lengthened (Valentinuzzi et al., 1997). Age dependent changes in circadian rhythms are also observed in insects. For example, sleep profile of fruit flies *D. melanogaster* changes with age (Koh et al., 2006b). In honeybee, workers start displaying robust activity rhythm with increasing age (Bloch and Robinson, 2001; Bloch, 2010). To the best of our knowledge such age dependent changes in circadian rhythms has never been reported in ants.

Two tropical species of carpenter ants *Camponotus compressus* and *Camponotus paria* live in dark underground nests, wherein light, temperature, and humidity are fairly constant (Hölldobler and Wilson, 1990). *C. paria* workers are day active, whereas *C. compressus* workers are night active, and both species colonize fairly similar habitat, often colonies of one species are found adjacent to the other. The *C. paria* ants are often attacked, injured, and killed by *C. compressus*, which suggests a clear dominance of *C. compressus* over *C. paria* (*Shahnaz Rahman Lone and Vijay Kumar Sharma, personal observation*). In our previous studies we shown that these ants display high level of developmental and circadian plasticity - possibly to meet the changing needs of the colony (Sharma et al., 2004a-c; Lone and Sharma, 2008; Lone et al., 2010, 2011). The minor workers (nurses) perform tasks around the clock and are found to be arrhythmic; major workers (foragers) bring food to the colony and are rhythmic, whereas major

workers who guard the colony are arrhythmic. The reproductives (virgin males and queens) are rhythmic and remain in-phase during the mating season (Sharma et al., 2004a). In the field, *C. compressus* males and queens mate when the ambient light intensity falls below 10 lux. We have observed that the reproductives of *C. paria* come out of their nests about 30-45 min before *C. compressus*. Males and queens from different colonies form groups and swarm near the mating site. In a previous study we have shown that virgin queens display rhythmic locomotor activity behaviour under constant laboratory conditions with circadian period (Sharma et al. 2004a-c). Much like the queens of other species of ants, *Camponotus* queens also become arrhythmic soon after mating (McCluskey, 1967), however, circadian rhythms reappear once the queens stop laying eggs (Sharma et al., 2004a). Notwithstanding the importance of mating and age related changes in circadian rhythms in facilitating social organization, these factors have never been rigorously and systematically studied in any social insect other than honeybees. Given that *Camponotus* ants are known to display considerable amount of plasticity in development (Lone and Sharma, 2008; Lone et al., 2010, 2011), and circadian behaviour (Sharma et al., 2004a-c), it would be interesting to know whether reproductive status and age have any effect on their circadian locomotor activity and phototactic behaviours.

In this chapter I have chosen two sympatric species of *Camponotus* ants – *C. compressus* and *C. paria* to assay the effect of mating and aging on their circadian locomotor activity and phototactic behaviours. I compared various circadian parameters of locomotor activity behaviour of virgin and mated queens of *C. compressus* species under 12:12 hr LD cycles to understand the effect of mating on the circadian clock. To

study the effect of aging and reproductive status on circadian rhythm I assayed locomotor activity rhythm of young and old virgin and mated queens of the *C. compressus* species.

5.2 Materials and methods

Virgin males and queens (winged) were collected from the campus of Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore (13°00′ N, 77°32′ E) while they were emerging out of their nests for mating flights. To additionally confirm the reproductive status of females in the laboratory the plates in which they were housed were continuously monitored for any fertile eggs. Similarly mated queens were collected while they were trying to dig into the soil to construct nests. They were identified by the absence of wings, and by their ability to lay fertile eggs. Ants were immediately transferred individually into petri plates (diameter × height = 90 mm × 15 mm) and introduced into an incubator with 12:12 hr LD, and constant temperature (25 ± 0.5 ^{o}C; mean ± SD), and humidity (~80%). The colonies were provided with *ad libitum* food in the form of Bhatkar diet (Bhatkar and Whitcomb, 1970) and 10% dilute honey solution. The petri-plates were cleaned with 5% ethanol on alternate days, at irregular hours. Care was taken to ensure minimal disturbance to the ants.

The locomotor activity behavior of ants was recorded individually using a recording device that uses two pairs of infrared emitters and receivers to track movements of small insects (Sharma, 2003c). Signal from the device was stored in 5 min bins for analysis using CLOCKLAB software from Actimetrics, USA (www.actimetrics.com). The activity of virgin males was recorded for 4-5 days, while those of queens for 7-10 days. The number of ants used in this study were - *C. paria* (CP) queens (n = 13), CP males (n = 10), *C. compressus* (CC) queens (n = 15), and CC

males (n = 11). Average activity (calculated using data collected over 4 days), species, and sex were taken as fixed factor for analysis of variance (ANOVA).

To study the effect of mating on locomotor activity rhythm we collected (n = 12) CP virgins, (n = 14) CP mated, (n = 14) CC virgins, and (n = 16) CC mated individuals, and transferred them individually into petri plates for recording their locomotor activity behaviour. Onset of locomotor activity was taken as the phase marker, and activity was averaged across the number of days and individuals to obtain average profiles. The coherence of phase of activity rhythm was estimated using circular vector, on a scale of 0 to 1, where 1 would mean perfect phase coherence, and 0 no coherence (Batschelet, 1981). The precision of activity rhythm was estimated by taking the reciprocal of standard deviation (SD) across the onsets of daily activity. Phase coherence of virgin males and females was calculated by generating five replicate sets of phase data by bootstrapping with replacement.

To estimate the effect of age on locomotor activity rhythm of queens, locomotor activity behavior of freshly caught virgin queens and mated *C. compressus* queens was recorded in LD and compared with those maintained under LD cycle of the laboratory for about a year. The reproductive status (virgin or mated) and age (young or old) were taken as fixed factors for ANOVA. A total of (n = 15 for virgins, n = 16 for mated) freshly caught queens and (n = 10 for virgins, n = 23 for mated) one year old *C. compressus* queens were used in this study.

For the phototaxis assay, virgin and mated queens of both species were kept individually in fresh petri plates with food and honey solution along with moist cotton to maintain humidity, in an incubator maintained under 12:12 hr LD cycles. Ants were

maintained under LD cycles for the first three days, and were subsequently introduced into DD for the assay. The assay was performed under dim red light ($\lambda > 650$ nm) in a Y-maze with two arms, each 7 cm long, joined at an angle of 45^{o} to a 5 cm long base. One of the arms of the Y-maze was illuminated at 10 lux using light emitting diode, while the other arm was kept dark. Ants were released from the base of Y-maze and given 1 min to chose between the illuminated and dark arms. If ants chose the illuminated arm they were classified as positively phototactic, and if they choose the dark arm they were classified as negatively phototactic. Eight groups, each comprising of six individuals were used at different circadian times (CTs) - CT00, 04, 08, 10, 14, 16, 18 and 22 (CT00 is taken as the onset of activity). At each time point, six queens were tested 4 times each to estimate the responses. The assay was repeated once again on the very next day with six fresh ants at each time point, and data from both the days were used as replicates for ANOVA, where time point was taken as a fixed factor. Ants unable to make a clear choice were given two more chances before being excluded from the data set. The percentages of ants who fail to make a clear choice were negligible (< 1%). In each trial, both arms, and base of the Y-maze were cleaned with 5% ethanol, and allowed to air dry to remove any odor. Separate apparatus were used for the two species.

5.3 Results

5.3.1 ***Activity rest rhythm of virgin males and queens:*** Activity of virgin males and queens of both species was similar at most time points tested except during lights-off (Figure 1a, b). Since the major differences occurred around the evening activity peak we have focused on this duration. To compare activity levels of ants, activity counts between ZT11 and ZT14 (ZT12 is taken as lights-off) was summed into 30 min bins for

the major part of this study. We find that activity of males of both species is comparable to females at all time points, except lights-off when *C. paria* males are more active than females (Figure 1a-b). Anticipation and startle responses to lights-off were estimated using formulae proposed in Stoleru et al. (2004). The virgin males and queens of *C. paria* anticipate lights-off better than *C. compressus*, which shows greater startle response than *C. paria* (Figure 1c, d). Furthermore, at all time points tested a significantly greater fraction of *C. paria* virgin queens are positively phototactic, while queens of *C. compressus* are negatively phototactic (Figure 3a).

ANOVA on the activity data revealed a significant effect of species ($p < 0.005$), sex ($p < 0.001$), time point ($p < 0.0001$), species × time point ($p < 0.0001$), and sex × time point interaction ($p < 0.0001$), however, effect of species × sex ($p = 0.16$), and species × sex × time point interactions are statistically not significant ($p = 0.81$). Post-hoc multiple comparisons using Tukey's test revealed that in *C. paria* activity of virgin males and queens are similar prior to lights-off, and afterwards males become more active than females ($p < 0.0005$) (Figure 1a). In *C. compressus*, activity of virgin males and queens are indistinguishably similar at all time points tested ($p > 0.05$) (Figure 1b).

The phase-relationship between the onset of activity rhythm and lights-off in *C. compressus* queens and males are + 0.31 hr and + 0.42 hr, respectively, and the activity rhythm in virgin males and queens show very high degree of phase coherence with *r* values of 0.98 and 0.99 (on a scale of 0-1), respectively. The phase-relationship between the onset of activity rhythm and lights-off in *C. paria* queens and males are + 0.88 hr and 1.79 hr, respectively, while the activity rhythm in virgin males and queens show very high degree of phase coherence with *r* values of 0.98 and 0.90. ANOVA on *r* values

showed a significant effect of species, sex, and species × sex interaction ($p < 0.0001$). Post-hoc comparisons using Tukey's test revealed that activity rhythm of *C. compressus* virgin queens is more coherent than that of *C. paria* ($p < 0.0005$), while that of males does not differ between the two species ($p = 0.97$) (Figure 2). ANOVA on the direction of phase coherence vector (a^o) showed significant effect of species, sex, and species × sex interaction ($p < 0.0001$). The a^o values do not differ between males and queens of *C. compressus* ($p = 0.42$), however, a^o values of *C. paria* males differ from that of queens ($p < 0.0005$). In *C. compressus*, phase distribution of males and queens does not differ as per Watson-Williams test ($p > 0.50$), while in *C. paria* it is found to be significantly different ($p < 0.0001$) (Figure 2a, b).

ANOVA on the precision data showed significant effect of species ($p < 0.0005$), however, effect of sex ($p = 0.73$), sex × species interaction is statistically not significant ($p = 0.36$). Post-hoc multiple comparisons revealed that precision of activity rhythm does not differ between males and queens of any given species ($p > 0.05$), however, activity rhythm of *C. compressus* is significantly more precise than *C. paria* ($p = 0.09$ for males; $p < 0.01$ for queens) (Figure 1e).

ANOVA on the phototactic response data showed significant effect of species ($p < 0.0001$), however, effect of time point ($p = 0.15$), and species × time point interaction is statistically not significant ($p = 0.88$). Post-hoc multiple comparisons using Turkey's test revealed that at all time points tested a significantly greater fraction of *C. paria* virgin queens are positively phototactic than *C. compressus* ($p < 0.05$; Figure 3a). Thus the two related species of *Camponotus* ants differ in their temporal distribution of circadian behaviours.

Figure 1

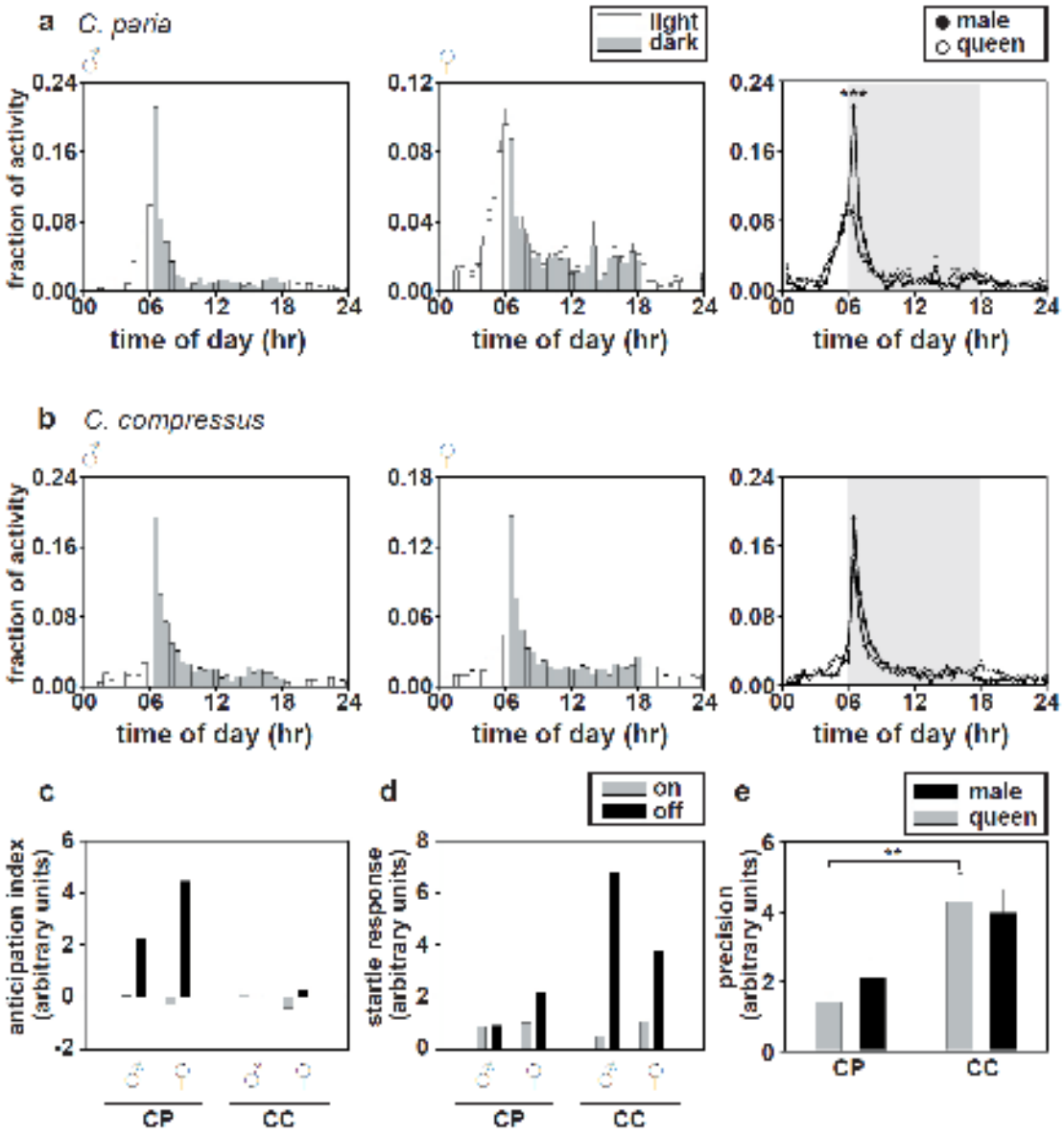


Figure 1 ***Activity rhythm of virgin males and queens.*** Activity profiles of *C. paria* (a) and *C. compressus* (b) virgin males and queens. Activity is plotted along ordinate and time of the day along abscissa. Light bars indicate activity during the light phase of light/dark (LD) cycles, and dark bars during darkness. In the combined plot, activity of males is shown in dark circles, and that of queens in open circles. Error bars are standard error around the mean (SEM). Anticipation (c) and startle responses (d) to lights-on and lights-off of *C. camponotus* (CC) and *C. paria* (CP), are shown as grey and dark bars respectively. The precision of activity rhythm of *C. paria* and *C. compressus* queens (grey bars) is significantly different ($p < 0.01$) (e), while that of CC males (black bars) is marginally greater than CP males ($p = 0.09$) (e). Statistical levels of significance $p < 0.05$ is denoted by one asterisk, $p < 0.01$ by two asterisks, and $p < 0.005$ by three asterisks.

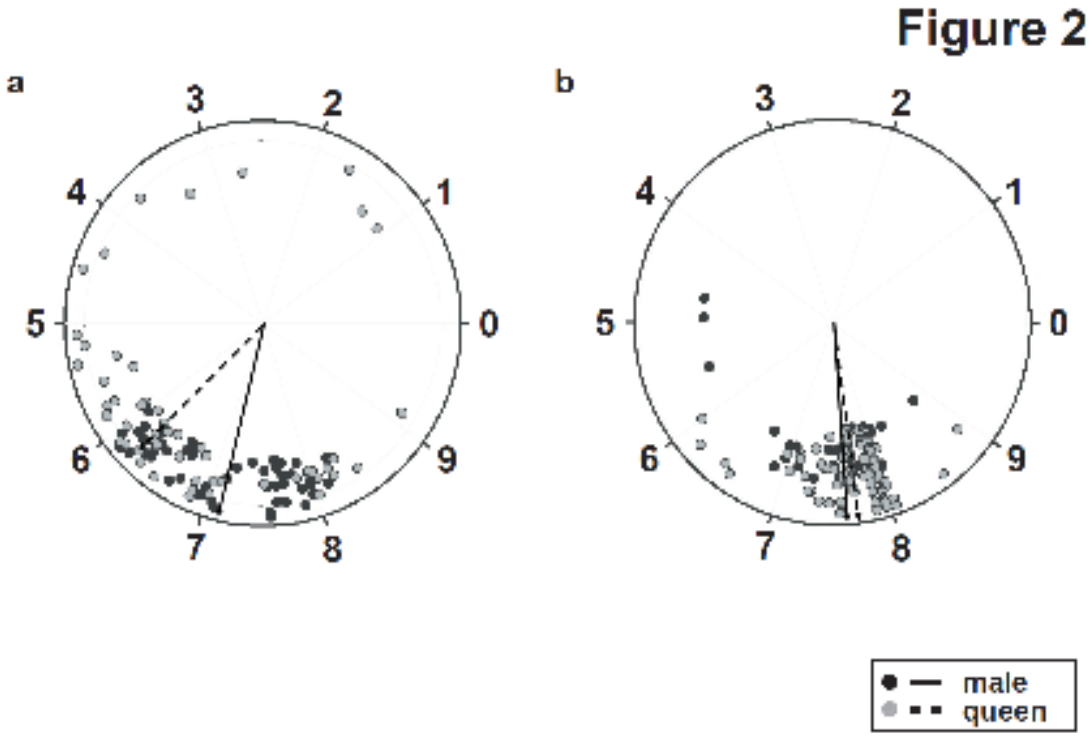


Figure 2 ***Phase distribution of virgin males and queens.*** Phase distribution of onset of locomotor activity rhythm of *C. paria* (a), and *C. compressus* (b), where dark circles and unbroken arrow denotes phase and phase coherence vector of males, and grey circles and broken arrow those of queens. The activity rhythm of *C. compressus* virgin queens is significantly more coherent than *C. paria* ($p < 0.0005$), while that of males does not differ between the two species ($p = 0.97$).

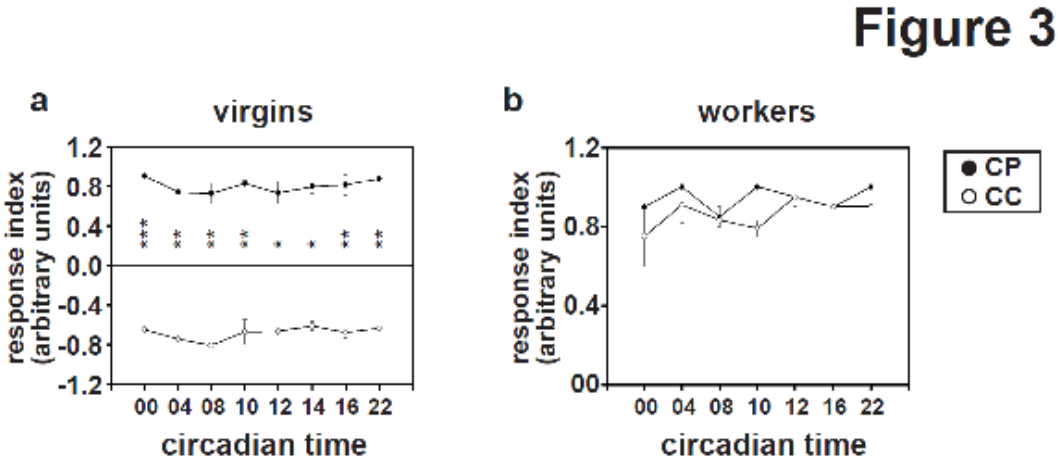


Figure 3 ***Phototactic response of virgin males and queens.*** The phototactic responses of queens (a) and workers (b) of *C. compressus* (CC) and *C. paria* (CP) ants plotted as a function of circadian time. Movements towards light are shown by positive values along the y-axis, and away from light by negative values. Other details same as in Figure 1. At all time points tested a significantly greater fraction of *C. paria* virgin queens are positively phototactic than *C. compressus* ($p < 0.05$). The phototactic response of the workers of the two species does not differ ($p > 0.05$).

5.3.2 ***Activity peak of ant queens is reduced after mating:*** The activity peaks of queens of both species decreases after mating, and most species-specific differences in activity and phototactic rhythms disappear (Figure 4a, b). The queens become poor in anticipating lights-off and show reduced startle response to lights-off (Figure 4a, b). *C. paria* queens become negatively phototactic, however, *C. compressus* queens remain negatively phototatctic (Figure 6).

ANOVA on the activity data showed significant effect of species (CP/CC) ($p < 0.05$), reproductive status (virgin/mated) ($p < 0.0001$), time point (ZTs) ($p < 0.0001$), species × time point ($p < 0.0001$), reproductive status × time point ($p < 0.0001$), species × time point × reproductive status interactions ($p < 0.0001$). However, effect of species × reproductive status interaction is statistically not significant ($p = 0.77$). Post-hoc multiple comparisons showed that following mating evening activity peak (at ZT12.5) of *C. compressus* queens decreases significantly ($p < 0.0001$) (Figure 4b). In *C. paria* queens also a similar decrement of activity is noticed at more than one phase - ZT11.5 ($p < 0.001$), ZT12 ($p < 0.0001$) and ZT12.5 ($p < 0.05$). The activity of virgin queens during light to dark transition (ZT11.5, ZT12 and ZT12.5) is significantly different between the two species ($p < 0.005$). However, after mating it becomes similar ($p > 0.05$) (Figure 4a, b).

ANOVA on the precision data revealed significant effect of species (CP/CC) ($p = 0.06$), species × reproductive status ($p < 0.01$) interaction, however, effect of reproductive status (virgin/mated) is statistically not significant ($p = 0.98$). Post-hoc multiple comparisons revealed that activity rhythm of mated *C. compressus* ($p = 0.13$) and *C. paria* queens ($p = 0.30$) is as precise as virgin queens (Figure 4d).

ANOVA on the activity peak data showed a significant effect of mating status (virgin/mated) ($p < 0.0001$), species × mating status interaction ($p < 0.05$), however, effect of species is statistically not significant ($p = 0.12$). Post-hoc multiple comparisons showed that activity peak of *C. compressus* ($p < 0.0005$) and *C. paria* ($p < 0.01$) queens is significantly reduced after mating (Figure 4c). *C. paria* queens stop anticipating lights-off, and start behaving in a manner similar to *C. compressus* queens. *C. compressus* queen's poor ability to anticipate lights-off is further reduced, and their startle response to lights-off is reduced to almost half (Figure 4e, f).

In *C. compressus*, phase-relationship of onset of activity with lights-off is - 0.14 hr in mated, and + 0.31 hr in virgin queens. Phase-relationship of activity onset in *C. paria* mated queens is + 0.28 hr, while that in virgin queens is +1.79 hr. ANOVA on the *r* values showed a significant effect of species, reproductive status, and species × reproductive status interaction ($p < 0.0001$). Post-hoc multiple comparisons revealed that *C. paria* mated queens are more synchronous ($p < 0.0005$) compared to virgin queens, while phase synchrony of virgin and mated *C. compressus* queens do not differ ($p = 0.96$) (Figure 5b). Following mating, direction of phase coherence vector of *C. compressus* and *C. paria* queens registered a significant change ($p < 0.0005$). The phase distributions of virgin and mated queens are significantly different as per Watson-Williams test ($p < 0.0005$) (Figure 5a, b).

ANOVA on the phototactic response data revealed a significant effect of species, reproductive status (virgin/mated) ($p < 0.0001$), and reproductive status × time point interaction ($p < 0.01$), however, effect of time point, species × reproductive status, species × time point and species × reproductive status × time point interactions is

Figure 4

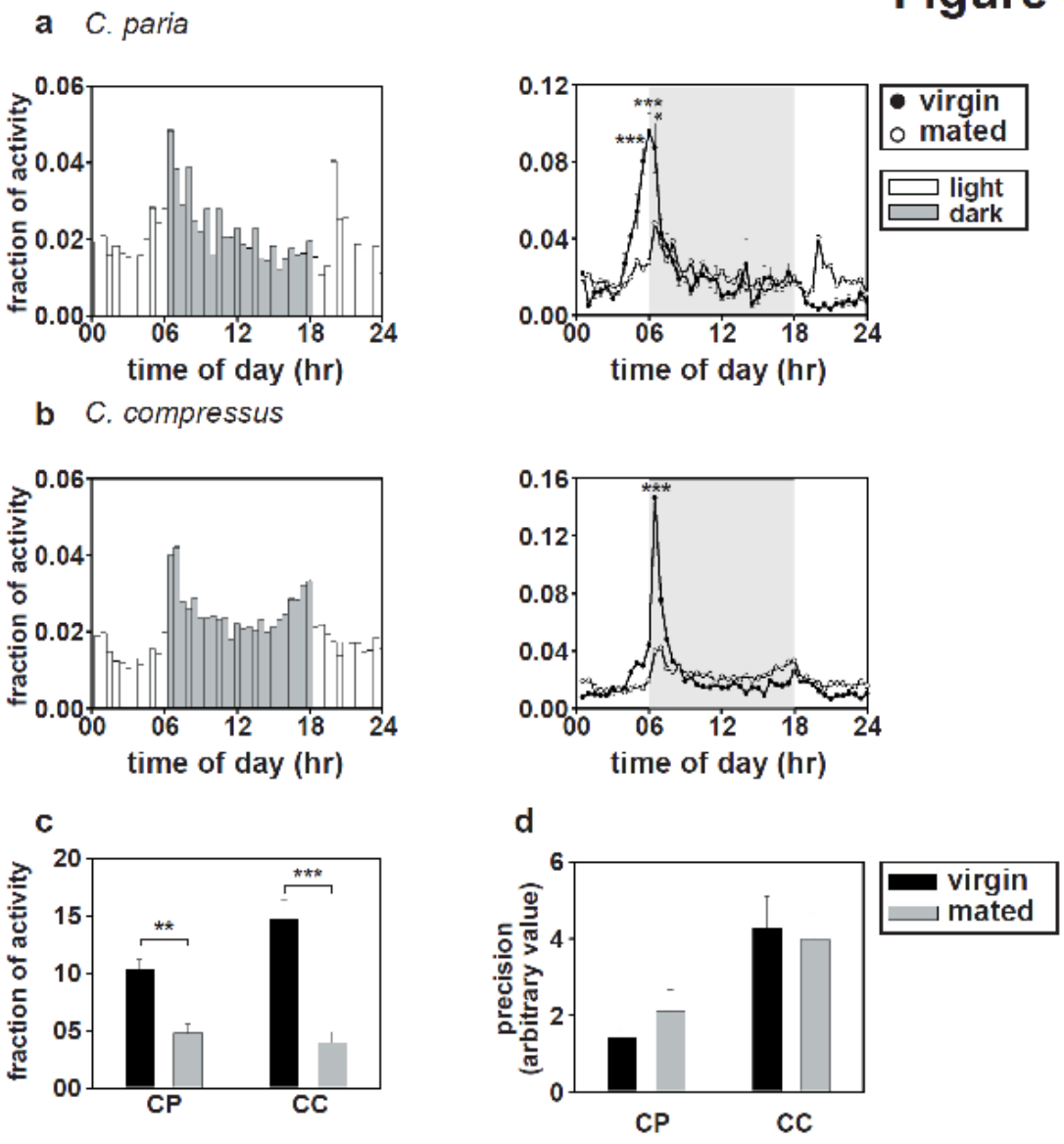


Figure 4 ***Effect of mating on the locomotor activity rhythm of queens.*** Activity profiles of *C. paria* (a) and *C. compressus* virgin and mated queens (b). Activity is plotted along ordinate and time of the day (in hours) along abscissa. Queens show reduction in activity following mating; *C. paria* queens show reduced activity at ZT11.5 ($p < 0.001$), ZT12 ($p < 0.0001$) and ZT12.5 ($p < 0.05$), while *C. compressus* only at ZT12.5 ($p < 0.0001$). As a result of mating queens show reduction in activity peak (*C. paria*; $p < 0.01$, and *C. compressus*; $p < 0.0005$) (c). Precision of locomotor activity rhythm of queens from both species remains unchanged after mating ($p > 0.05$) (d).

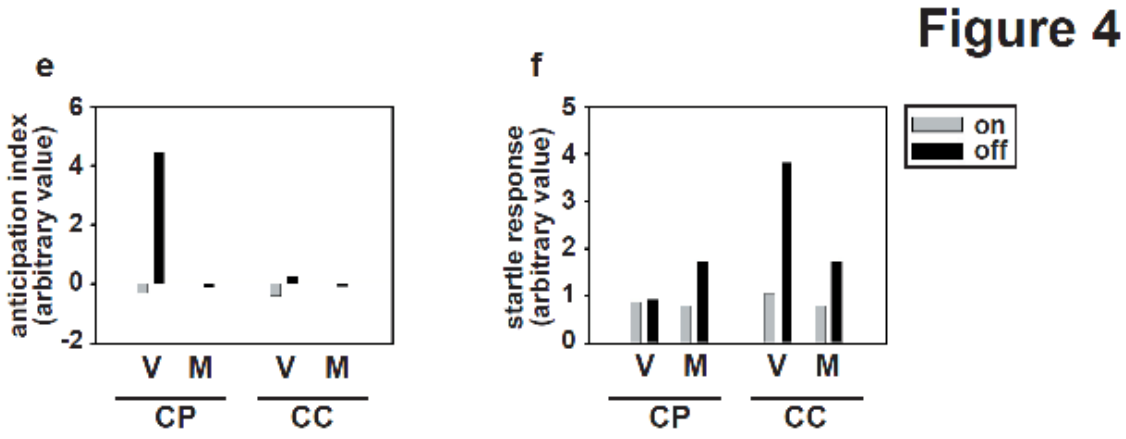


Figure 4 ***Effect of mating on the locomotor activity rhythm of queens.*** Anticipation to lights-off in *C. paria* (e) and startle response in *C. compressus* queens is reduced after mating (f). Other details same as in Figure 1.

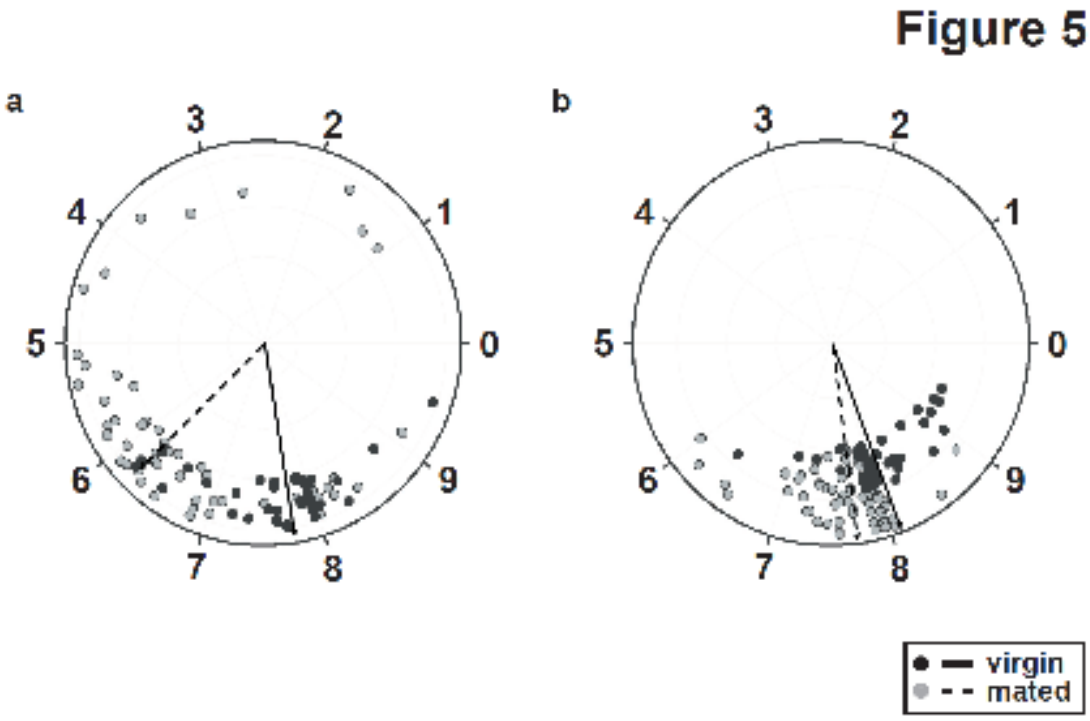


Figure 5 ***Effect of mating on the phase coherence of locomotor activity rhythm of queens.*** There is a significant increase in phase synchrony ($p < 0.0005$) of *C. paria* queens following mating (a), while in *C. compressus* queens it remains unchanged ($p = 0.96$) (b). There is also a change in the direction ($p <$

0.0005) of phase coherence vector in both the species. The phase distributions of virgin and mated queens are significantly different in both species as per Watson-Williams test ($p < 0.0005$) (a, b).

Figure 6

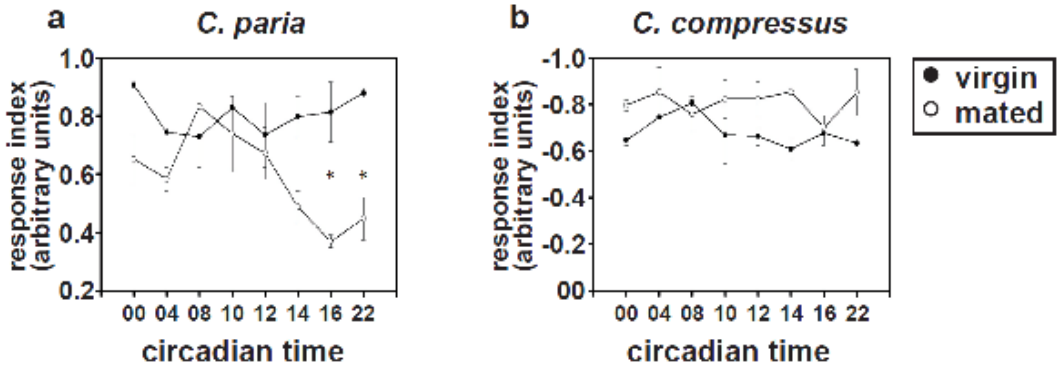


Figure 6 ***Effect of mating on phototactic behaviour of queens.*** Phototactic response in *C. paria* at CT16 ($p < 0.05$) and CT22 ($p < 0.05$) becomes negative after mating (a), whereas there is no change in the photic preferences in *C. compressus* queens (b). Other details same as in Figures 1 and 3.

statistically not significant ($p > 0.05$). Post-hoc multiple comparisons revealed that post mating *C. paria* queens become less phototactic at two time points (ZT16 and ZT22) ($p < 0.05$), while at other time points there is no change in the phototactic response ($p > 0.05$). In *C. compressus* there is no effect of mating on phototactic behaviour ($p < 0.05$) (Figure 6a, b).

5.3.3 ***Effect of age on activity rhythm of virgin and mated C. compressus queens:***

While mated queens do not show any change in activity with age, morning and evening peaks of activity of virgin queens undergo significant age related changes (Figure 7a, b). With age, morning activity peak of virgin queens increases, and their evening peak of activity decreases. ANOVA on the activity data showed significant effect of reproductive status (virgin/mated) ($p < 0.0001$), time point (ZTs) ($p < 0.0001$), reproductive status × time point ($p < 0.0001$), age × time point ($p < 0.0001$), reproductive status × time point × age interactions ($p < 0.0001$). However, effect of age ($p = 0.19$), and reproductive status × age interaction is statistically not significant ($p = 0.82$). Post-hoc multiple comparisons showed that while age had no effect on the locomotor activity rhythm of mated queens, in virgin queens morning peak of activity (at ZT0.5) increases significantly, and evening peak of activity (at ZT12.5) decreases significantly ($p < 0.0001$) (Figure 7a,b).

5.4 Discussion

Our studies reveal interesting differences in the behaviour of two related species of ants – *C. paria* and *C. compressus*. The workers of *C. paria* are day active, while those of *C. compressus* are night active, however, virgin males and queens of both species mate in the evening within a span of an hour. The *C. paria* virgin queens go out on their mating

Figure 7

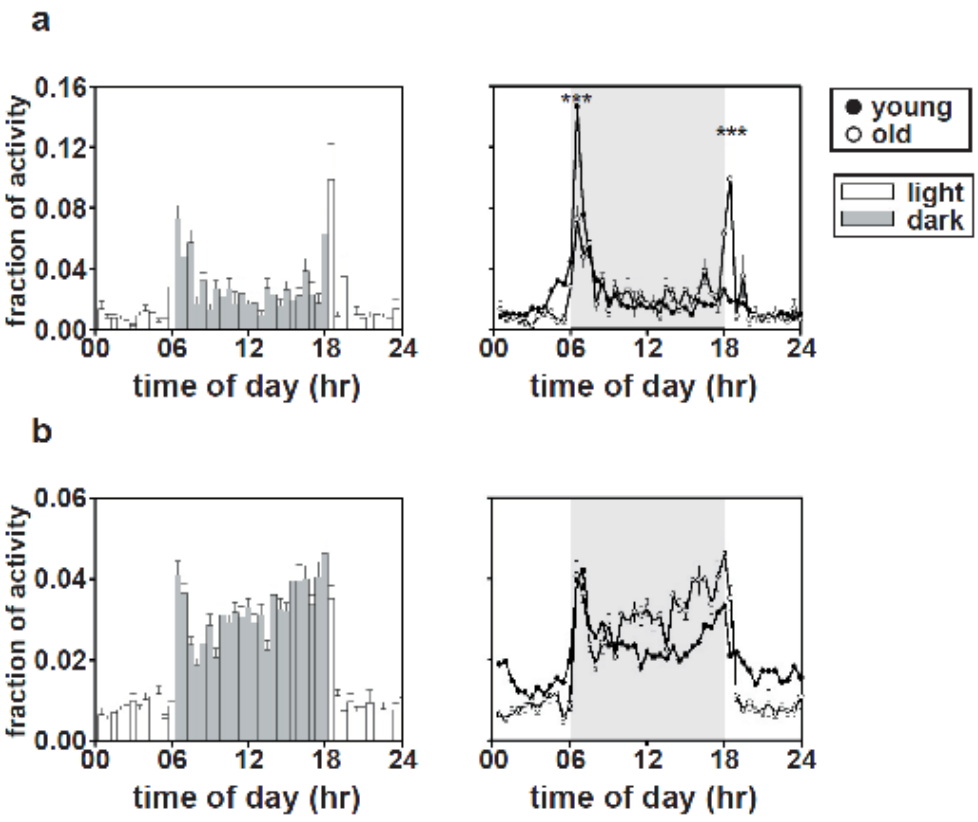


Figure 7 ***Effect of aging on the activity profiles of virgin and mated queens.*** The figure illustrates activity profiles of young and old virgin (a) and mated queens (b) of *C. compressus* queens under 12:12 hr light/dark (LD) cycles. The evening activity peak of virgin queens decreases significantly after one year ($p < 0.0001$), and a novel morning peak emerges ($p < 0.0001$). Other details same as in Figure 1.

flights about 30 - 45 min prior to *C. compressus* queens (*Shahnaz Rahman Lone and Vijay Kumar Sharma, personal observation*). The queens of *C. paria* are positively phototactic, while queens of *C. compressus* are negatively phototactic, at all time points tested (Figure 3a). Under laboratory LD cycles, activity profiles of *C. compressus* virgin males and queens are quite similar, while those of C. *paria* males and queens differ but only slightly. Immediately after lights-off (ZT12.5) *C. paria* males exhibit relatively higher levels of activity than queens (Figure 1). As indicated by the positive phase-relationship between activity onset and time of lights-off in laboratory LD cycles, *C. paria* virgin queens start activity about an hour and half prior to lights-off (+ 1.79 hr), while males about an hour later (+ 0.88 hr). The reproductives of *C. compressus* start activity just before lights-off maintaining a relatively less positive phase-relationship with LD cycles (+ 0.31 hr for queens, and + 0.42 hr for males) (Figures 2a, b). The *C. paria* virgin males and queens anticipate lights-off much better than *C. compressus*, who exhibit a prominent startle response to lights-off (Figures 1c, d). Furthermore, activity rhythm of *C. compressus* queens is more precise and coherent than *C. paria* queens, while there is no difference between the precision of activity rhythm in males of the two species (Figure 1e). The properties of the activity rhythm of virgin males and females of both species are in close agreement with those observed under natural conditions. After

mating, activity profiles and activity peaks of queens are severely altered in both the species; *C. paria* queens stop anticipating lights-off and *C. compressus* queens undergo reduced level of startle response to lights-off. After mating *C. paria* queens change their photic preferences, particularly during subjective nights (CT16 and CT22), when they become relatively less photophilic (Figure 6a). Such changes in photic preferences are not seen in the queens of *C. compressus*; who were photophobic as virgins and remain so even after mating (Figure 3a, b). The *C. compressus* queens exhibit age related changes in locomotor activity rhythm (Figure 7). While activity rhythm of mated queens remains invariant with age, those of virgin queens changed from being unimodal to bimodal in the course of one year.

The ability to keep time is of prime importance to social insects especially for the production of sexual castes, for scheduling their nuptial flights, and for establishing new colonies (Tauber et al., 1986). Further, in eusocial ants critical events such as growth, development and reproduction need to be tightly regulated with regard to time of the day and year (Hölldobler and Wilson, 1990). For example, among army ants *Neivamymex*, males of sixteen species are found to undertake mating flights at different times of the year (Baldridge et al., 1980). Similarly, African *Dorylus* ants go out on their mating flights at species-specific times of the day, males of *D. moestus* species fly out at dawn, *D. burmeisteri* species at sunset, while others at different times during the night (Haddow et al., 1966). Mating in *Camponotus* ants is seasonal, it occurs during the months of April and May, soon after the first rains, following a prolonged dry season (Lone and Sharma, 2008). The virgin males and queens of *C. paria* are seen to shuttle in and out of their nests prior to their mating flights, probably to sample light. They eventually fly out

of their nests in large numbers, and are seen swarming near mating sites. This is consistent with the fact that in the laboratory recordings mated queens of *C. paria* started activity prior to lights-off (Figure 5a), and *C. compressus* queens only after lights-off (Figure 5b). These ants mate during evenings possibly to avoid the risk of desiccation due to day time heat, predation by bats, dragonflies, birds, workers of same or different species, and game birds or by some unknown predators (Levin et al., 2009; Whitcomb et al., 1973). We have seen in field observations that *C. paria* virgin males and queens start their mating flight 30-45 min before *C. compressus*, which is quite consistent with the results on locomotor activity behaviour under laboratory LD cycles (Figures 2a, b). We found that under laboratory LD cycles, *C. paria* virgin queens start activity an hour or so earlier than males (Figure 1a), which suggests that probably queens are normally active in the nest much before the males but are somehow prevented from going out on mating flight by the workers (Hölldobler and Wilson, 1990). The workers of *Camponotus* ants (*C. herculeans*) are known to prevent the reproductives from making unscheduled take-off for mating flights (Hölldobler and Wilson, 1990). This could be because workers are likely to be better informed about the local surroundings such as light, temperature, humidity, predators, and habitat conditions, and therefore may be in a better position to decide about the suitable time for nuptial flights of the reproductive castes. The activity profile of *C. compressus* ants (Figure 1b) is found to match well with the timing of their mating flights in nature, which is often close to dusk. The *C. paria* males and females anticipate lights-off much more efficiently than *C. compressus*. In nature *C. compressus* and *C. paria* ants are found to fight fiercely, and most often it is the *C. compressus* which

wins, and probably this may be the reason why *C. paria* ants have evolved the ability to schedule their mating flight much before its competitor *C. compressus*.

Ants like many other insects are attracted towards light, a behaviour which is believed to be of great adaptive value to insects (Gottfried and Gunn, 1961). The *C. paria* queens are positively phototactic as virgins, and their mating flight occurs when there is enough light in the environment. The *C. compressus* queens are negatively phototactic (Figure 3a), and they undertake mating flight when the ambient light intensity falls below 10 lux. Importance of such differences in phototactic responses can be gauged by the fact that the phototactic behavior of workers is similar even though their activity onset times are almost 12 hr apart (Figure 3b). These results clearly suggest a close link between the circadian and mating behaviours in *Camponotus* ants.

In several species of ants, winged males and queens mate at species-specific times of the day. Soon afterwards males die while inseminated queens leave their aerial mating sites, land on the ground, and start digging into the soil to construct a new nest (McCluskey, 1992, Hölldobler and Wilson, 1990). The *C. paria* queens, who were positively phototactic as virgins may perhaps rely more on vision for mating than olfaction, and became negatively phototactic after mating (Julian and Gronenberg, 2002). On the other hand, *C. compressus* queens were negatively phototactic as virgins and remained so even after mating (Figure 6). In ants, post-mating behavioural changes are seen within a few minutes of mating, and changes in hydrocarbon profiles is found to occur within 30 min, rendering the queens unattractive for courting males (Oppelt and Heinze, 2009). Post-mating shedding off of wings and initiation of egg-laying by queens has primarily been attributed to changes in juvenile hormone titer (Kearney et al., 1977;

Vargo and Laurel, 1994). After mating, queens of both species undergo decrease in activity peaks associated with decrease in lights-off anticipation in *C. paria*, and startle response in *C. compressus* (Figure 4). Post-mating changes in behaviour are well documented in honeybees (Fahrbach et al., 1995; Richard et al., 2007), and ants (McCluskey, 1967; Sharma, 2004a), and are believed to be of great relevance for the social organization of insects (Julian and Gronenberg, 2002). Such long-lasting changes in behaviour are found to be associated with concurrent re-modeling of neural network, and reduction in brain volume, mostly of the regions supposed to be vestigial under the dark conditions of colony where queens spend most of their lives.

Social insects also undergo age dependent changes in their circadian rhythms (Moore, 2001). For example, freshly emerged honeybees workers (during the first 2 – 3 weeks of emergence) are mostly confined to the nest, and their activity, oxygen consumption, and temperature regulatory behaviours occur without any rhythmicity (Moore, 2001). Older bees (4 - 7 weeks), bring food for the colony, and have well developed behavioural rhythms (Toma et al., 2000; Moore, 2001). In ants, older workers occupy different zones of the nest and are capable of performing wider range of tasks suggesting that division of labor in ants is relatively flexible (Sendova-Franks and Franks, 1993). With age, activity profile of virgin *C. compressus* queens changes, while those of mated queens remain mostly unchanged (Figures 7a, b). Such changes in the activity of queens are marked by significant reduction in the evening activity peak, and an appearance of morning activity peak (Figure 7a). This suggests that virgin queens of *Camponotus* ants undergo age-related changes in circadian behaviour. Although, we do not yet understand what the adaptive significance could be of bimodal activity in older

queens, or why mated young do not display bimodal activity patterns in *Camponotus* ants, we speculate that unimodal peak may reflect activity associated with mating flight, and bimodal activity may be related to digging of nest during nights, and behaviours associated with tasks inside the colony.

In summary, I have shown that reproductives of two species of *Camponotus* ants have temporally segregated their activity/rest rhythms, well-tuned to suit their mating behaviour. The *C. paria* virgin queens mate half an hour before dusk and are positively phototactic. Under laboratory LD conditions they start activity much before lights-off, while *C. compressus* queens are negatively phototactic as virgin and do not anticipate lights-off but show startle effect to lights-off. The virgin males and queens of night active species (*C. compressus*) display highly precise activity rhythm confined to the light to dark transition, while those of day active species (*C. paria*) are active for a larger stretch of time marked by relatively less precise activity rhythm. Such species-specific behavioural signatures of circadian clocks displayed by these ants disappear soon after mating. Such changes are likely to be adaptive as they may help queens to cope with the challenging conditions in dark underground nests, where consolidated activity would be of no use. Furthermore, the activity rhythm of virgin queens undergoes age-related changes, a feature not seen in mated queens. Following mating queens of day active species become negatively phototactic, while night active species remains negatively phototactic. Post-mating and age-dependent modulations of circadian behaviours are likely to conserve energy from being spent on futile behaviours and help ants in adapting to constant conditions of their nest.

Chapter 6

Socio-Sexual Interactions (SSI)

6.1 Introduction

Circadian clocks time a multitude of physiological and behavioral processes in a wide variety of organisms ranging from bacteria to humans. These clocks use several time cues to keep track of time in the local environment, among which social cues are of special importance. In fruit flies *Drosophila melanogaster*, social interactions especially among heterosexuals have been shown to have significant effect on circadian timing system (Fujii et al., 2007). Such interactions are rhythmic and occur predominantly in the night resulting in intense locomotor activity, and involve stereotypic behaviors such as chasing, avoiding, singing, dancing, licking, and copulation (Hall, 1994). In *Drosophila*, core circadian clock genes have been implicated in the regulation of rhythms in some socio-sexual behaviours such as courtship and mating (Wheeler et al., 1991; Tauber et al., 2003). Mating is the most important activity in the life of a sexually breeding organism - an ultimate determinant of its fitness. Although we know a fair deal about the regulation of locomotor activity and adult emergence rhythms, our understanding of the mechanisms underlying mating and egg laying rhythms are still in its infancy. Mating in *Drosophila* comprise a complex set of behaviors involving intense locomotor activity by both males and females (Hall, 1994). Circadian clocks regulate inter-pulse intervals in the courtship song of *Drosophila* (Kyriacou and Hall, 1980), an important ritual in courtship and mating (Hall, 1994). Not just the song but also the process of courtship itself is rhythmic at least in some species of *Drosophila* - *D. melanogaster*, *D. yakuba* and *D. kikkawai*, with a peak occuring just before lights-on (Hardeland, 1972). Mating in *Drosophila* is also rhythmic (Hardeland, 1972; Loher and Zervas, 1979), and is believed to be primarily dictated by females (Sakai and Ishida,

2001). A recent study showed that nocturnal sex drive (NSD), which is reflected in terms of close association between males and females, and quantified as enhanced night time activity is rhythmic and dictated primarily by males (Fujii et al., 2007). NSD is olfaction mediated as flies with loss of function mutation in a widely expressed olfactory receptor *Or83b* (Larsson et al., 2004) display severely compromised NSD (Fujii et al., 2007). In *D. melanogaster*, several pheromone receptors reside in the trichoid sensillae (Vosshall and Stocker, 2007), of which four (*Or65a*, *Or88a*, *Or47b*, and *Or67d*) are considered to be important for socio-sexual communications between males and females (van der Goes van Naters and Carlson, 2007). Although there is no doubt about the involvement of olfaction in socio-sexual interactions between males and females (Levine et al., 2002; Fujii et al., 2007; Krupp et al., 2008), specific olfactory receptor(s) involved in NSD are still unknown.

In *Drosophila*, circadian clocks regulate oscillations of genes responsible for vision, learning and memory, synaptic transmission, olfaction, locomotion, detoxification, metabolic stress, metabolism, mating, and egg laying, and at the molecular level are comprised of transcription-translational feedback loops of mRNA and protein levels of core clock genes such as *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*) (Allada and Chung, 2010; Claridge-Chang et al., 2001). The circadian organization is multi-oscillatory; central pacemaker cells located in the fly brain, and peripheral oscillators in various organs work in tandem to regulate rhythmicity in behavior and physiology (Plautz et al., 1997; Giebultowicz, 2000). The central pacemakers in *D. melanogaster* is comprised of six neuronal groups – Pigment Dispersing Factor (PDF)-positive small and large ventral lateral neurons (sLN_v and ILN_v), PDF-negative 5^{th} sLN_v,

dorsal lateral neurons (LN_d), three groups of dorsal neurons (DN1, DN2 and DN3), and lateral posterior neurons (LPN) (Kaneko and Hall, 2000; Helfrich-Förster, 2003, 2004; Sheeba et al., 2008). Electrical silencing or genetic ablation of PDF positive neurons results in arrhythmicity under constant dark (DD) conditions (Renn et al., 1999; Blanchardon et al., 2001; Nitabach et al., 2002). Overexpression of core clock protein PER in these neurons also cause arrhythmicity in locomotor activity behavior (Blanchardon et al., 2001; Yang and Sehgal, 2001). Besides neurons in the fly brain functional clocks are also located in peripheral organs such as antennae (Krishnan et al., 1999; Tanoue et al., 2004), compound eye photoreceptors (R1-R6)(Cheng and Hardin, 1998), epidermis (Ito et al., 2008), wings, legs, and malpighian tubules (Giebultowicz and Hege, 1997; Plautz et al., 1997; Hall, 2003). Although some peripheral oscillators are capable of generating self sustained oscillations, others such as those located in prothoracic gland required for development of fly, and fat bodies involved in feeding, rely mostly on central pacemakers in the fly brain for their proper functioning (Myers et al., 2003; Xu et al., 2008).

Here we report the results of our study aimed at identifying specific olfactory receptor(s), and clock neurons involved in mediating socio-sexual interactions (SSI) between males and females of fruit flies *D. melanogaster*. For this, we monitored locomotor activity behavior of pairs of flies from several wild type and mutant strains with specific defects in their olfactory or circadian timing systems. The logic of using locomotor activity to assess SSI or NSD is that the amount of locomotor activity of couples is positively correlated with courtship (Hall, 1994). We adapted a simple approach of recording locomotor activity behaviour of two flies together in locomotor

activity tubes (7 mm diameter × 80 mm length) designed for larger insects, using Drosophila Activity Monitoring system (DAM), Trikinetics, USA. The advantage of using DAM system over any other tracking device is that it allows us to make direct observation on a large number of individuals and allows estimation of sleep levels. We used locomotor activity and sleep of a pair of flies as read outs to quantify SSI. The male-female (MF) couples of wild type Canton S (CS) flies show enhanced night time activity and reduced sleep. The activity patterns of wild type MF couples closely match those reported by Fujii et al. (2007). Furthermore, our results suggest that the circadian neurotransmitter PDF, and the LN_v and LN_d clock neurons are not required for SSI. We show that such interactions are primarily male driven, and mediated by a subtype of the olfactory receptor *47b* (*Or47b*) and its peripheral circadian clock system.

6.2 Materials and methods

Different strains of fruit flies *D. melanogaster* were maintained as pre-adults and subsequently as virgin adults under 12:12 hr light/dark (LD) cycles with 500 lux white fluorescent light during the light phase and dim red light ($\lambda > 650$ nm) during the dark phase. Fly strains used in our study were: *CS*, *w*, *OR*, *yw*, $Or83b^0$, *Or65aGAL4*, *Or47bGAL4*, *Or88aGAL4*, *Or67dGAL4*, *pdfGAL4*, *cryGAL4*, *Mai179GAL4*, pdf^0, *UASdti*, *UASdORKC1*, *UASdORKNC1*, and *UASkir2.1*. Flies were segregated as male-male (MM), female-female (FF), and male-female (MF) pairs and introduced into activity tubes of 7 mm diameter and 80 mm length for locomotor activity recording. The locomotor activity tubes had corn-meal food at one end and cotton plug on the other. These tubes were placed in the DAM system and locomotor activity data was collected and stored in 5 min bins. Food in the activity vials was changed every day to avoid any

possible disturbance due to larval movement and physiological effects of presence of eggs and larvae. The activity data was analyzed using CLOCKLAB software from Actimetrics, USA. For visual comparison of activity patterns, actograms - a plot of activity, with time of the day plotted along abscissa and day in chronological manner along ordinate, were drawn. We used locomotor activity and sleep as read outs for SSI between males and females. A couple was considered to be asleep only when no activity count was registered for a duration of 5 min (Shaw et al., 2000). The activity and sleep profiles of the couples were computed by averaging data collected over a period of 24 hr, using data for the first five successive days. In addition, we estimated and compared the total activity, and sleep, during the light, dark, and entire LD cycles. Total, day time and night time sleep of MF couples was analyzed and compared with those of controls (MM and FF couples). To compare cumulative locomotor activity of MM, FF and MF pairs we excluded data collected during the light/dark and dark/light transitions (i.e., one hour before and after lights-on and lights-off) to avoid any confounding effect of startle activity due to sudden presence and absence of light. The amount of activity and sleep of couples were analyzed using analysis of variance (ANOVA) followed by post-hoc multiple comparisons using Tukey's test where $p < 0.05$ was considered as statistical level of significance. Statistical analysis of data was implemented using STATISTICA^{TM} for Windows Release 5.0 B (StatSoft., 1995).

6.3 Results

6.3.1 *Male-female (MF) Canton-S couples exhibit SSI related circadian phenotypes:*

During the nights MF couples are more active than MM or FF couples (Figs. 1a-c). ANOVA on the locomotor activity data showed significant effect of pairing

(MM/FF/MF), time of the day (day/night), and pairing × time of the day interaction. Post-hoc multiple comparisons using Tukey's test revealed that night time activity of MF couples is significantly higher than MM ($p < 0.005$) or FF ($p < 0.0005$) couples (Figs. 1d, e), whereas day time activity of MF and MM couples is significantly lower than FF couples ($p < 0.0005$), however, day time activity of MF and MM couples does not differ ($p = 0.20$) (Figs. 1d, e).

ANOVA on the sleep data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons showed that MF couples in general sleep significantly lesser compared to MM or FF couples ($p < 0.0005$). Also, during the night MF couples sleep significantly lesser compared to MM ($p < 0.005$) or FF ($p < 0.0005$) couples, whereas during the day, MF couples sleep significantly lesser than MM ($p < 0.005$) but as much as FF couples ($p = 0.81$) (Figs. 1f, g).

6.3.2 ***Enhanced night time activity is not an artifact of summation of male and female activity:*** The males, females were tagged, and their activity was recorded individually. The time series data of two corresponding males, or two females, or a male and a female was added, and the average activity of MM, FF, and MF pairs was obtained. ANOVA on the summed activity data showed that there is significant effect of pairing ($p < 0.05$), time of the day ($p < 0.0001$), and pairing × time of the day interaction ($p < 0.05$). Post-hoc multiple comparisons using Tukey's test suggest activity levels are similar among MM, FF and MF when compared during night ($p = 0.99$) and day ($p > 0.33$). The day time activity of FF couples was significantly higher compared to MM ($p < 0.01$), but not different than MF couples ($p > 0.05$) (Figs. 2d, e).

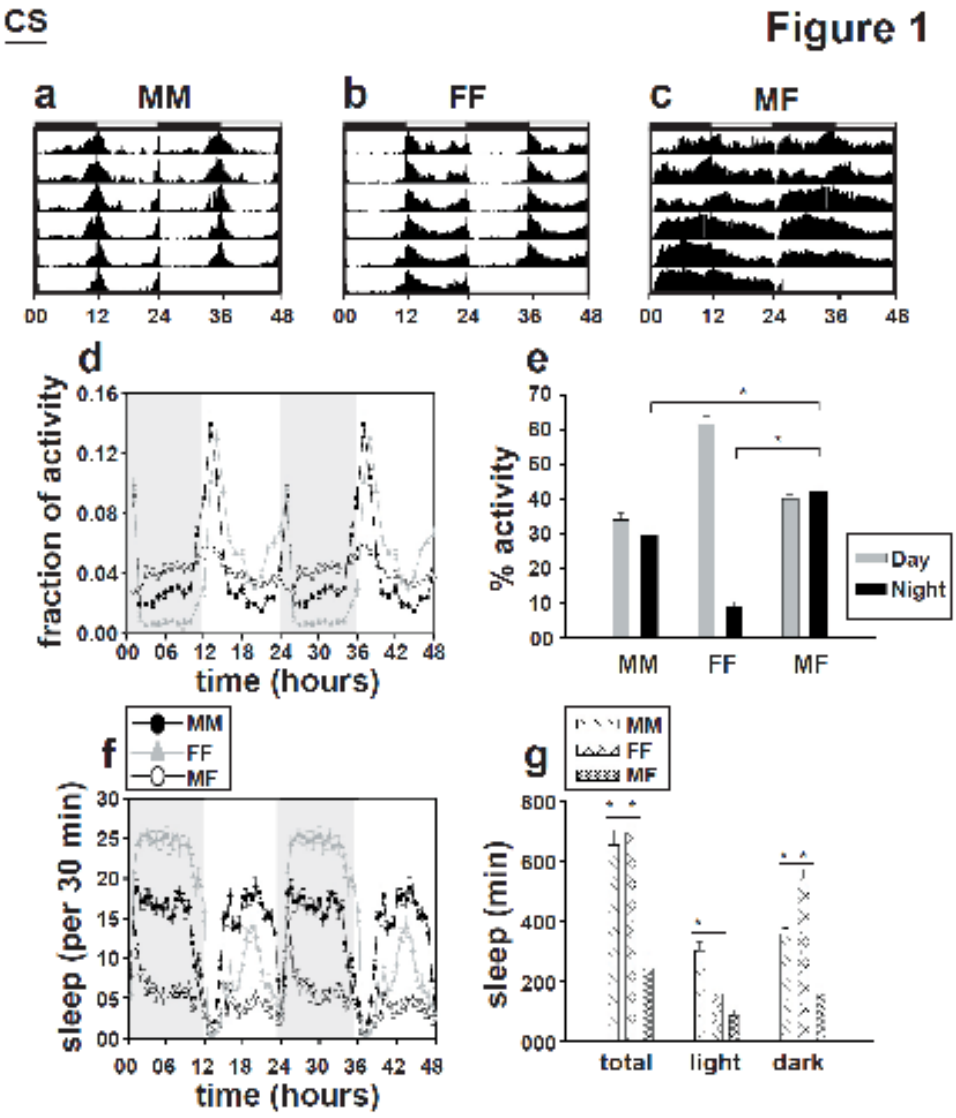


Figure 1 ***SSI in CS flies.*** Double plots of actograms (c) show higher night time activity in male-female (MF) couples compared to male-male (MM) (a) or female-female (FF) (b) couples. Profiles of the activity (d) show greater night time activity in MF couples which when analyzed (e) revealed that night time activity in MF couples is significantly higher than MM ($p < 0.005$) or FF couples ($p < 0.0005$). During the day, MF couples are as active as MM, but significantly less active than FF couples ($p < 0.0005$). There is also a reduction in total sleep which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep data (g) indicates that there is a decrease in total sleep of MF couples relative to MM or FF couples ($p < 0.0005$); during the night MF couples sleep lesser compared to MM ($p < 0.005$) or FF ($p < 0.0005$) couples, whereas during the day they sleep significantly lesser compared to MM ($p < 0.005$) but as much as FF ($p = 0.81$) couples. The light bars (a-c) over the actograms and areas in the activity and sleep profiles represent time of the day, and gray bars signify night time under 12:12 hr light/dark (LD) cycles (d and e). Data was analyzed by ANOVA followed by post-hoc multiple comparisons using Tukey's test. A number of MM ($n = 28$), FF ($n = 28$), MF ($n = 28$) couples of CS flies were used in this experiment. Asterisk signifies $p < 0.05$.

6.3.3 ***Male-female (MF) white eye couples exhibit enhanced night time activity and reduced sleep:*** Actograms show higher night time activity in MF couples (Fig. 3c) compared to MM (Fig. 3a) or FF couples (Fig. 3b). ANOVA on the activity data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons using Tukey's test suggest that night time activity of MF couples is significantly higher than MM or FF couples ($p < 0.0005$). During the day, activity of MF couples is comparable to MM ($p = 0.99$) but lower than FF ($p < 0.0005$) couples (Figs. 3d, e).

ANOVA on the sleep data showed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons suggest that there is a significant reduction in the overall sleep of MF couples compared to MM or FF couples ($p < 0.0005$). During the day time, MF couples sleep significantly lesser compared to MM ($p < 0.005$) but as much as FF couples ($p = 0.81$). However in the night, MF couples sleep significantly lesser compared to MM or FF couples ($p < 0.0005$) (Figs. 3f, g).

6.3.4 ***Male-female (MF) Oregon R couples exhibit enhanced night time activity and reduced sleep:*** Actograms showed higher night time activity in MF couples (Fig. 4c) compared to MM or FF couples. ANOVA on the activity data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons using Tukey's test revealed that night time activity of MF couples is significantly higher than MM ($p < 0.05$) or FF ($p < 0.001$) couples, however, during the day time activity of MF couples is significantly higher than MM ($p = 0.05$) but as much as FF ($p = 0.09$) couples (Figs. 4d, e).

Figure 2

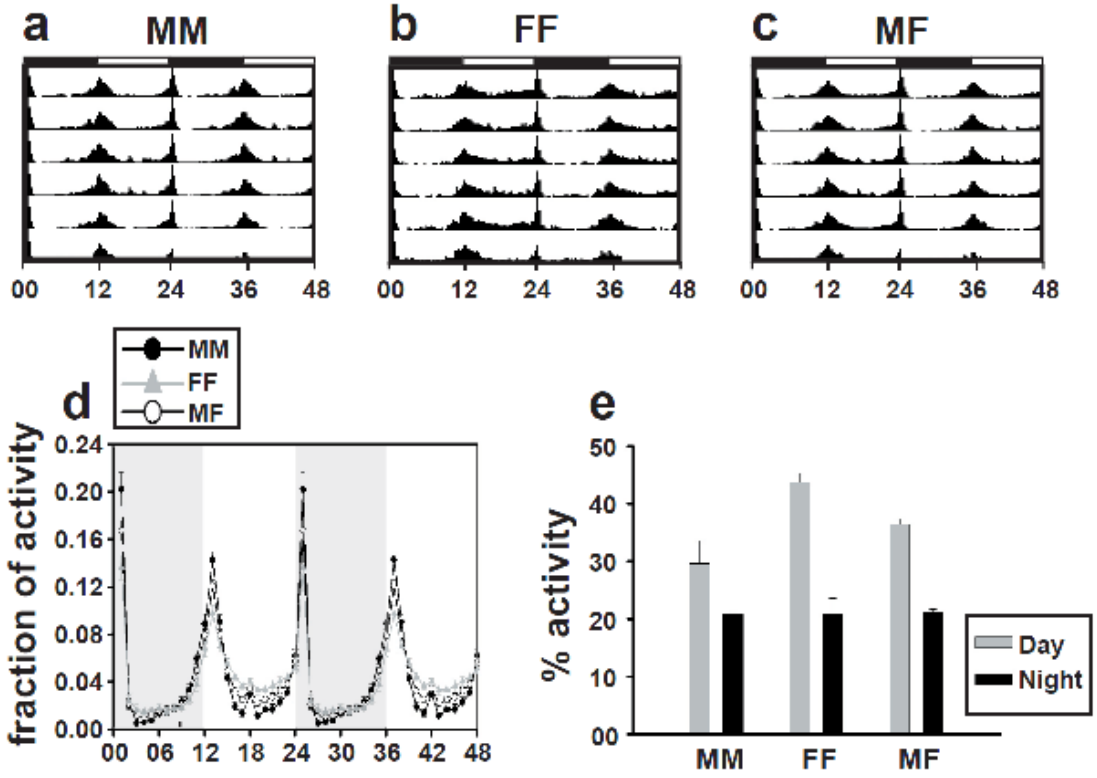


Figure 2 ***Increase in night time activity and loss of sleep in male-female (MF) couples not an artifact of summation of their activities.*** Double plots of actograms using pooled time series data of two males (MM: a), two females (FF: b), and one male and one female (MF: c). Activity profiles of MM, FF and MF (d). Analysis of activity data indicates that night and day time activity of MF does not differ statistically from MM and FF ($p > 0.05$). A total of MM ($n = 16$), FF ($n = 16$), and MF ($n = 16$) were used in this experiment. All other aspects same as Fig. 1.

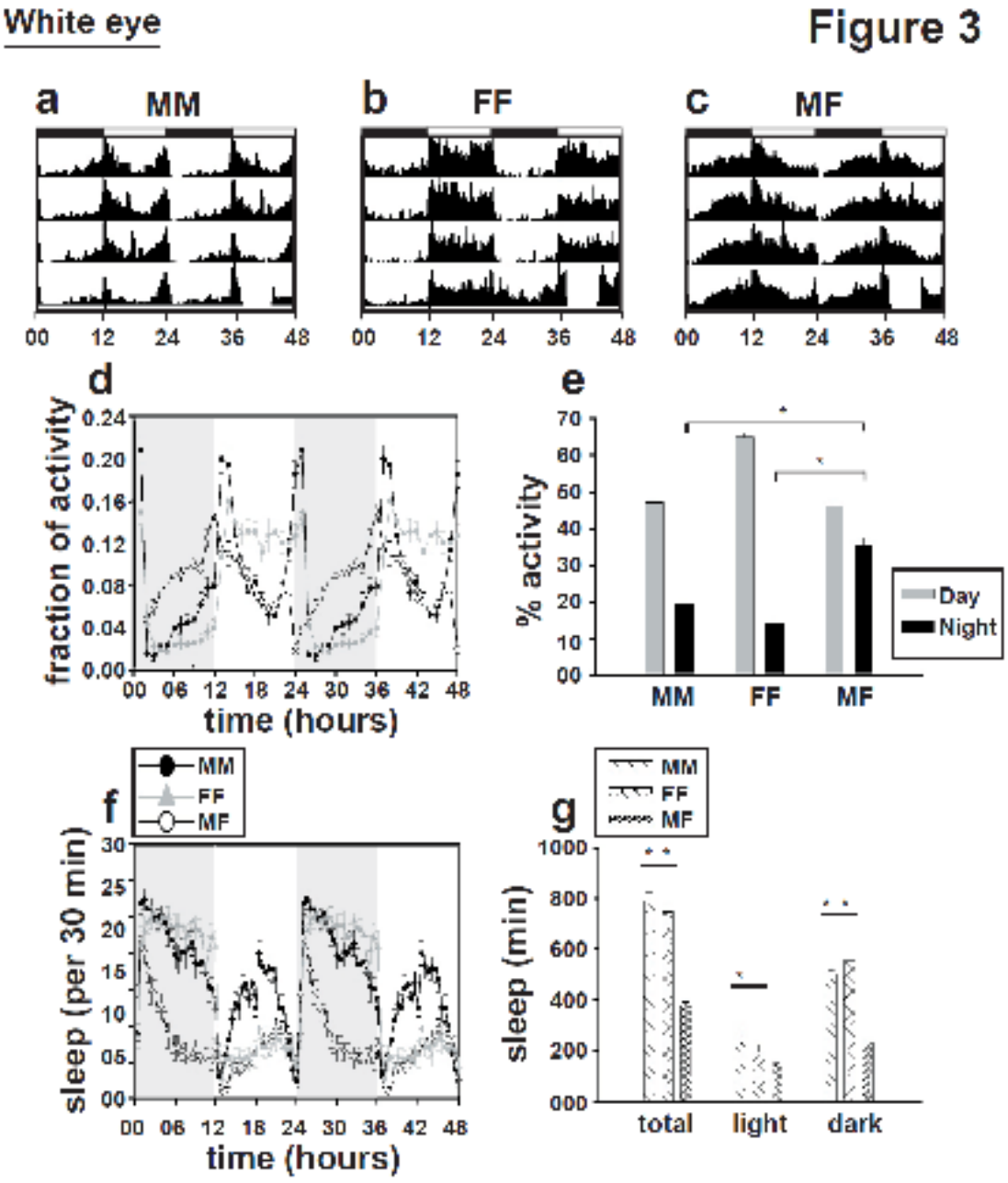


Figure 3 ***Increase in night time activity and loss of sleep in white eye flies due to male-female (MF) interactions.*** Double plots of actograms (c) show higher night time activity in MF couples compared to MM (a) or FF (b) couples. Profiles of activity (d) indicate that night time activity in MF couples is higher than MM or FF couples. Analysis (e) revealed that night time activity in MF couples is significantly greater than MM or FF couples ($p < 0.0005$), while their day time activity is similar to MM ($p = 0.99$) but significantly lesser than FF ($p < 0.0005$) couples. There is also a concurrent reduction in the total sleep which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep data (g) indicates that there is decrease in total sleep of MF couples relative to MM or FF ($p < 0.0005$) couples. During the night, MF couples sleep significantly lesser compared to MM and FF couples ($p < 0.0005$), whereas during the day they sleep significantly lesser compared to MM ($p < 0.005$) but as much as FF ($p = 0.81$) couples. As many as ($n = 22$) MM, ($n = 15$) FF, and ($n = 24$) MF couples were used in this experiment. All other aspects same as Fig. 1.

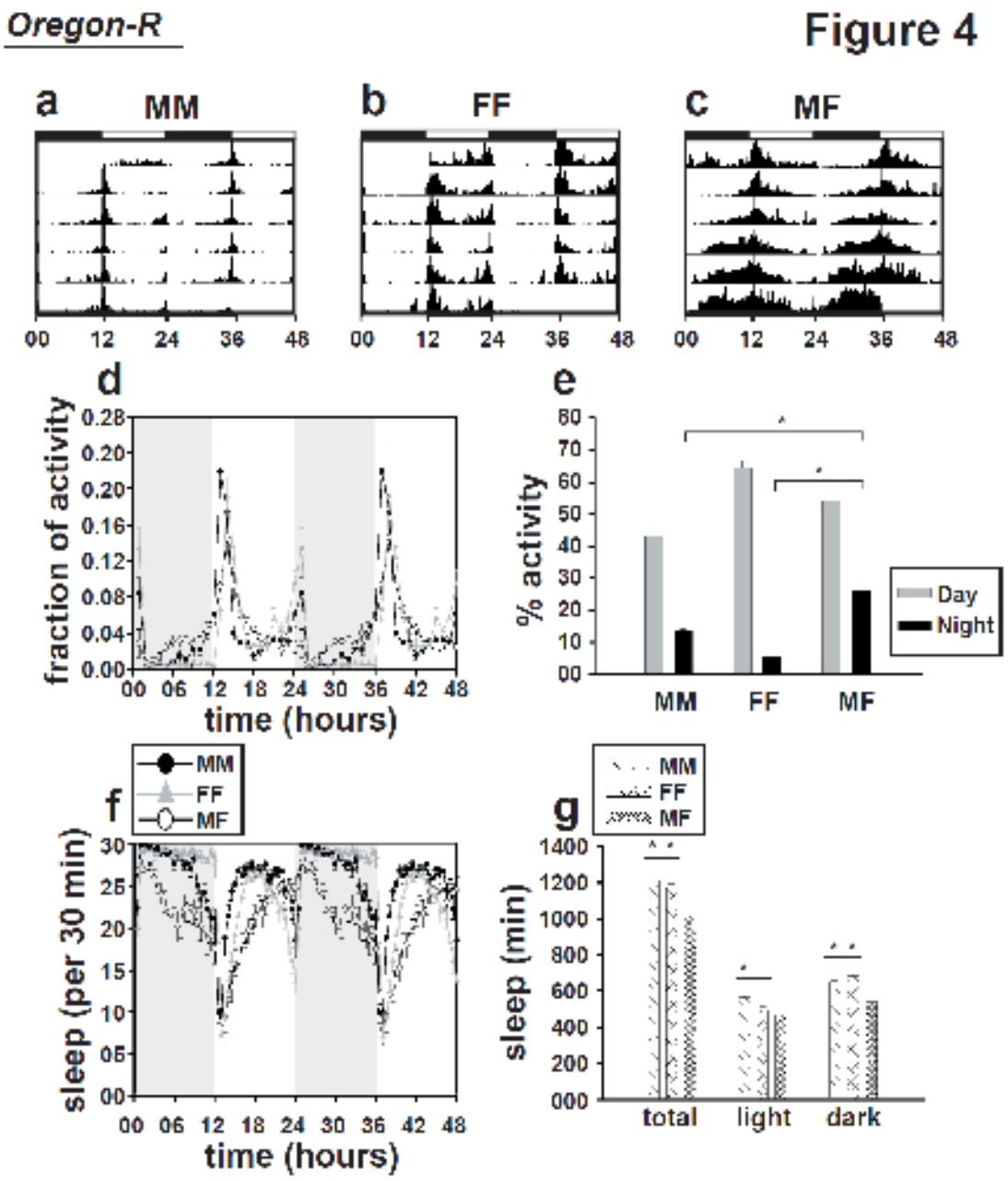


Figure 4 ***Increase in night time activity and loss of sleep in Oregon R flies due to male-female (MF) interactions.*** Double plots of actograms (c) show higher night time activity in male-female (MF) couples compared to MM or FF (a and b) couples. Profile of the activity (d) indicates that MF couples are more active in the night which when analyzed (e) shows that night time activity in MF couples is significantly higher than MM ($p < 0.05$) and FF ($p < 0.001$) couples. The day time activity of MF couples is similar to FF ($p = 0.09$) but significantly higher than MM ($p < 0.05$) couples. There is also a reduction in total sleep which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep data (g) indicates that there is a decrease in total sleep in MF couples relative to MM or FF couples ($p < 0.0005$). During the night, MF couples sleep lesser compared to MM ($p < 0.005$) or FF ($p < 0.0005$) couples, while during the day time they sleep significantly lesser compared to MM ($p < 0.005$) but as much as FF ($p = 0.91$) couples. As many as ($n = 15$) MM, ($n = 15$) FF, and ($n = 16$) MF couples were used in this experiment. All other aspects same as Fig. 1.

ANOVA on the sleep data showed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc comparisons revealed that in MF couples overall sleep is significantly lesser comparison to MM or FF couples ($p < 0.0005$), however, total sleep in MM and FF couples does not differ ($p = 0.59$). During the night, MF couples have significantly reduced sleep compared to MM ($p < 0.005$) or FF ($p < 0.0005$) couples, while during the day they sleep significantly lesser than MM ($p < 0.005$) but as much as FF ($p = 0.91$) couples (Figs. 4f, g).

6.3.5 ***Male-female (MF) yellow white couples exhibit enhanced night time activity and reduced sleep:*** Actograms showed higher night time activity in MF couples (Fig. 5c) compared to MM or FF couples. ANOVA on the activity data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons using Tukey's test revealed that night time activity of MF couples is significantly higher than MM and FF ($p < 0.0005$) couples, however, during the day time activity of MF couples is significantly lower than FF ($p < 0.005$) but as much as MM ($p = 0.98$) couples (Figs. 5d, e).

ANOVA on the sleep data showed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc comparisons revealed that in MF couples overall sleep is significantly lesser comparison to MM or FF couples ($p < 0.0005$), however, total sleep in MM and FF couples does not differ ($p = 1$). During the night, MF couples have significantly reduced sleep compared to MM ($p < 0.05$) or FF ($p < 0.005$) couples, while during the day there are no significant differences in sleep when MF is compared with MM ($p = 0.11$) and FF ($p = 0.97$) couples (Figs. 5f, g).

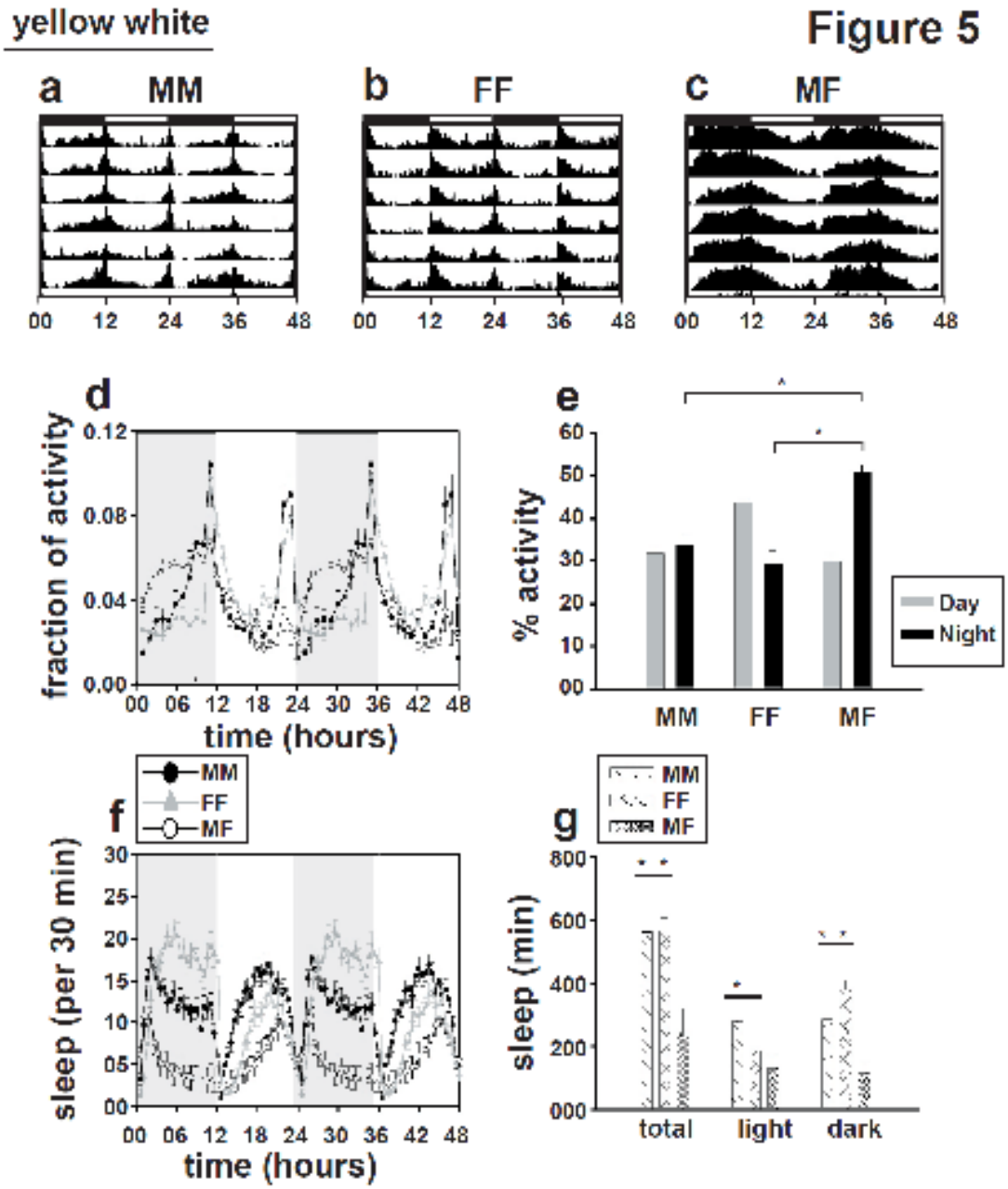


Figure 5 ***Increase in night time activity and loss of sleep in yellow white flies due to male-female (MF) interactions.*** Double plots of actograms (c) show higher night time activity in male-female (MF) couples compared to MM or FF (a and b) couples. Profile of the activity (d) indicates that MF couples are more active in the night which when analyzed (e) shows that night time activity in MF couples is significantly higher than MM and FF ($p < 0.0005$) couples. The day time activity of MF couples is lower than FF ($p < 0.005$) but insignificant from MM ($p = 0.98$) couples. There is also a reduction in total sleep which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep data (g) indicates that there is a decrease in total sleep in MF couples relative to MM or FF couples ($p < 0.0005$). During the night, MF couples sleep lesser compared to MM ($p < 0.05$) or FF ($p < 0.005$) couples, while during the day time there are no differences in sleep when MF couples are compared with MM ($p = 0.11$) and FF ($p = 0.97$). As many as ($n = 18$) MM, ($n = 17$) FF, and ($n = 14$) MF couples were used in this experiment. All other aspects same as Fig. 1.

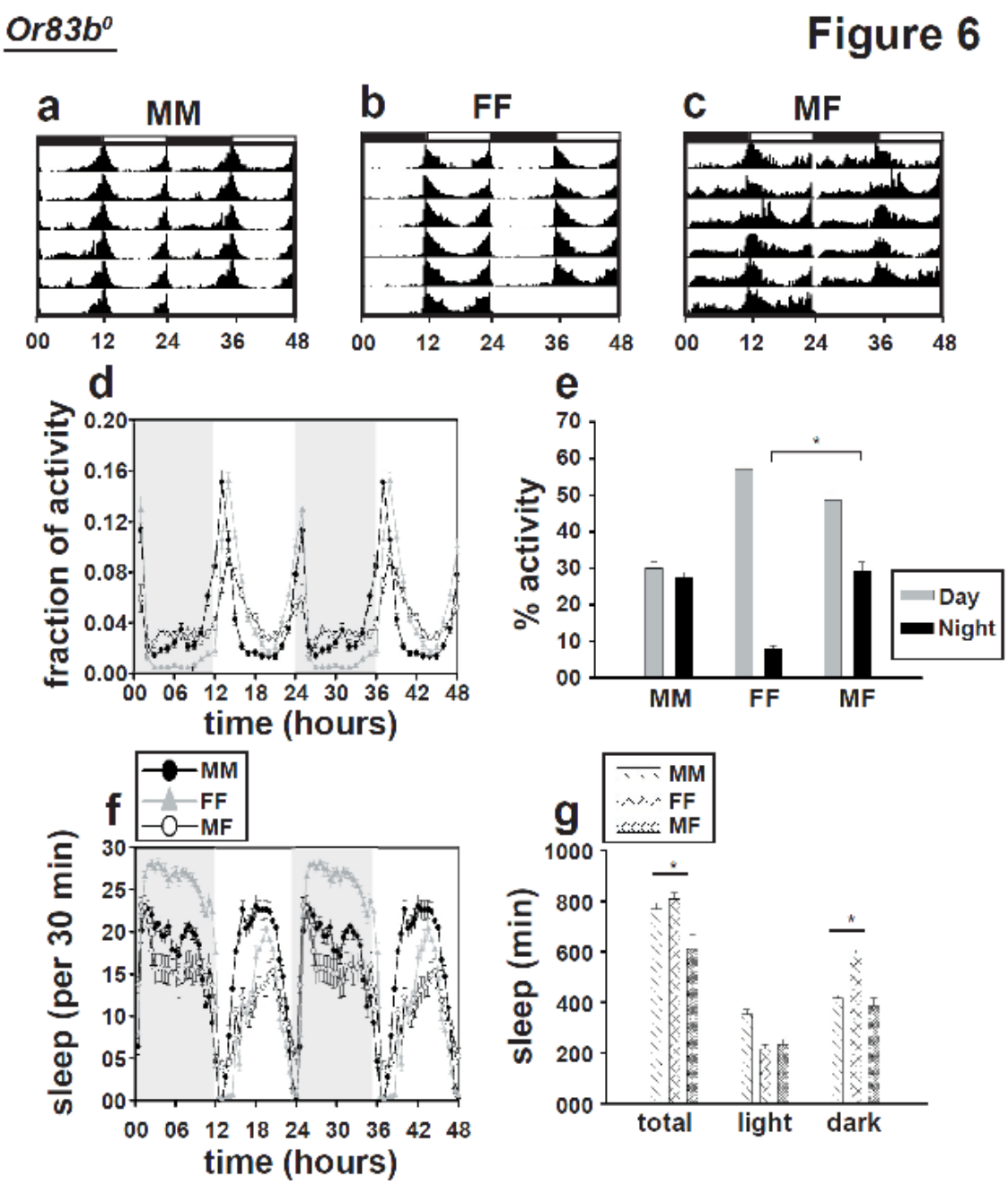


Figure 6 ***SSI is olfaction mediated.*** Double plots of actograms (c) of *Or83b* flies show that night time activity male-female (MF) couples is similar to male-male (MM) but higher than female-female (FF) couples (a and b). Profiles of activity (d) indicate that night time activity in MF couples is similar to MM or FF couples. Analysis shows that night time activity in MF pairs (e) is not different from MM ($p = 0.97$) but significantly greater than FF ($p < 0.0005$) couples. During the day, activity of MF couples is significantly lesser than FF ($p < 0.05$) but higher than MM ($p < 0.0005$) couples. Sleep profile (f) suggests that sleep levels are comparable between MF and MM couples. Analysis of sleep data (g) indicates that total sleep in MF couples is similar to MM ($p > 0.05$) but lesser than FF ($p < 0.05$) couples. During the day MF couples sleep as well as MM ($p = 0.32$) or FF ($p = 0.99$) couples, while during the night they sleep as much as MM ($p = 0.99$) but significantly lesser than FF ($p < 0.01$) couples. As many as ($n = 28$) MM, ($n = 28$) FF, and ($n = 28$) MF couples were used in this experiment. All other aspects same as Fig. 1.

6.3.6 ***SSI is olfaction-mediated:*** Actograms of couples with loss of function mutation in a widely expressed olfactory receptor *Or83b* (Larsson et al. 2004) indicate that night time activity of MF couples (Fig. 6c) is comparable to MM (Figs. 6a) or FF (Figs. 6b) couples. ANOVA on the activity data showed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons revealed that night time activity of MF couples is comparable to MM ($p = 0.97$) but significantly higher than FF ($p < 0.0005$) couples, whereas during the day, MF couples are significantly more active than MM ($p < 0.0005$) but less active than FF ($p < 0.05$) couples (Figs. 6d, e).

ANOVA on the sleep data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons suggest that overall sleep in $Or83b^0$ MF couples is comparable to MM ($p = 0.09$) but significantly lesser than FF ($p < 0.05$) couples. Day time sleep in MF couples does not differ statistically from MM ($p = 0.32$) or FF ($p = 0.99$) couples, while night time sleep is significantly lesser than FF ($p < 0.01$) but similar to MM ($p = 0.99$) couples (Figs. 6f, g).

6.3.7 ***Moderate night time activity and loss of sleep in Or65a ablated (Or65a/UASdti) flies:*** Actograms show moderately higher night time activity in *Or65a* ablated (*Or65aGAL4/UASdti*) MF couples (Fig. 7c) relative to MM or FF couples (Figs. 7a, b). ANOVA on the activity data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons using Tukey's test showed that night time activity of MF and MM couples is statistically not different ($p = 0$ .13), whereas that of FF couples is significantly lower compared to MF ($p < 0.0005$)

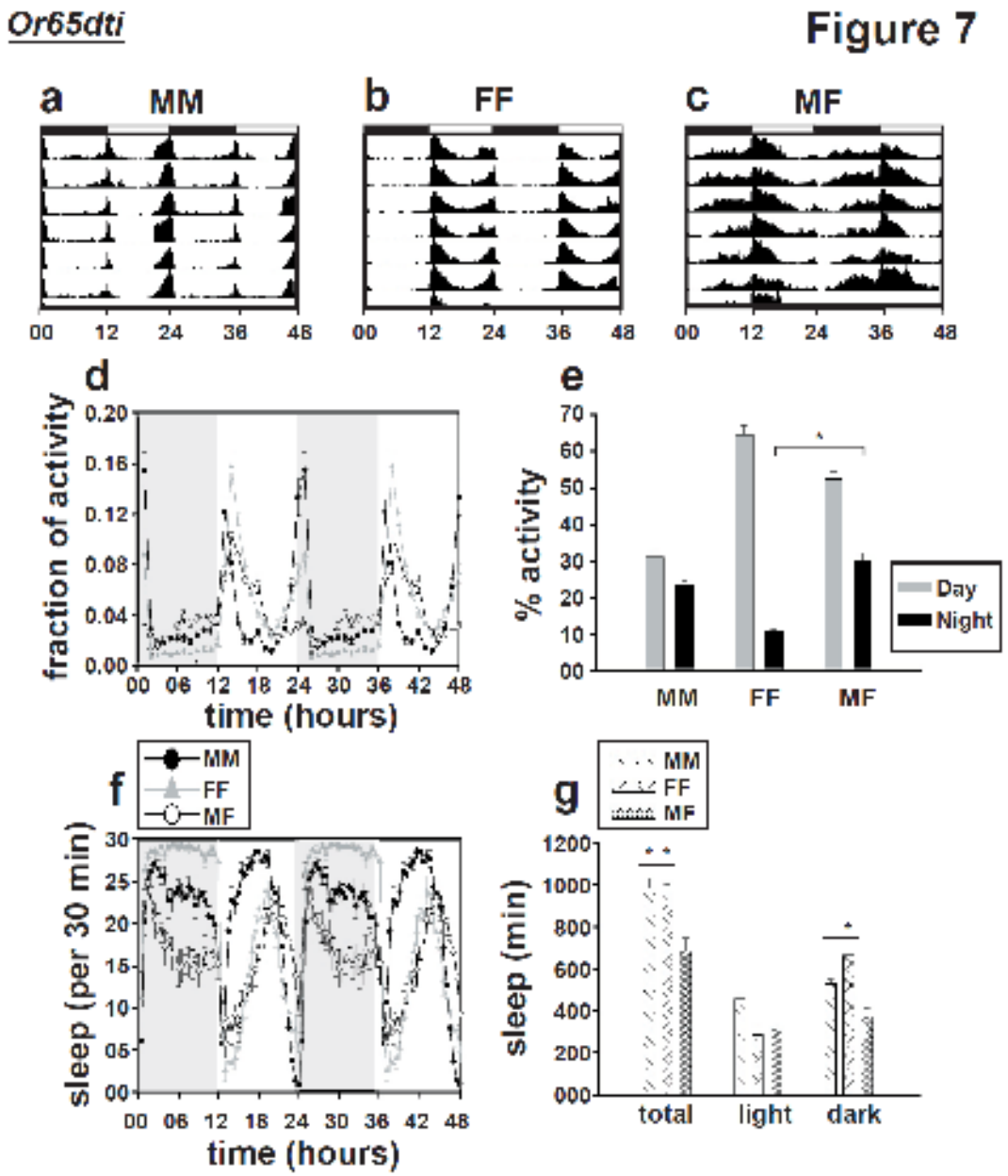


Figure 7 ***Moderate decrease in night time activity and recovery of night time sleep in Or65a ablated flies (Or65a-Gal4/UASdti) male-female (MF) couples.*** Double plots of actograms (c) show moderately higher night time activity in male-female (MF) couples compared to MM (a) or FF (b) couples. Profiles of activity (d) indicate that night time activity in MF couples is marginally higher than MM and FF couples. Analysis (e) shows that night time activity in MF couples is comparable to MM ($p = 0.13$) but greater than FF ($p < 0.0005$) couples. During the day activity of MF couples is lesser than FF ($p < 0.005$) but greater than MM ($p < 0.0005$) couples. Due to moderate increase in night time activity there is a reduction in overall sleep in MF couples which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep data (g) indicates that there is a decrease in total sleep in MF pairs relative to MM ($p < 0.001$) or FF ($p < 0.005$) couples. During night MF couples sleeps significantly lesser compared to FF ($p < 0.001$) but as much as MM ($p = 0.19$) couples, whereas during the day they sleep as much as MM ($p = 0.26$) or FF couples ($p = 0.99$) (f and g). As many as ($n = 21$) MM, ($n = 19$) FF, and ($n = 19$) MF couples were used in this experiment. All other aspects same as Fig. 1.

or MM ($p < 0.001$) couples. During the day, MF couples are more active than MM couples ($p < 0.0005$), but both are less active than FF couples ($p < 0.005$) (Figs. 7d, e).

ANOVA on the sleep data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons showed that total sleep in MF couples is lesser compared to FF ($p < 0.005$) or MM ($p < 0.001$) couples, and but those in MM and FF couples does not differ ($p = 0.99$). The day time sleep of the all couples ($p > 0.05$) and night time sleep of MF and MM ($p = 0.19$), MM and FF ($p = 0.30$) couples does not differ, however, MF couples sleep significantly lesser than FF ($p < 0.001$) couples (Figs. 7f, g).

6.3.8 ***Moderate night time activity and loss of sleep in Or88a ablated (Or88a/UASdti) flies:*** Actograms (Figs. 8a-c) show moderately higher night time activity in *Or88a* ablated (*Or88aGAL4/UASdti*) MF couples (Fig. 8c) compared to MM or FF couples (Figs. 8a, b). ANOVA on the activity data showed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc comparisons revealed that night time activity of MF and MM ($p = 0.50$), and MM and FF ($p = 0.11$) couples does not differ statistically, whereas that of MF couples is significantly higher than FF couples ($p < 0.005$). During the day, activity of MF or FF couples is significantly higher than MM couples ($p < 0.001$), whereas that of MF and FF couples does not differ ($p = 0.99$). Day time activity of FF is higher than that of MM ($p < 0.001$). (Figs. 8d, e).
ANOVA on the sleep data revealed significant effect of pairing, and time of the day, however, pairing × time of the day interaction is only marginally significant. Post-hoc comparisons suggest that MF couples sleep lesser compared to MM ($p < 0.0005$) or FF

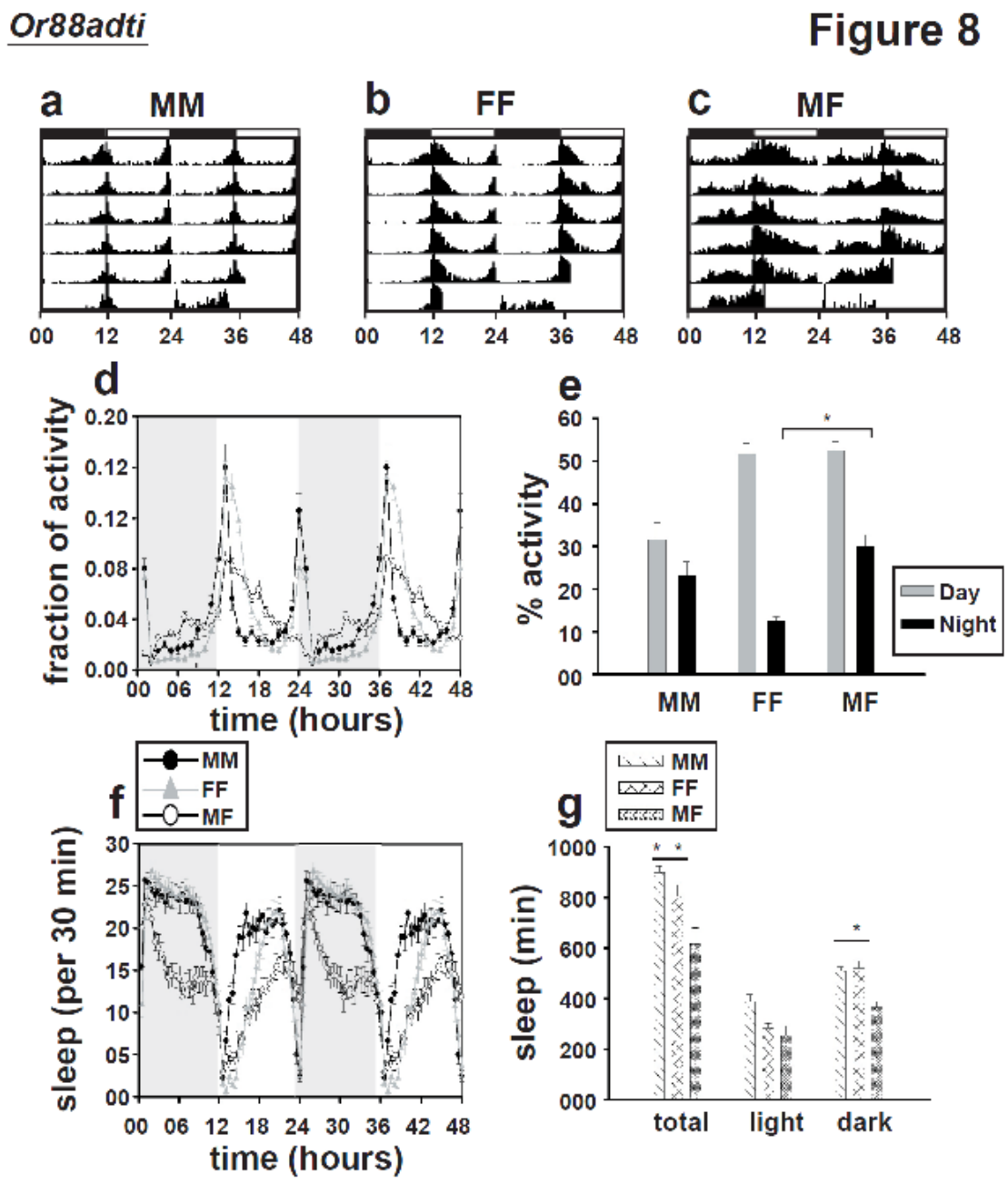


Figure 8 ***Moderate decrease in night time activity and recovery of night time sleep in Or88a ablated flies (Or88a/UASdti) male-female (MF) couples.*** Double plots of actograms (c) show slightly higher night time activity in male-female (MF) couples compared to MM (a) or FF (b) couples. Profiles of activity (d) indicate that there is slight increase in night time activity in MF couples. Analysis (e) shows that night time activity in MF pairs is comparable to MM ($p = 0.50$) but greater than FF ($p < 0.005$) couples, whereas during day time activity in MF couples is similar to FF ($p = 0.99$) but greater than MM ($p < 0.001$) couples. There is also reduction in total sleep which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep (g) indicates that there is decrease in total sleep in MF couples relative to MM ($p < 0.0005$) or FF ($p < 0.05$) couples. Day time sleep of all three couples does not differ statistically ($p > 0.05$), whereas night time sleep of MF couples is comparable to MM ($p = 0.11$) but slightly lesser than FF ($p = 0.06$) couples (f and g). As many as ($n = 20$) MM, ($n = 22$) FF, and ($n = 21$) MF couples were used in this experiment. All other aspects same as Fig. 1.

($p < 0.05$) couples, while MM and FF couples sleep equally well ($p = 0.59$).Daytime sleep in MF couples does not differ from MM ($p = 0.15$) or FF ($p = 0.99$) couples, while night time sleep is similar to MM ($p = 0.11$) but lesser than FF ($p = 0.06$) couples (Figs. 8f,g).

6.3.9 ***Male-female (MF) Or67d loss of function (Or67dGAL4) couples exhibit increased night time activity and reduced sleep:*** The *Or67dGAL4* strain is a knock-in line for the *olfactory receptor 67d* (*Or67b*) gene and is receptor for 11-cis-vaccenylacetate (cVA) (Kurtovic et al., 2007). Actograms (Figs. 9a-c) show higher night time activity in MF couples (Fig. 9c) compared to MM or FF couples (Figs. 9a, b). ANOVA on the activity data showed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons revealed that during night activity levels of MF couples is greater compared to MM or FF couples ($p < 0.0005$), whereas during the day MF couples are as active as MM or FF ($p > 0.05$) couples (Figs. 9d, e).

ANOVA on the sleep data showed effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc comparisons revealed that total, night time ($p < 0.0005$) as well as day time ($p < 0.05$) sleep in MF couples is significantly lesser compared to MM or FF couples (Figs. 9f, g).

6.3.10 ***Reduced SSI in Or47b ablated MF couples:*** Actograms (Figs. 10a-c) show that night time activity in *Or47b* ablated (*Or47bGAL4/UASdti*) MF couples (Fig. 10c) does not differ from MM but is relatively greater than FF couples (Figs. 10a, b). ANOVA on the activity data revealed significant effect of pairing, time of the day, and pairing × time

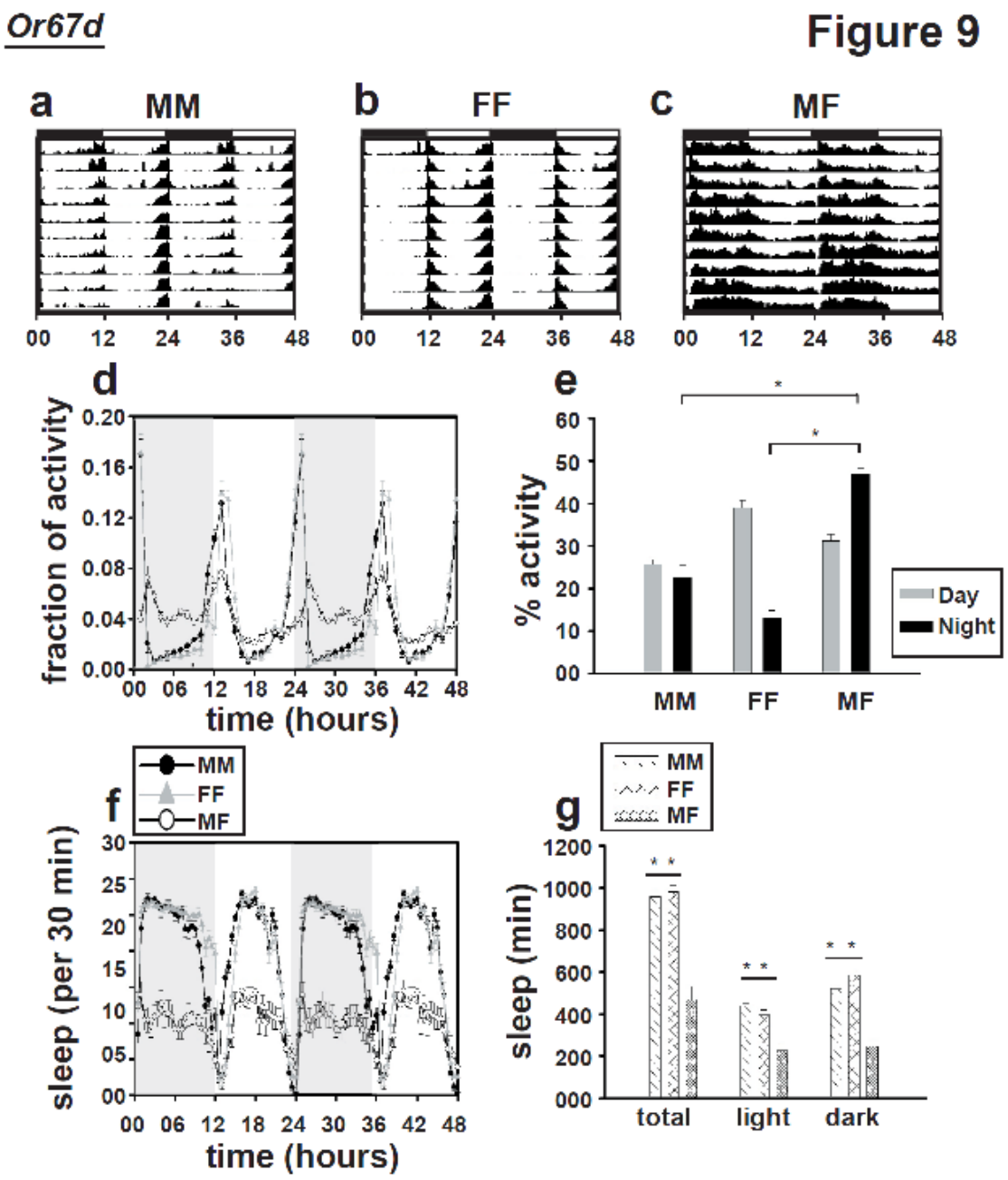


Figure 9 ***Night time activity and loss of sleep in Or67dGAL4 knock-in flies due to male-female (MF) interactions.*** Double plots of actograms of Or67dGAL4 knock-in flies (S9a-c) show higher night time activity in male-female (MF) couples (c) compared to MM or FF couples (a and b). Profiles of activity (d) also indicate that there is higher night time activity in MF couples. Analysis of activity (e) shows that night time activity in MF couples is significantly higher than MM or FF couples ($p < 0.0005$). During the day activity of MF couples is similar to MM ($p = 0.33$) or FF ($p = 0.08$) couples. Due to increased night time activity there is also a reduction in total sleep which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep data (g) indicates that there is a decrease in total sleep in MF couples relative to MM or FF couples ($p < 0.0005$). During the night they sleep significantly lesser compared to MM or FF couples ($p < 0.0005$), whereas during the day MF couples sleep significantly lesser compared to MM ($p < 0.005$) or FF ($p < 0.05$) couples. As many as ($n = 16$) MM, ($n = 22$) FF, and ($n = 16$) MF couples were used in this experiment. All other aspects same as Fig. 1.

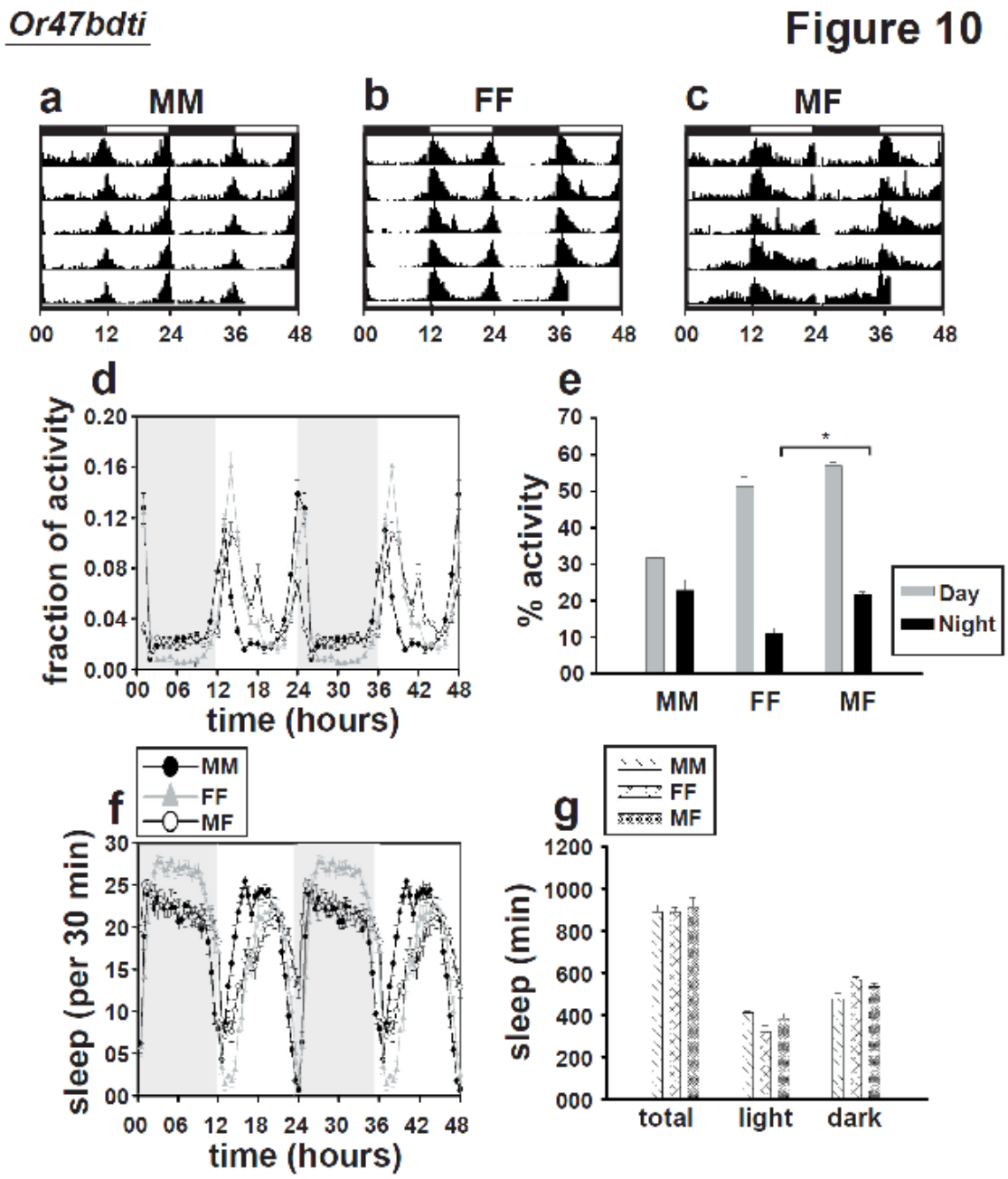


Figure 10 ***SSI is Or47b ablated flies.*** Double plots of actograms (c) show that night time activity in male-female (MF) couples is similar to MM (a) but is higher than FF (b) couples. Profiles of activity (d) also indicate that night time activity in MF couples is comparable to MM ($p = 0.99$) but significantly greater than FF couples ($p < 0.05$), whereas day time activity is comparable to FF ($p = 0.36$) but significantly greater than MM ($p < 0.0005$) couples. The night time sleep profiles of MF couples match closely with MM couples and day time sleep profiles match with FF couples (f and g). As many as ($n = 24$) MM, ($n = 24$) FF, and ($n = 24$) MF couples were used in this experiment. All other aspects same as Fig. 1.

of the day interaction. Post-hoc analysis suggest that night time activity in MF couples does not differ from MM couples ($p = 0.99$), but is significantly greater than FF couples ($p < 0.05$). Day time activity of MF couples is significantly higher than MM ($p < 0.0005$) but comparable to FF ($p = 0.36$) couples (Figs. 10d, e).ANOVA on the sleep data revealed that the effect of time of the day, and pairing × time of the day interaction is statistically significant; however, effect of pairing is statistically not significant. Post-hoc comparisons revealed that total sleep, day, and night time sleep in MF couples is comparable to MM or FF couples ($p > 0.70$) (Figs. 10f, g).

6.3.11 ***Male-female (MF) control (Or47bGAL4) couples exhibit enhanced night time activity and reduced sleep:*** We decided to study activity and sleep in couples of this strain to rule out the possibility of any genetic background related effect that may have caused loss of socio-sexual interaction phenotype in the *Or47b* ablated flies. Actograms show higher night time activity is higher in control (*Or47bGAL4*) MF couples (Fig. 11c) compared to MM or FF couples (Figs. 11a, b). ANOVA on the activity data suggests significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc analysis indicates that night time activity in MF couples is significantly higher than MM or FF couples ($p < 0.0005$), whereas day time activity is similar to MM ($p = 0.34$) but lower than FF ($p < 0.0005$) couples (Fig. 11d, e).

ANOVA on the sleep data shows significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc analysis suggests that total, and night time sleep is significantly lower in MF couples compared to MM or FF couples ($p < 0.0005$), whereas day time sleep in MF couples is significantly lesser compared to MM ($p < 0.0005$) but as much as FF ($p = 0.23$) couples (Figs. 11f, g).

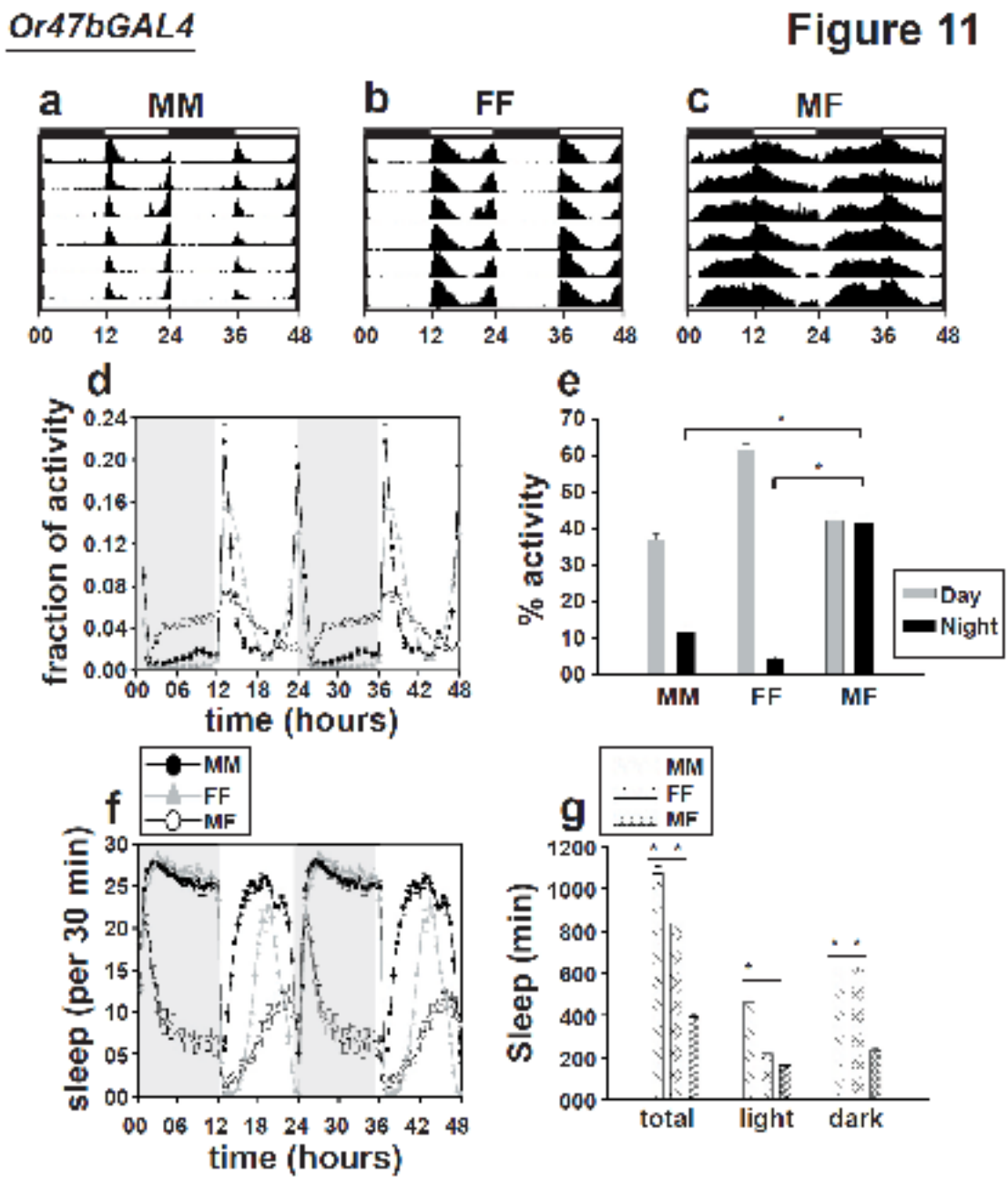


Figure 11 ***Night time activity and loss of sleep in control (Or47bGAL4) flies due to male-female (MF) interactions.*** Double plots of actograms (c) show higher night time activity in male-female (MF) couples compared to MM (a) or FF (b) couples. Profiles of activity (d) indicate that MF couples are more active in the night. Analysis of activity (e) shows that night time activity of MF couples is significantly greater than MM or FF couples ($p < 0.0005$). During the day, activity of MF couples is comparable with MM ($p = 0.34$) but significantly lesser than FF ($p < 0.0005$) couples. Due to increased night time activity there is a reduction in the overall sleep which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep data (g) indicates that there is a decrease in total sleep in MF couples relative to MM or FF couples ($p < 0.0005$). During the night, MF couples sleep lesser compared to MM or FF couples ($p < 0.0005$), whereas during the day they sleep significantly lesser compared to MM ($p < 0.0005$) but as much as FF ($p = 0.23$) couples. As many as ($n = 24$) MM, ($n = 24$) FF, and ($n = 24$) MF couples were used in this experiment. All other aspects same as Fig. 1.

Or47bdORKC1/NC1

Figure 12

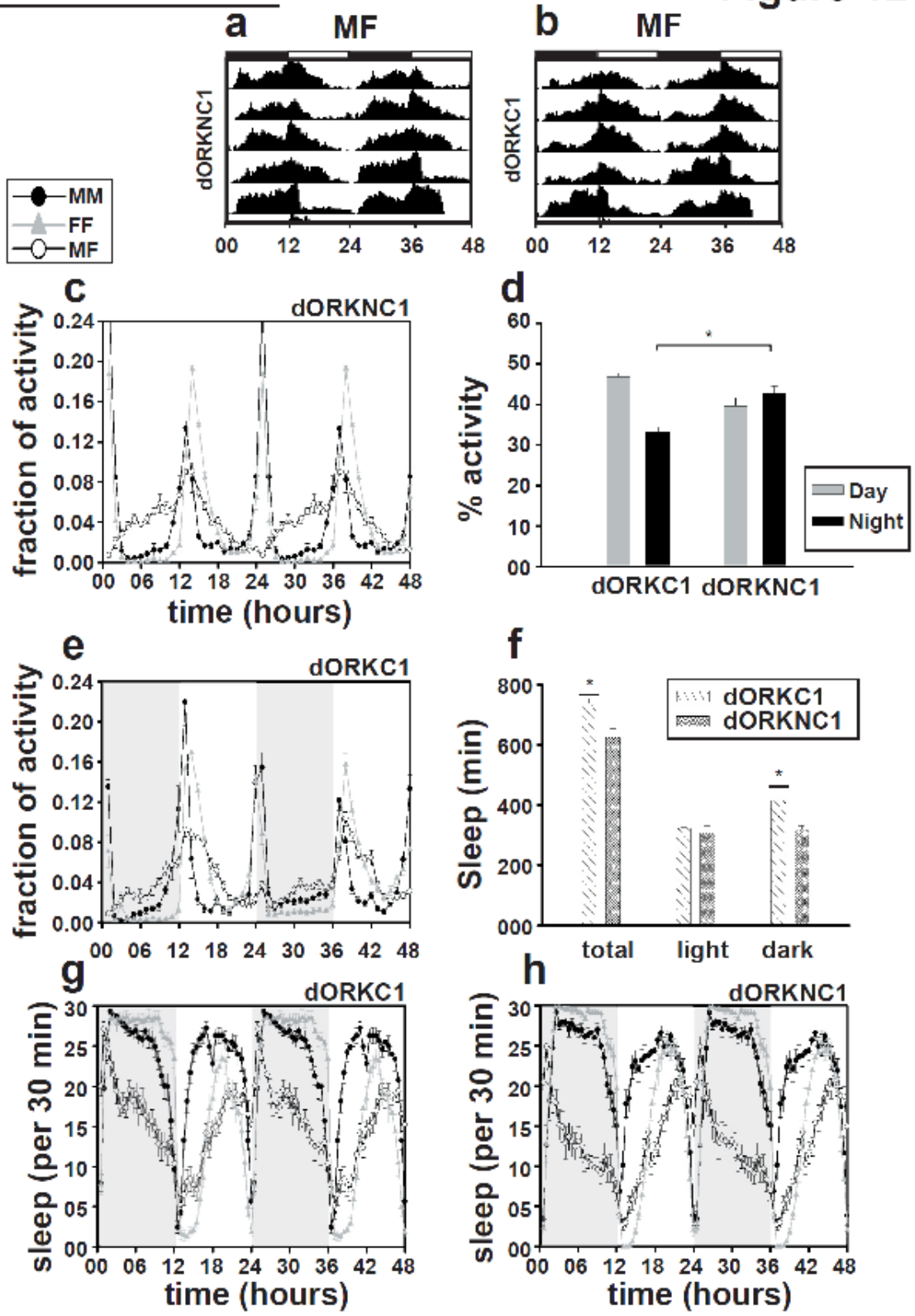


Figure 12 ***SSI in Or47b silenced flies.*** Double plots of actograms show lower night time activity in silenced (*Or47bGAL4*/ UASDorkC1) male-female couples (b) compared to the controls (*Or47b-GAL4/UASDorkNC1*). Profiles of activity (c and d) indicate that there is significant reduction in the night time activity of silenced couples. Analysis of activity data (e) revealed that night time activity of silenced couples is significantly reduced compared to controls ($p < 0.05$). Sleep profiles suggest that silenced couples sleep relatively more compared to control couples see (f and g). Analysis of sleep suggests that total sleep in silenced couples is significantly greater than non-silenced controls ($p < 0.01$). The night time sleep of silenced couples is significantly greater than controls ($p < 0.05$), while there are differences in day time sleep is statistically not significant ($p = 0.98$) (f-h). As many as ($n = 16$) silenced, and ($n = 16$) non-silenced male-female couples were used in this experiment. All other aspects same as Fig. 1.

6.3.12 ***Reduced SSI in Or47b silenced MF couples:*** During the nights, *Or47b* silenced (*Or47bGal4/UASDorkC1*) MF couples exhibit significantly lesser activity compared to control (*Or47bGal4/UASDorkNC1*) MF couples. ANOVA on the activity data showed that the effect of time of the day, and strain × time of the day interaction is statistically significant, however, effect of strain is statistically not significant. Post-hoc multiple comparisons revealed that in silenced MF couples night time activity is significantly lower, and day time activity is significantly higher compared to control MF couples ($p < 0.05$) (Figs. 12c-e).

ANOVA on the sleep data showed significant effect of strain, time of the day, and strain × time of the day interaction. Post-hoc multiple comparisons revealed that silenced MF couples sleep significantly more than control couples ($p < 0.01$), and the differences are mostly due to night time sleep ($p < 0.05$), because during the day couples of both strains sleep equally well ($p = 0.98$) (Figs. 12f-h).

6.3.13 ***SSI is male driven:*** Actograms (Figs. 13a, b) show higher night time activity in couples comprising *CS* males and time blind ($glass^{60j};cry^{01}$) females (Fig. 13a) compare to couples with time blind males and *CS* females (Fig. 13b). ANOVA on the activity data revealed significant effect of time of the day, and pairing × time of the day interaction, however, effect of pairing is statistically not significant. Post-hoc multiple comparisons revealed that night time activity is significantly higher when males in the MF couple are *CS* and females are time blind ($p < 0.05$), while day time activity of both types of couples is comparable ($p = 0.42$) (Figs. 13c, d).

ANOVA on the sleep data showed significant effect of pairing, and time of the day, however, effect of pairing × time of the day interaction is statistically not significant.

The day ($p = 0.47$) and night ($p = 0.34$) time sleep do not differ among the two types of couples, however, total sleep in couples with wild type males and time blind females was significantly lesser than couples with time blind males and wild type females ($p < 0.05$) (Figs. 13e, f).

6.3.14 ***Olfaction in males is critical for SSI:*** Actograms (Fig. 14a, b) indicate that MF couples with *Or83b*^{0} males and *CS* females ($M^{o}F^{+}$) have reduced night time activity compared to couples with *CS* males and *Or83b*^{0} females ($M^{+}F^{o}$). ANOVA on the activity data revealed significant effect of time of the day, and pairing × time of the day interaction, however, effect of pairing is statistically not significant. Post-hoc multiple comparisons revealed that during the night activity in $M^{o}F^{+}$ couples is significantly lower compared to $M^{+}F^{+}$ ($p < 0.0005$) or $M^{+}F^{o}$ ($p < 0.05$) couples, however, it is not different than $M^{o}F^{o}$ couples ($p = 0.97$), while during the day, $M^{o}F^{+}$ couples are as active as $M^{o}F^{o}$ ($p = 0.25$) but more active than $M^{+}F^{+}$ ($p < 0.0005$) or $M^{+}F^{o}$ ($p < 0.05$) couples (Figs. 14c, d).

ANOVA on the sleep data revealed significant effect of pairing, time of the day; however, pairing × time of the day interaction is statistically not significant. Post-hoc multiple comparisons suggests that $M^{o}F^{+}$ couples sleep more compared to $M^{+}F^{+}$ ($p < 0.0005$) or $M^{+}F^{o}$ ($p < 0.05$) couples, whereas there is no difference between the sleep of $M^{o}F^{+}$ and $M^{o}F^{o}$ couples ($p = 0.98$). During the night, $M^{o}F^{+}$ couples sleep significantly more than $M^{+}F^{+}$ ($p < 0.05$) but as much as $M^{+}F^{o}$ or $M^{o}F^{o}$ couples ($p > 0.05$) (Figs. 14e and f). During the day, MF couples of all genotypes sleep equally well ($p > 0.05$).

6.3.15 ***Or47b receptor in males is important for SSI:*** Actograms of *Or47b* ablated male and intact female ($M_{-}F_{+}$) couples (Fig. 15a) exhibit significantly lower night time activity compared to couples with males and *Or47b* ablated females ($M_{+}F_{-}$) (Fig. 15b). ANOVA

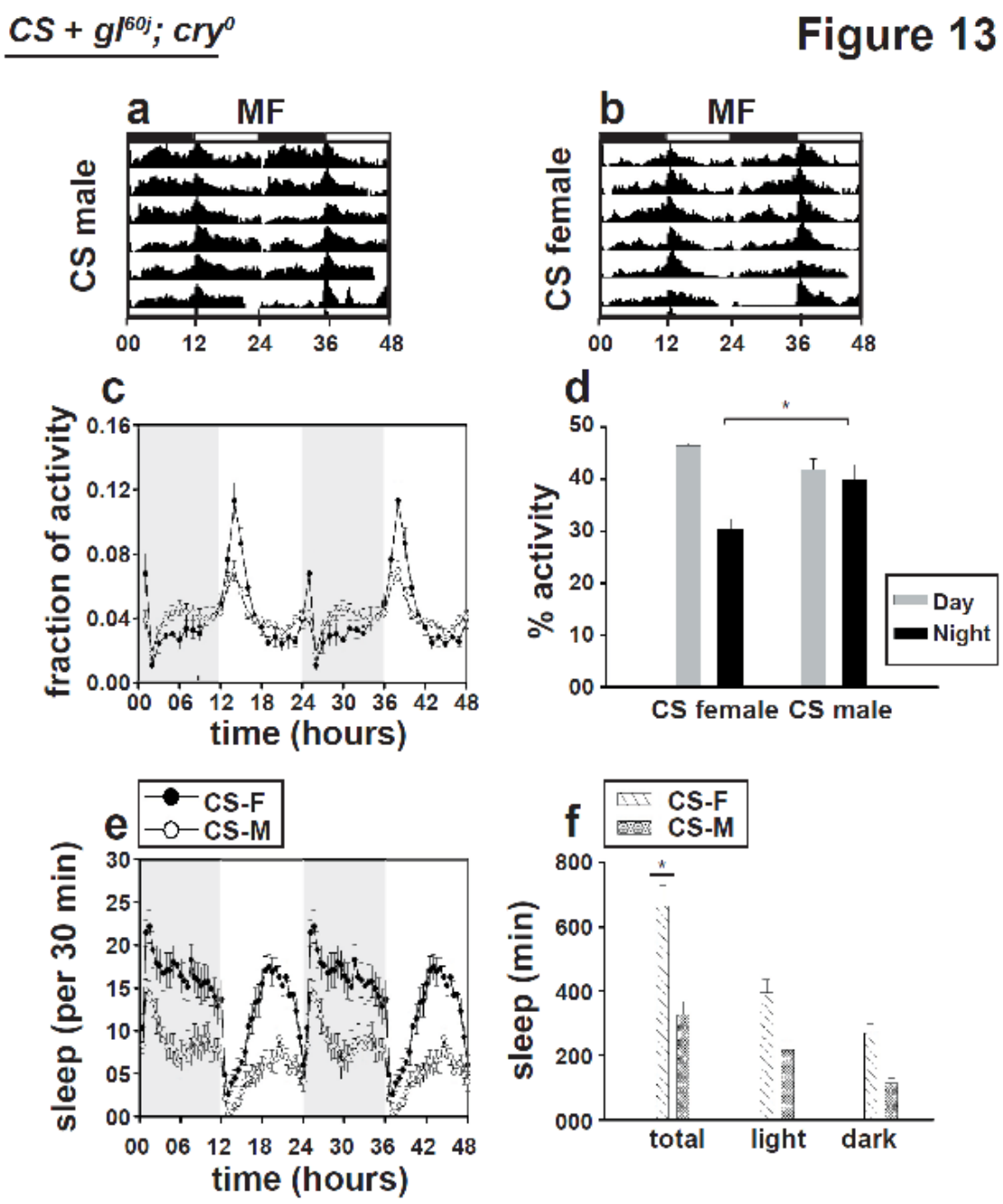


Figure 13 ***SSI is male driven.*** Double plots of actograms (a) show higher night time activity when *CS* males are paired with time-blind ($gl^{60j};cry^{01}$) females compared to when time-blind males are paired with *CS* females (b). Profiles of activity (c) also indicates that there is a significantly greater night time activity in couples with wild type males and time-blind females (d) compared to time-blind male and wild type female couples ($p < 0.05$). Due to increased night time activity in MF couples with wild type males there is a concurrent reduction in the overall sleep (e and f). Analysis of sleep data (f) indicates that total sleep in couples with wild type males is significantly lesser than couples with time-blind males ($p < 0.05$) (f). As many as ($n = 16$) *CS* male and $gl^{60j};cry^{01}$ female, and ($n = 16$) $gl^{60j};cry^{01}$ male and *CS* female couples were used in this experiment. All other aspects same as Fig. 1.

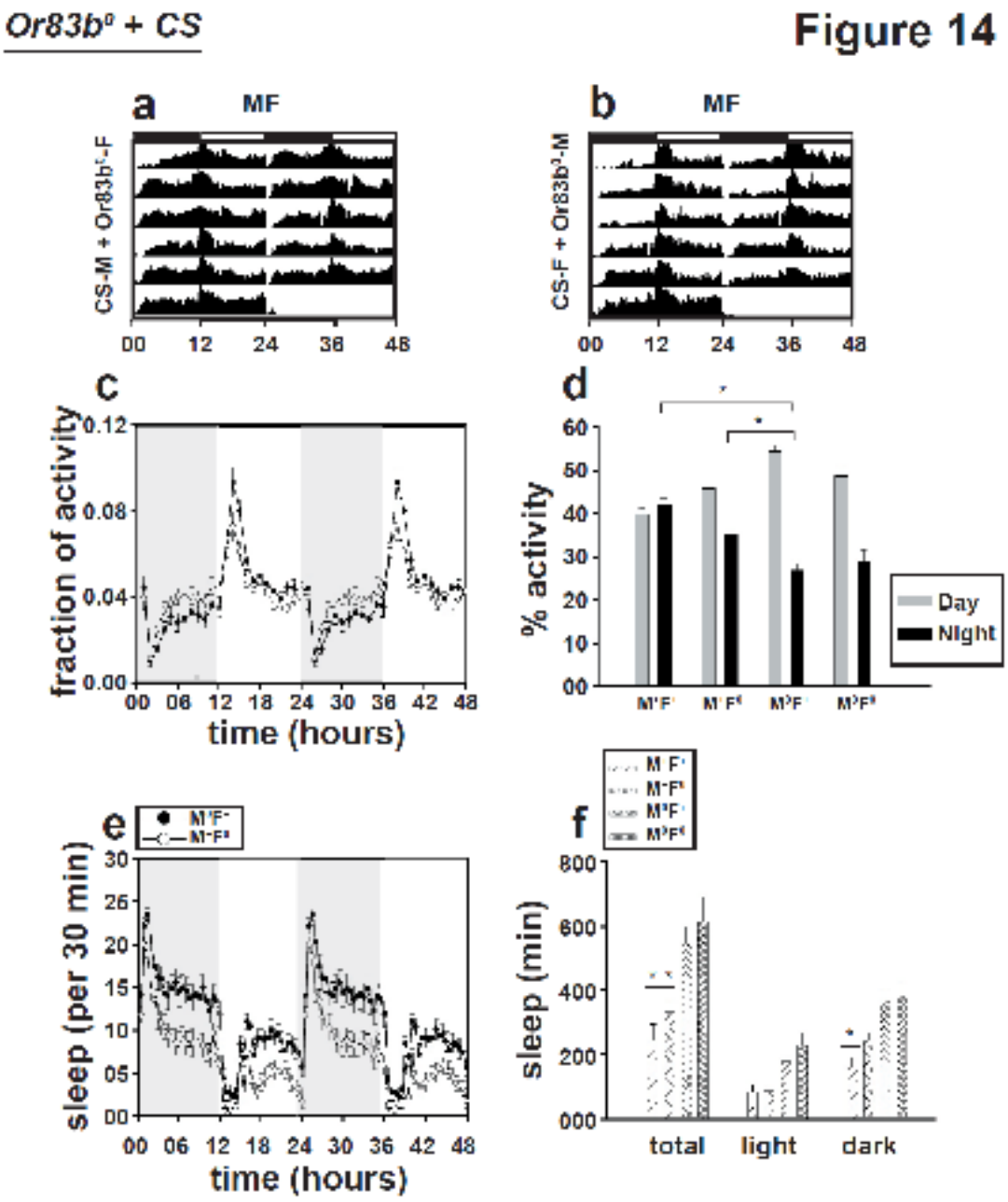


Figure 14 ***Olfaction in males is necessary for SSI.*** Double plots of actograms (a) show higher night time activity as a result of pairing of *CS* males and *Or83b*^{0} females ($M^{+}F^{o}$) compared to *Or83b*^{0} male and *CS* female couples ($M^{o}F^{+}$) (b). Profiles of the activity (c) indicate that $M^{+}F^{o}$ couples are more active during night than $M^{o}F^{+}$ couples. Analysis of activity data (d) shows that night time activity of $M^{o}F^{+}$ couples is significantly lower than $M^{+}F^{+}$ ($p < 0.0005$) or $M^{+}F^{o}$ ($p < 0.05$) but similar to $M^{o}F^{o}$ couples ($p = 0.97$). During the day, activity of $M^{o}F^{+}$ couples is greater than $M^{+}F^{+}$ ($p < 0.0005$) or $M^{+}F^{o}$ ($p < 0.05$) but similar to $M^{o}F^{o}$ ($p = 0.25$) couples. Sleep profiles (e) indicate that $M^{o}F^{+}$ couples sleep significantly more than $M^{+}F^{o}$ ($p < 0.05$) or $M^{+}F^{+}$ ($p < 0.0005$) couples. During the day time $M^{o}F^{+}$ couples sleep as much as the other two couples, whereas during the night they sleep as much as $M^{+}F^{o}$ ($p = 0.59$) but significantly more than $M^{+}F^{+}$ ($p < 0.05$) couples. As many as ($n = 28$) $M^{o}F^{+}$ ($n = 28$) $M^{+}F^{o}$, and ($n = 28$) $M^{+}F^{+}$ couples were used in this experiment. All other aspects same as Fig. 1.

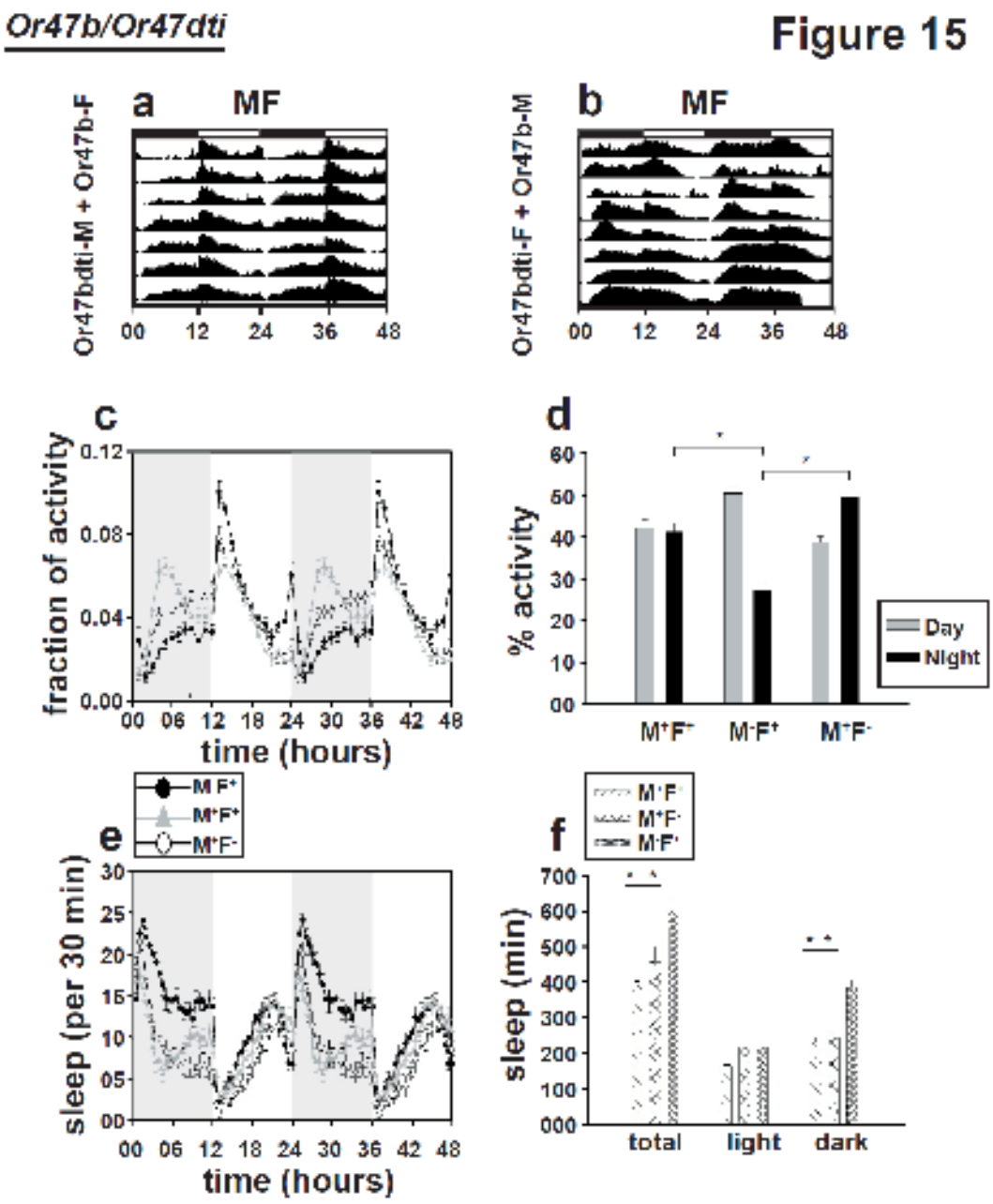


Figure 15 ***Or47b in males is necessary for SSI.*** Double plots of actograms (a) show lower night time activity in male-female (MF) couples with *Or47b* ablated (*Or47b-GAL4/UASdti*) males and intact (*Or47bGAL4*) females (M^-F^+) compared to couples with intact males and ablated females (M^+F^-) (b). Profile of activity (c) also indicates that night time activity M^-F^+ couples is lower than M^-F^+ or M^+F^+ couples ($p < 0.0005$). During the day time M^-F^+ couples are more active than M^+F^- ($p < 0.005$) or M^+F^+ couples ($p < 0.05$). Due to decrease in night time activity there is also some amount of sleep recovery in M^-F^+ couples (e). Analysis of sleep data suggests that M^-F^+ couples sleep significantly more compared to M^+F^- ($p < 0.005$) or M^+F^+ ($p < 0.0005$) couples. During the night M^-F^+ couples sleep more than M^+F^- or M^+F^+ ($p < 0.005$) couples, whereas during the day all three couples sleep equally well. As many as ($n = 24$) M^+F^+, ($n = 24$) M^+F^-, and ($n = 24$) M^-F^+ couples were used in this experiment. All other aspects same as Fig. 1.

on the activity data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons suggest that night time activity in M_-F_+ couples is significantly lower than M_+F_- couples ($p < 0.0005$), while their day time activity is significantly higher than M_+F_- couples ($p < 0.001$) (Figs. 15c, d).

ANOVA on the sleep data suggests significant effect of pairing, time of day, and pairing × time of the day interaction. Post-hoc comparisons revealed that there is recovery of sleep when ablated males are paired with intact or ablated females. Sleep in M_-F_+ couples is significantly greater than M_+F_+ ($p < 0.0005$) or M_+F_- ($p < 0.005$) couples. During the day, there are no differences in their sleep, while during nights M_-F_+ couples sleep significantly more than M_+F_+ or M_+F_- couples ($p < 0.005$) (Figs. 15e, f).

Role of PDF and clock neurons in the regulation of SSI:

6.3.16 ***SSI related night time activity in MF couples free-runs in DD:*** To see whether SSI in *Drosophila* is clock controlled we decided to study locomotor activity rhythm of MF, MM, and FF couples under DD conditions. While the locomotor activity of MM and FF couples is primarily restricted to the subjective day and free-runs with species-specific circadian periods (Figs. 16a, b), that of MF couples it is spread well into the subjective night (Fig. 16c).

6.3.17 ***Circadian neurotransmitter PDF is not involved in SSI:*** Actograms indicate that pdf^0 MF couples exhibit higher night time activity compared to MM or FF couples (Figs. 17a, b). ANOVA on the activity data revealed that the effect of time of the day, and pairing × time of the day interaction is statistically significant; however, effect of pairing is statistically not significant. Post-hoc multiple comparisons revealed that night

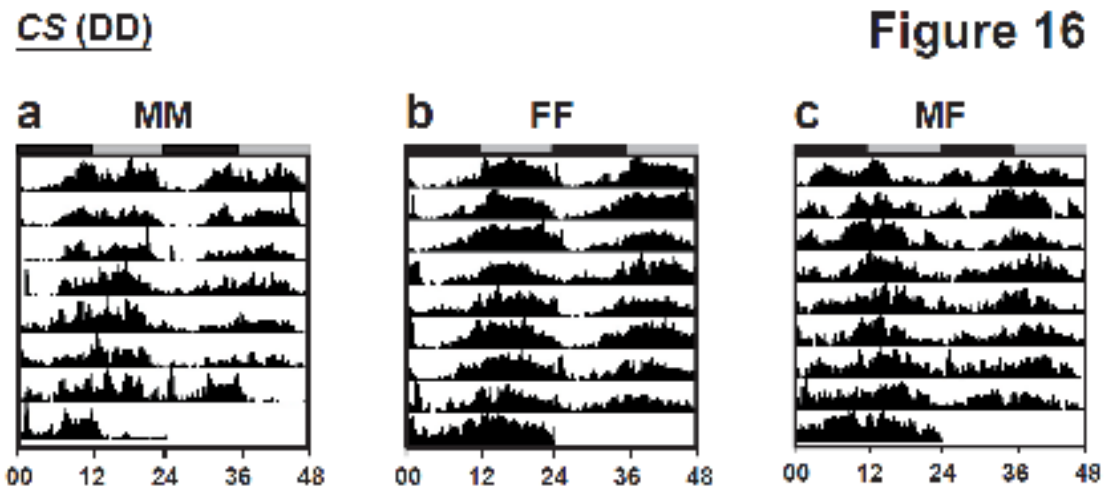


Figure 16 ***Night time activity of MF couples persists under DD.*** Double plots of actograms (a-c) of wild type CS flies indicates that male-male (MM: a) and female-female (FF: b) couples are active during the light phase under 12:12 hr light/dark (LD) cycles, while male-female (MF) couples start activity with lights-OFF and continue until late in the evening. Under DD conditions, activity rhythm of MM and FF couples free-run with activity mostly restricted to subjective day, whereas MF couples exhibit activity even during subjective night (c). As many as (n = 16) MM, (n = 16) FF, and (n = 16) MF couples were used in this experiment. All other aspects same as Fig. 1.

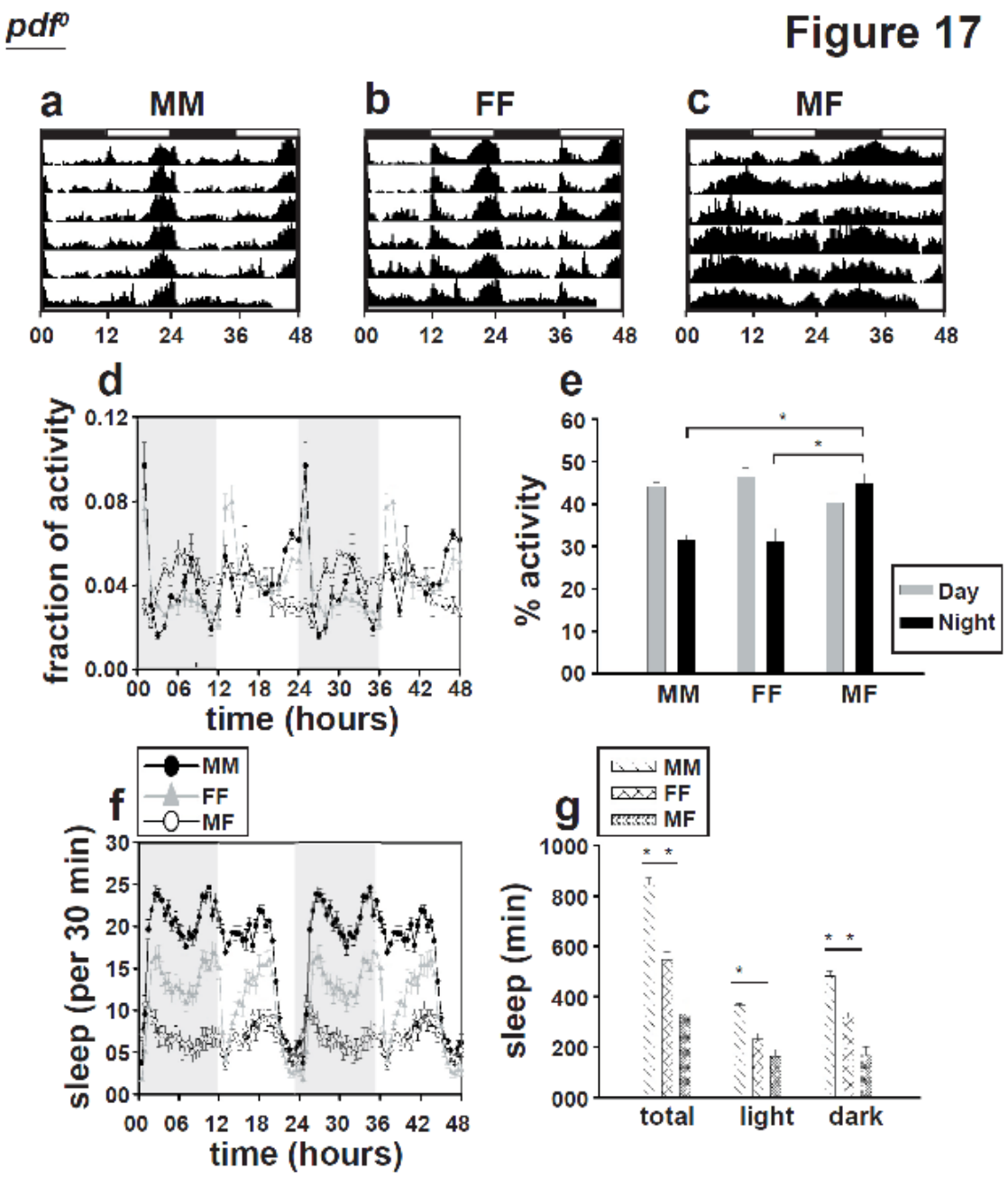


Figure 17 ***Circadian neurotransmitter PDF is not required for SSI.*** Double plots of actograms of flies with loss of function for circadian neurotransmitter *pigment dispersing factor* (*pdf*^{0}) (c) show that male-female (MF) couples exhibit higher night time activity compared to MM (a) or FF (b) couples. Profiles of activity (d) indicate that there is greater night time activity in MF couples. Analysis (e) shows that night time activity in MF couples is significantly greater than MM or FF couples ($p < 0.005$), however, during day time, MF couples are as active as MM ($p = 0.80$) or FF ($p = 0.39$) couples. Due to increased night time activity there is a reduction in total sleep which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep (g) indicates that there is decrease in total sleep in MF couples relative to MM ($p < 0.0005$) or FF ($p < 0.001$) couples. During the night MF couples sleep lesser compared to MM ($p < 0.0005$) or FF ($p < 0.05$) couples, whereas during the day MF couples sleep significantly lesser compared to MM ($p < 0.005$) but as much as FF ($p = 0.78$) couples. As many as ($n = 20$) MM, ($n = 22$) FF, and ($n = 22$) MF couples were used in this experiment. All other aspects same as Fig.1.

time activity in MF couples is significantly higher compared to MM or FF couples ($p <$ 0.005), while their day time activity does not differ from MM ($p = 0.80$) or FF ($p = 0.39$) couples (Figs. 17d, e).

Analysis of sleep data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons showed that MF couples sleep less compared to MM ($p < 0.0005$) or FF ($p < 0.001$) couples. During the night, MF couples sleep significantly less compared to MM ($p < 0.0005$) or FF ($p < 0.05$) couples, while during the day they sleep significantly lesser than MM ($p < 0.005$) but as much as FF ($p = 0.78$) couples (Figs. 17f,g).

6.3.18 ***Silencing PDF-positive clock neurons do not have any significant impact on SSI:*** Actograms of electrically silenced (*pdfGAL4/UASkir2.1*) flies show higher night time activity in MF couples compared to MM or FF couples (Figs. 18a-c). ANOVA on the activity data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc comparisons indicate that night time activity is higher in MF couples compared to MM ($p < 0.005$) or FF ($p < 0.0005$) couples, while during the day MF couples are less active than FF ($p < 0.005$) but as active as MM ($p = 0.23$) couples (Figs. 18d, e).

ANOVA on sleep data showed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc analysis suggests that MF couples sleep less compared to MM or FF couples ($p < 0.0005$). During the night, MF couples sleep significantly less compared to MM ($p < 0.01$) or FF ($p < 0.0005$) couples, while during the day they sleep lesser than MM ($p < 0.005$) but as much as FF couples ($p = 0.99$) (Figs. 18f, g).

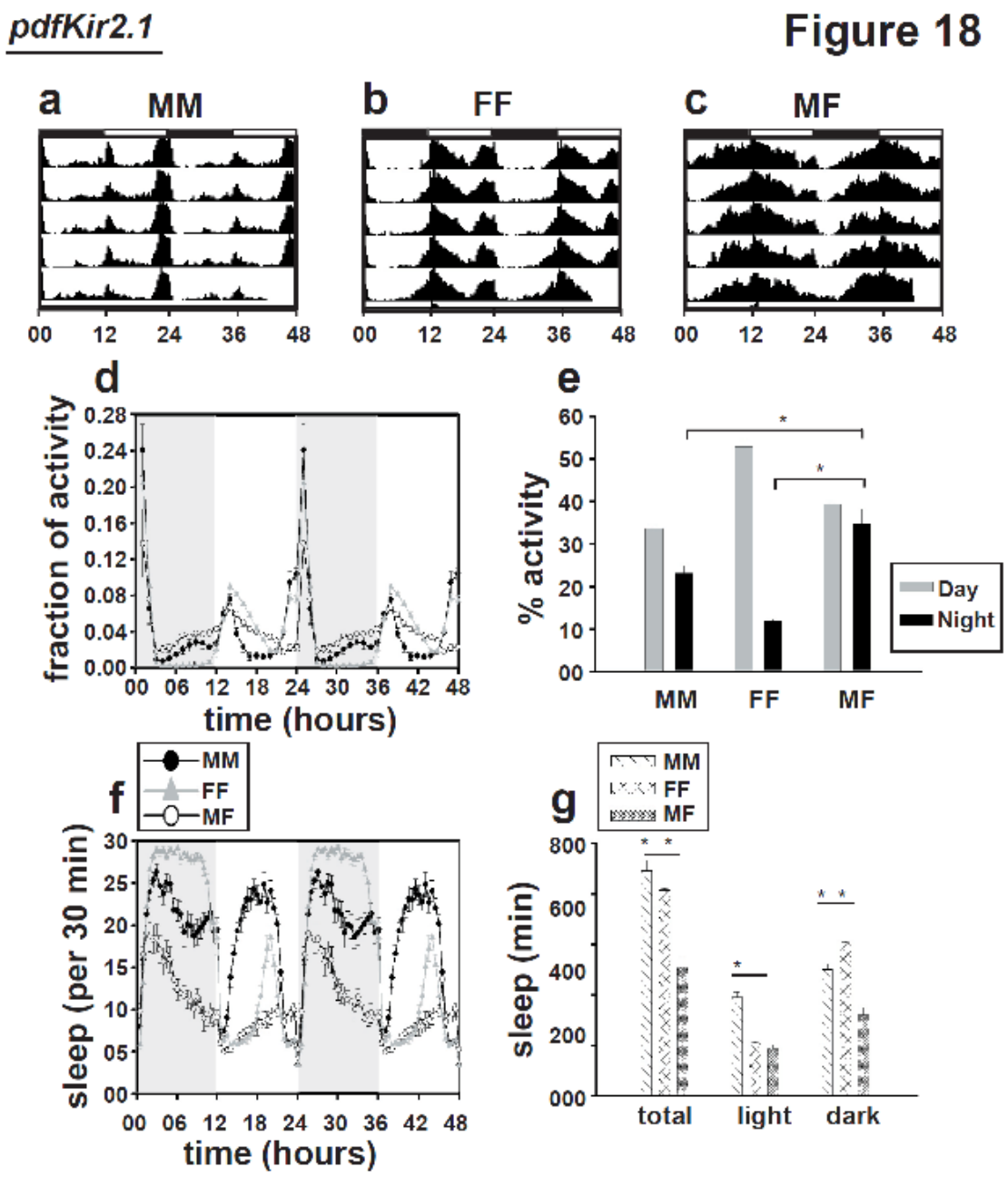


Figure 18 ***Electrical silencing of PDF-positive neurons does not have any impact on SSI.*** Double plots of actograms of electrically silenced flies (*pdfGAL4/UASkir2.1*) (c) show that night time activity of male-female (MF) couples is higher than MM (a) or FF (b) couples. Profiles of activity (d) also indicate that there is greater night time activity in MF couples. Analysis of activity data (e) shows night time activity in MF couples is significantly higher than MM ($p < 0.005$) or FF ($p < 0.0005$) couples, while during the day they are as active as MM ($p = 0.23$) but relatively less active than FF ($p < 0.005$) couples. Due to increased night time activity there is reduction in total sleep which is mostly accounted for by the reduction in night time sleep (f). Analysis of sleep data (g) indicates that there is decrease in total sleep in MF couples relative to MM or FF couples ($p < 0.0005$). During the night MF couples sleep lesser compared to MM ($p < 0.01$) or FF ($p < 0.0005$) couples, whereas during the day MF couples sleep significantly lesser compared to MM ($p < 0.005$) but as much as FF ($p = 0.99$) couples. As many as ($n = 16$) MM, ($n = 16$) FF, and ($n = 16$) MF couples were used in this experiment. All other aspects same as Fig. 1.

pdf^{0} (DD)

Figure 19

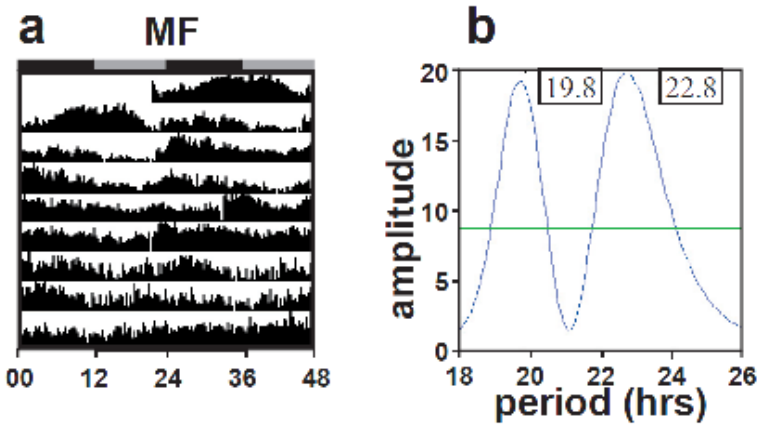


Figure 19 MF couples are active subjective night in *pdf*^{0}. Male –female couples become active during subjective night after first day (a). Average periodogram of MF couples (b) (n=32).

6.3.19 ***pdf^{0} are active during subjective night in constant darkness:*** *pdf^{0}* male females were entrained in LD10-22hr for 4 days as isolates and then they were kept together before placing them in constant darkness.Fig.19a shows in the first day, they are active in the subjective day and on second day onwards it is shifted towards subjective night.

6.3.20 ***Silencing CRY-positive clock neurons has significant impact on SSI:***

Actograms indicate that electrically silenced (*cryGAL4/UASkir2.1*) MF couples are overall more active as compared to MM or FF couples (Figs. 20a, b). ANOVA on the activity data revealed that the effect of pairing, and time of the day is statistically not significant, however, effect of pairing × time of the day interaction is significant. Post-hoc multiple comparisons showed that during the night MF couples are as active as MM ($p = 0.77$), but more active than FF ($p < 0.005$) couples, while during the day they are more active than MM ($p = 0.07$), but less active than FF ($p < 0.01$) couples (Figs. 20d, e).

Analysis on sleep data showed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons showed that MF couples sleep significantly lesser than MM ($p < 0.01$) or FF ($p = 0.06$) couples. During the night, MF couples sleep as much as MM ($p = 0.68$) but marginally lesser than FF ($p = 0.06$) couples whereas during the day they sleep as much as MM ($p = 0.23$) or FF ($p = 1$) couples (Figs. 20f, g).

6.3.21 ***Functional clock in the peripheral Or47b neurons is necessary for SSI:***

Actograms of flies where clock function in the *Or47b* neurons is disrupted by PER overexpression (*Or47bGAL4/UASper*) indicates that in the night MF couples are as active as MM or FF couples (Figs. 21a-c). ANOVA on the activity data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc

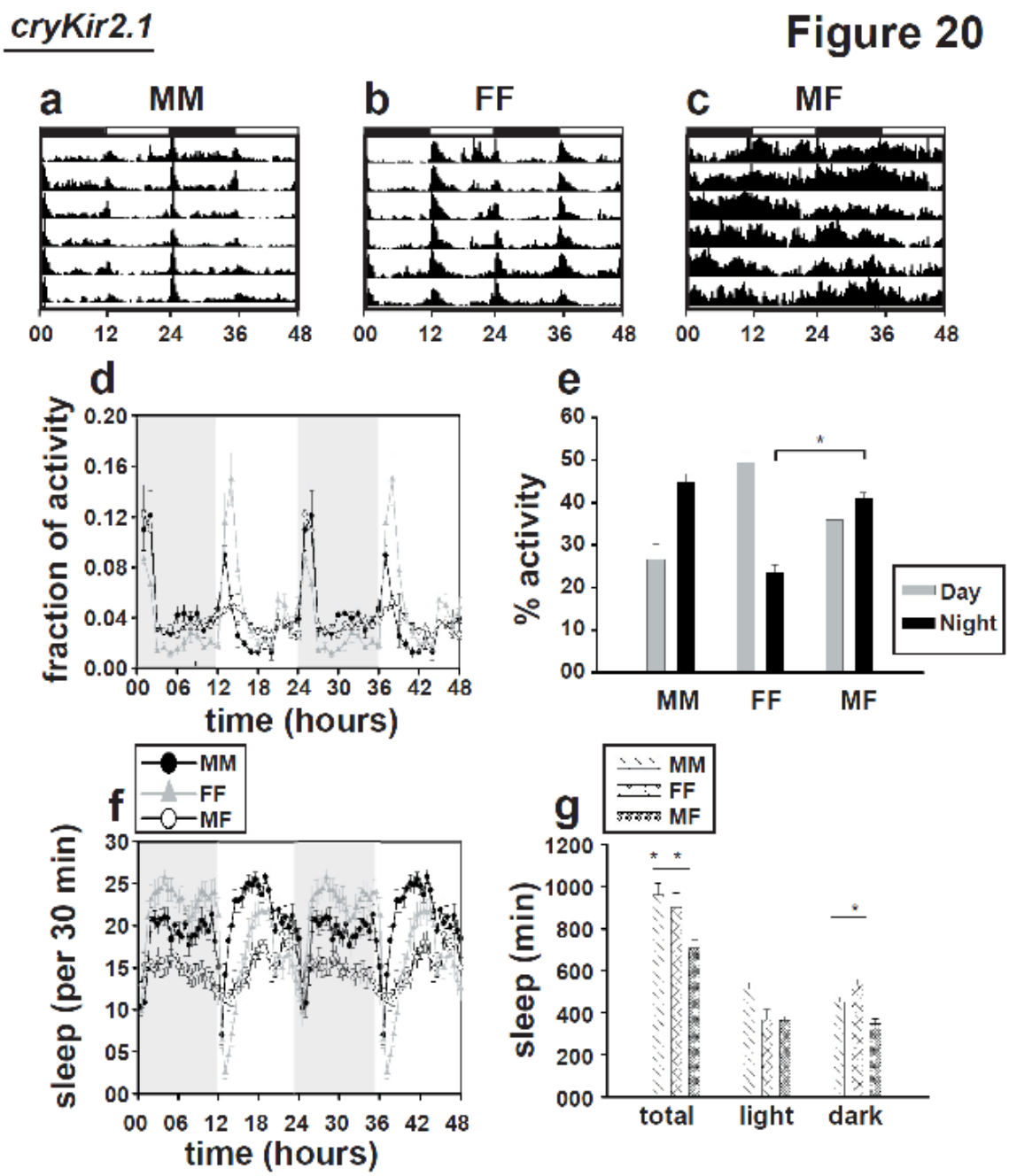


Figure 20 ***Electrical silencing of CRY-positive neurons results in reduction in SSI.*** Double plots of actograms of silenced flies (*cry-GAL4/UASkir2.1*) (c) show that male-female (MF) couples exhibit marginally higher night time activity compared to MM (a) or FF couples (b). Profiles of activity (d) also indicate that night time activity in MF couples is more or less similar to MM couples. Analysis of activity data (e) shows that night time activity in MF couples is similar to MM ($p = 0.77$) but significantly higher than FF couples ($p < 0.005$), while during day time they are more active than MM ($p = 0.07$) but lesser than FF ($p < 0.01$) couples. There is also a significant recovery of sleep in the MF couples (f). Analysis of sleep data (g) indicates that although total sleep in MF couples is significantly lesser compared to MM ($p < 0.01$) or FF ($p = 0.06$) couples, their night time sleep is comparable to MM ($p = 0.68$) or FF ($p > 0.05$) couples. The day time sleep of MF couples is also comparable to MM ($p = 0.23$) or FF ($p = 1.00$) couples. As many as ($n = 16$) MM, ($n = 16$) FF, and ($n = 16$) MF couples were used in this experiment. All other aspects same as Fig. 1.

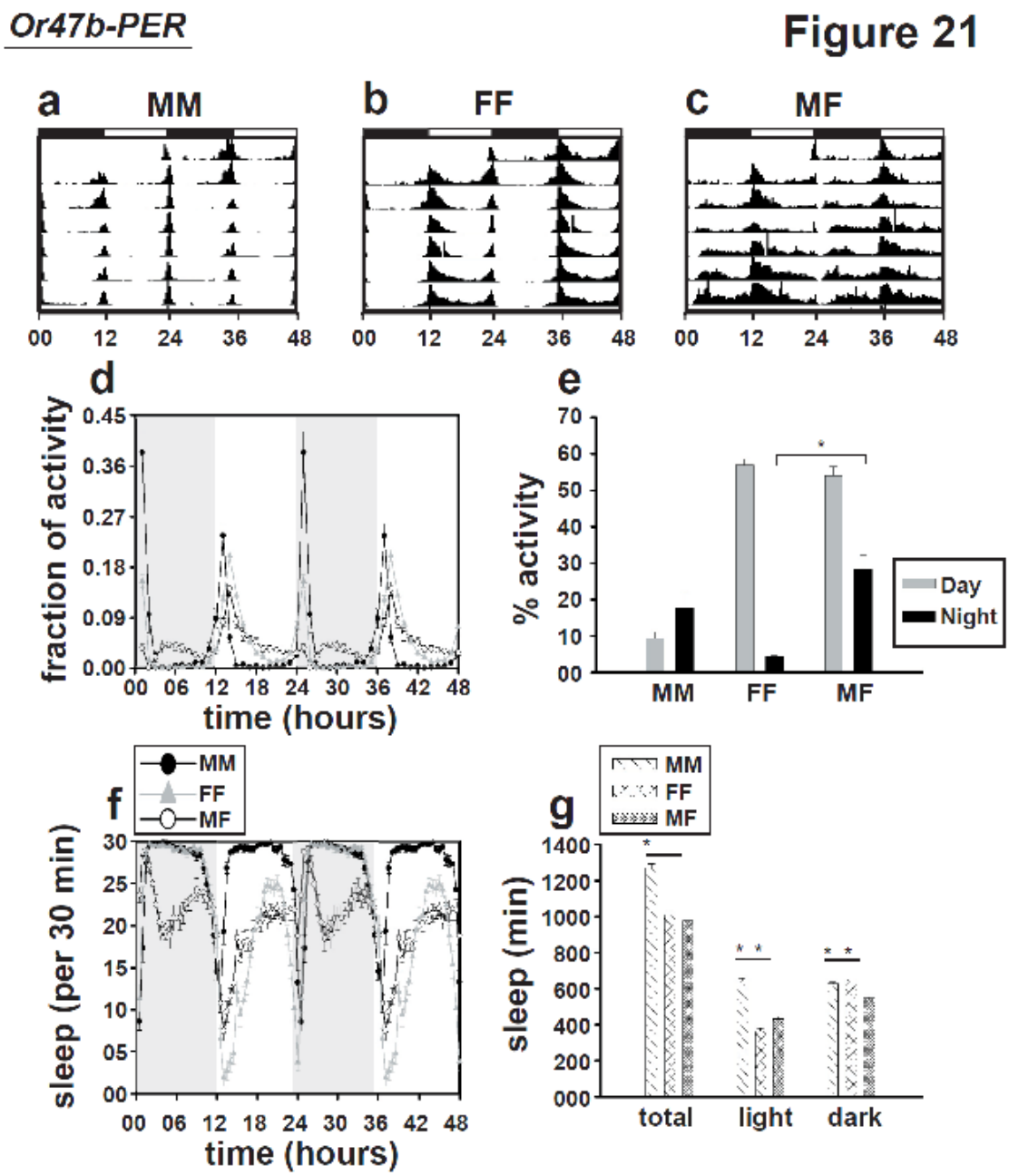


Figure 21 ***Overexpression of PER in Or47b neurons results in reduced SSI.*** Double plotted actograms (c) of flies with PER over-expression in *Or47b* neurons (*Or47bGAL4/UASper*) show that night time activity of MF couples is more or less similar to MM (a) or FF (b) couples. Profiles of activity (d) indicate that night time activity of MF couples is similar to MM but significantly higher than FF couples. Analysis of activity data (e) shows that night time activity of MF couples is similar to MM ($p = 0.09$) but higher than FF ($p < 0.0005$) couples, whereas day time activity is more than MM ($p < 0.0005$) but as much as FF ($p = 0.96$) couples. There is also reduction in total sleep in MF couples (f). Analysis of sleep data (g) indicates that total sleep in MF couples is lesser than MM ($p < 0.0005$) but comparable to FF ($p = 0.69$) couples. During the night MF couples sleep lesser compared to MM ($p < 0.01$) or FF ($p < 0.0005$) couples, whereas during the day they sleep lesser than MM ($p < 0.0005$) but more than FF ($p < 0.05$) couples (f and g). As many as ($n = 19$) MM, ($n = 20$) FF, and ($n = 18$) MF couples were used in this experiment. All other aspects same as Fig. 1.

multiple comparisons suggest that night time activity in MF couples is similar to MM ($p = 0.09$) but higher than FF couples ($p < 0.0005$), while their day time activity is higher than MM ($p < 0.0005$) but similar to FF ($p = 0.96$) couples (Figs. 21d, e).

ANOVA on the sleep data revealed significant effect of pairing, time of the day, and pairing × time of the day interaction. Post-hoc multiple comparisons suggest that sleep in MF couples does not differ from FF couples ($p = 0.69$) but is significantly lesser than MM couples ($p < 0.0005$). Night time sleep in MF couples is significantly lesser compared to MM ($p < 0.01$) or FF ($p < 0.0005$) couples but day time sleep is significantly greater than FF ($p < 0.05$) but lesser than MM ($p < 0.0005$) couples (Figs. 21f, g).

6.3.22 *Overexpression of PER in Or47b neurons has greater impact on SSI than Mai179 neurons:* Actograms indicate that MF couples with PER overexpression in *Or47b* neurons (*Or47bGAL4/UASper* - *Or47b*-PER) have significantly lower night time activity compared to couples with PER overexpression in *Mai179* neurons (*Mai179GAL4/UASper – Mai179*-PER) (Fig. 22a). ANOVA on the activity data revealed significant effect of time of the day, and strain × time of the day interaction, however, effect of strain is statistically not significant. Post-hoc multiple comparisons using Tukey's test suggest that night time activity in *Mai179*-PER couples is significantly higher than *Or47b*-PER couples ($p < 0.05$), whereas their day time activity does not differ statistically ($p = 0.13$) (Figs. 22c, d).

ANOVA on sleep data showed significant effect of strain, time of the day, however, strain × time of the day interaction is statistically not significant. Post-hoc comparisons suggest that *Or47b*-PER couples sleep more than *Mai179*-PER couples ($p < 0.0005$). The night time sleep in *Or47b*-PER couples is marginally higher than

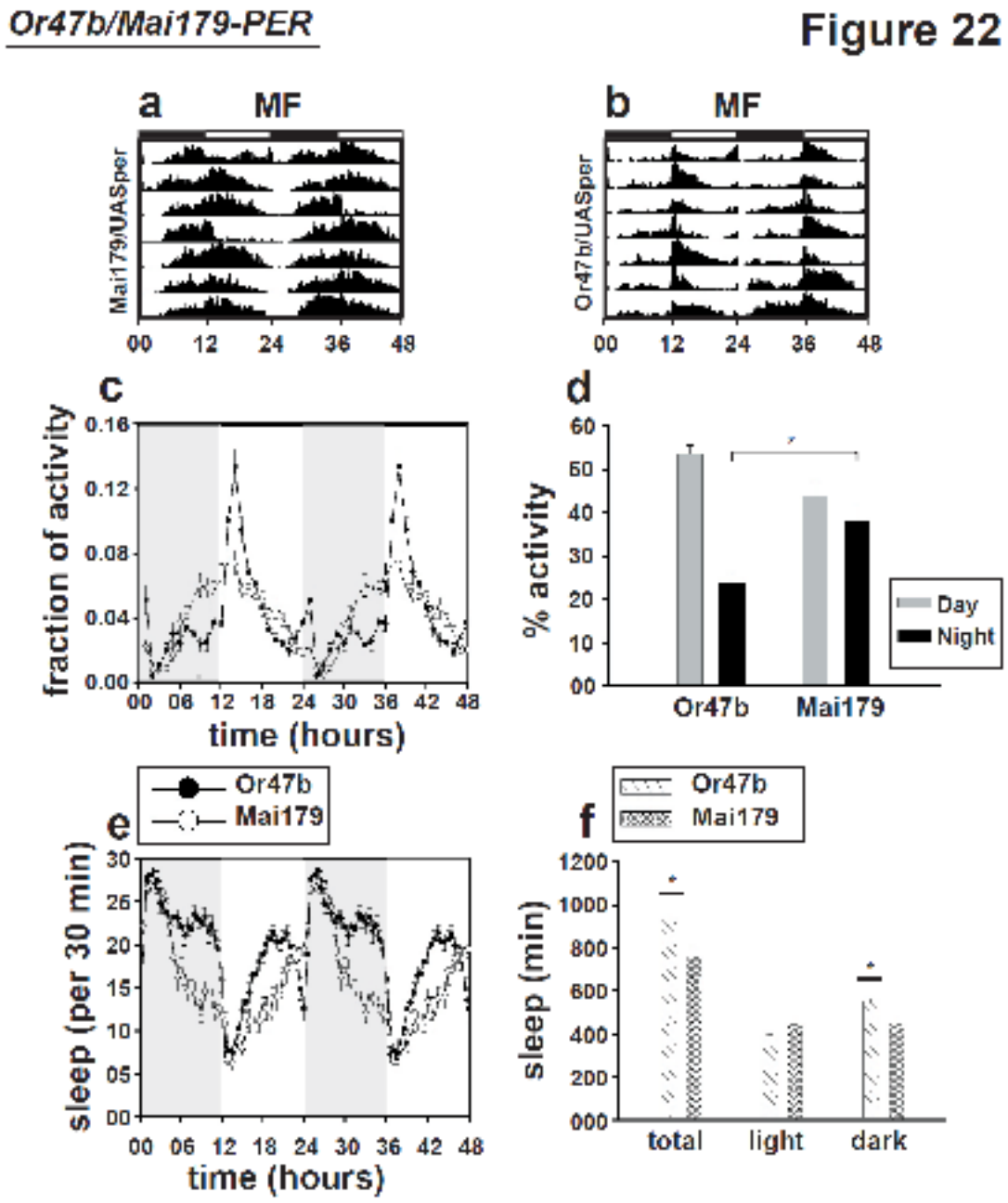


Figure 22 ***Couples with PER over expressed in Or47b neurons display reduced SSI compared to those in Mai179 neurons.*** Double plots of actograms (b) show that male-female (MF) couples of flies with PER overexpressed in *Or47b* neurons (*Or47b-GAL4/UASper)* (*OR47b-PER*) show lower night time activity compared to couples with PER overexpressed in *Mai179* neurons (*Mai179-GAL4/UASper*) (*Mai179-PER*) (a). Profiles of activity (c) also indicates that night time activity is lower in *OR47b-PER* couples compared to *Mai179-PER* couples (d). Analysis of activity suggests that night time activity of *OR47b-PER* couples (d) is lower than *Mai179-PER* couples ($p < 0.05$), whereas their day time activity does not differ ($p = 0.13$). Due to relatively low night time activity, *Or47b-PER* couples sleep more compared to *Mai179-per* couples (e). Analysis of sleep data suggests that total sleep ($p < 0.0005$) as well night time sleep in *Or47b-PER* couples is greater than *Mai179-PER* couples ($p = 0.06$), whereas there are no differences in their day time sleep ($p = 0.43$) (e and f). A total of ($n = 24$) *Or47b-PER* and ($n = 24$) *Mai179-PER* couples were used in this experiment. All other aspects same as Fig. 1.

Table 1a

		Activity		Sleep		
		Day	Night	Light	Night	Total
CS	MM	34.0 ± 3.8	29.6 ± 2.9**	300.5 ± 61.7**	355.2 ± 44.2**	655.7 ± 47.8***
	FF	61.6 ± 4.7***	08.7 ± 1.5***	157.2 ± 21.0	542.2 ± 61.3***	699.4 ± 45.2***
	MF	40.0 ± 2.4	41.9 ± 1.7	086.2 ± 39.8	158.5 ± 61.7	244.7 ± 99.8
W	MM	47.0 ± 1.9	19.2 ± 1.6***	289.8 ± 39.1**	499.6 ± 35.3***	789.5 ± 71.6***
	FF	64.9 ± 1.8***	14.0 ± 2.3***	192.5 ± 54.6	553.1 ± 02.4***	745.8 ± 56.8***
	MF	46.2 ± 2.1	35.5 ± 3.3	143.1 ± 15.1	234.3 ± 20.7	377.5 ± 27.5
yw	MM	31.8 ± 2.5	33.8 ± 4.3***	277.2 ± 06.3	284.1 ± 09.6*	661.3 ± 14.2***
	FF	43.5 ± 1.9**	29.4 ± 5.0***	184.8 ± 30.9	380.6 ± 51.1**	565.4 ± 80.5***
	MF	30.1 ± 2.6	51.0 ± 2.9	130.7 ± 74.6	114.3 ± 70.9	245.0 ± 144.6
OR	MM	43.1 ± 4.0*	13.3 ± 0.7*	567.1 ± 16.5**	647.6 ± 16.0**	1214.8 ± 27.0***
	FF	64.3 ± 4.1	05.3 ± 1.5***	490.9 ± 44.4	680.9 ± 14.5***	1171.9 ± 45.4***
	MF	54.2 ± 5.9	26.1 ± 5.3	461.8 ± 30.1	537.6 ± 36.87	999.4 ± 31.7

Table 1b

Or47bGAL4/ UASdtl	MM	31.8 ± 4.8***	22.7 ± 5.8	411.0 ± 15.5	477.6 ± 52.2	888.7 ± 63.4
	FF	51.1± 5.2	10.8 ± 3.0*	319.3 ± 56.6	568.9 ± 20.2	888.2 ± 44.1
	MF	56.9 ± 1.8	21.6 ± 1.3	383.1 ± 51.7	533.1 ± 61.7	916.2 ± 85.5
Or88bGAL4/ UASdtl	MM	31.4 ± 8.1***	23.1 ± 06.5	389.9 ± 53.5	509.5 ± 28.9	899.5 ± 47.8***
	FF	51.7 ± 4.8	12.4 ± 02.1**	283.8 ± 36.0	521.3 ± 53.6	805.1 ± 86.6*
	MF	52.5 ± 2.1	30.0 ± 05.1	252.1 ± 82.2	364.1 ± 49.0	616.3 ± 125.1
Or65aGAL4/ UASdtl	MM	31.0 ± 3.4***	23.4 ± 2.0	455.1 ± 56.6	526.1 ± 48.0	981.2 ± 98.8***
	FF	64.3 ± 5.4**	10.4 ± 1.1***	285.5 ± 86.7	664.6 ± 20.1***	950.1 ± 103.1**
	MF	52.3 ± 3.7	30.0 ± 3.7	311.5 ± 52.8	373.1 ± 79.1	684.7 ± 122.2
Or67dGAL4 Knockin line	MM	25.7 ± 1.8	22.5 ± 5.0***	440.7 ± 14.1**	519.7 ± 35.2***	960.4 ± 44.2***
	FF	39.0 ± 2.9	13.1 ± 2.9***	397.6 ± 39.8*	583.6 ± 33.4***	981.3 ± 52.6***
	MF	31.2 ± 2.6	46.8 ± 2.4	224.9 ± 51.8	245.5 ± 51.7	470.4 ± 103.5

$p < 0.05$ =*, $p < 0.01$ = ** and $p < 0.001$ = ***. Acivity levels are in percentage and sleep in minutes. MM (male + male), FF (female + female) are compared with MF (Male + Female).

Mai179-PER couples ($p = 0.06$) but their day time sleep is comparable ($p = 0.43$) (Figs. 22e, f).

6.4 Discussion

Night time activity of MF couples is enhanced when males and females are co-housed together; MF couples display higher night time activity and reduced sleep compared to MM or FF couples (Figs. 1, 3-5). Given that amount of activity and courtship of MF couples are known to be positively correlated, this implies that heterosexual couples are involved in courtship during the night (Hall, 1994), which is consistent with the fact that *Drosophila* courtship follows a daily rhythm with a distinct peak in the night (Hardeland, 1972; Fujii et al., 2007). The MM couples also exhibit slight but non-negligible night time activity, which is consistently found in many strains of flies (CS, *yw*, *w* and *OR*) that we studied (Figs. 1, 3-5). This increase in night time activity is not artifact as there is no increase in night time activity when male and female data sets are summed (Figs. 2). *Drosophila* females allow the same male to copulate with her not more than thrice in four days (Fujii et al., 2007), however, males do try to court tirelessly, which is quite apparent from the activity levels of MF couples. The SSI mediated circadian phenotype (enhanced night time activity and sleep loss) persists in MF couples even after being together for 10 days, which suggests that SSI does not depend upon the actual act of mating, rather reflects mating associated locomotor activity.

The phenomenon of increased night time activity and reduced night time sleep in MF couples was observed in many strains of *Drosophila* (CS, *yw*, *w* and *OR*) indicating that it is a consistent behaviour in *Drosophila melanogaster* heterosexual couples (Figs. 1, 3-5; Table 1). Increased night time activity and sleep loss in MF couples is primarily

attributed to courtship, which involves several sensory modalities such as auditory, visual, and chemosensory (Fujii et al., 2007). The results of our study suggest that olfaction plays a major role in SSI (Figs. 6a-g). Our results are in agreement with several previous studies (Levine et al., 2002; Fujii et al., 2007; Krupp et al., 2008) which showed that most social interactions in *Drosophila* are mediated by olfaction. Although olfaction plays a key role in SSI, it is not clear which olfactory receptor(s) are involved in the communication of social signals. We have shown that in *Or83b* loss of function mutants that completely lack olfactory ability, night time activity is significantly decreased and sleep loss recovered (Figs. 6a-e). The olfactory receptor *Or67d*, which is known to be involved in the reception of male-specific pheromone 11-*cis*-vaccenyl acetate (cVA) (Kurtovic et al., 2007) does not have any role as *Or67d* knock-in couples exhibit normal SSI (Figs. 9a-g). Although, ablation of two other olfactory receptor neurons (ORNs), *Or65a* (Figs. 7a-e) and *Or88a* (Figs. 8a-e) does not alter night time activity in MF couples it causes measurable impact on night time sleep (Figs. 7, 8f, g), suggesting that these receptors may also have some role in SSI. Upon ablation of *Or47b* neurons, night time activity of MF couples reduced to levels similar to MM or FF couples (Figs. 10a-e). *Or47b* ablated couples started sleeping as well as MM or FF couples (Figs. 10f, g). Therefore, manipulation of *Or47b* alone seems to be sufficient in blocking SSI in MF couples, which suggests that *Or47b* is right at the core of olfactory signaling cascade involved in the communication of social signals during SSI. Reduction in night time activity and sleep recovery in MF couples is associated with the absence of *Or47b* neurons and not with any genetic artifact of the parental strains because MF couples of parental control strains display significantly higher night time activity and sleep loss

compared to same sex couples (Figs. 11a-g). Importance of *Or47b* is further confirmed when we examined flies with electrically silenced *Or47b* neurons by ectopically expressing *dORKC1* or *Kir2.1* channels (Nitabach et al., 2002). The night time activity of silenced MF couples is significantly reduced (Figs. 12a-e) and sleep loss considerably recovered (Figs. 12f-h). SSI mediated circadian phenotypes of MF couples is primarily dictated by males because MF couples with time-blind, or $Or83b^{0}$, and *Or47b* ablated males display reduced SSI compared to couples with wild type males (Figs. 13-15).

The locomotor activity behavior of MF couples is rhythmic under 12:12 hr LD cycles with activity spread over most part of night and first half of day, while those of MM or FF couples is mostly restricted during the day. Similarly under DD, activity of all three couples show circadian rhythm in activity; activity of MF couples is confined to subjective night and early part of subjective day, while that of same sex couples are confined to subjective day (Fig. 16). Interestingly, MF couples with loss of function mutation in the circadian neurotransmitter PDF (pdf^{0}) exhibit normal SSI (Fig. 17). The activity of pdf^{0} MF couples free-run under DD (Renn et al., 1999), and they show enhanced activity during subjective night (Fig. 19), which suggests that PDF may not be involved in SSI. Moreover, electrical silencing of PDF-expressing LN_v neurons does not affect SSI, and silenced MF couples continue to display higher night time activity and reduced sleep compared to MM or FF couples, suggesting that electrical activity of LN_v clock neurons is not necessary for SSI (Fig. 18). However, when a larger subset of clock neurons that express CRY (LN_v, LN_d, and DN neurons) is electrically silenced, MF couples display significantly reduced activity and normal night time sleep, almost similar to MM or FF couples (Fig. 20), suggesting that CRY-positive, PDF-negative clock

neurons (DNs) govern SSI. However, it is likely that reduced SSI in flies with electrically silenced CRY-positive neurons could be due to silencing of peripheral oscillators harboring CRY and not due to that of core clock neurons in the circadian network.

Overexpression of core clock protein PER in the clock neurons of *Drosophila* results in arrhythmic locomotor activity behaviour (Blanchardon et al., 2001; Yang and Sehgal, 2001), while its constitutive expression in R1-R6 photoreceptor cells represses intracellular *per* mRNA cycling but does not cause any adverse effect on the circadian locomotor activity rhythm of *Drosophila* (Zeng et al., 1994), suggesting that effect of ectopic PER expression is intracellular, and is restricted only to the zone of expression. To elucidate the role of peripheral oscillators located in the olfactory system of *Drosophila*, we expressed PER protein constitutively in the *Or47b* ORNs. This resulted in a significant reduction of night time activity and a considerable recovery of sleep in the MF couples, indicating that disruption of circadian clocks in peripheral *Or47b* oscillators has significant effect on SSI (Fig. 21). To evaluate the role of circadian pacemakers in the fly brain we additionally overexpressed PER in LN_v and LN_d neurons using *Mai179* driver. The MF couples with manipulated core clocks continued to exhibit enhanced night time activity and reduced sleep compared to couples with similar dysfunction in peripheral *Or47b* oscillators (Figs. 22). These results suggest that functional clocks in *Or47b* neurons are necessary for SSI in *Drosophila*. Furthermore, our study suggests that circadian neurotransmitter PDF, and LN_v and LN_d clock neurons are dispensable for SSI. These results are consistent with the findings of a recent study (Hamasaka et al., 2010), which showed that neuropeptide-F-positive LN_d and PDF-positive clock cells are not

necessary for heterosexual interactions in *Drosophila*. During our studies an article by Fujji and Amrein (Fujii and Amrein, 2010) appeared claiming that lateral neurons regulate SSI, a result which is in striking contrast to the finding of our study. The only way we could explain this is that in the Fujii and Amrein (2010) study courtship ratio was used as an estimate for SSI, whereas we use amount of activity and sleep. Moreover, the results of our study are consistent with one of the results in Fujii and Amrein (2010) study, where the authors found no detectable effect of loss of clock function in the PDF-expressing neurons (*pdfGAL4/UASCYC*Δ) on courtship ratio.

It is likely that *Or47b* mediated interactions between males and females modify circadian clocks of couples by altering transcription and hence protein levels of core clock genes in the circadian molecular clockwork (Krupp et al., 2008). Taken together the results on PER overexpression, our study suggests that PDF-positive LN_v, PDF-negative 5^{th} sLN_v, and some of the LN_d neurons do not participate in the regulation of SSI, at least not under LD conditions, however, they may become prominent in DD in terms of sustenance of SSI mediated circadian phenotypes.

In summary, the results of my study suggest that male-female couples of wild type flies display socio-sexual interaction mediated increase in night time activity and sleep loss compared to male-male and female-female couples. This behavior is clearly male driven and involves olfactory means of communication. Among the olfactory receptors, *Or47b* plays a major role. The SSI mediated night time activity in wild type flies free-runs in DD. While the circadian neurotransmitter PDF and functional clocks in LN_v and LN_d neurons are not necessary for its persistence, clocks in the peripheral *Or47b* neurons and DN neurons seem to be critical.

Chapter 7

After-Effects of Socio-Sexual Interactions

7.1 Introduction

In fruit flies *Drosophila melanogaster*, circadian clocks regulate rhythms in behaviour such as locomotor activity, adult emergence, olfaction, mating, and egg laying (Dunlap et al., 2004; Howlader and Sharma, 2006; Allada and Chung, 2010), and in metabolic processes resulting in rhythms in metabolism, concentration of cytosolic Ca^{2+}, cyclic nucleotides, cAMP, phospholipids and kinases (O'Neill et al., 2008; Nakahata et al., 2009; Allada and Chung, 2010). These clocks have also been implicated in the regulation of various cyclic events during reproduction (Ikeda, 1976; Sakai and Ishida, 2001; Beaver et al., 2002; Tauber et al., 2003; Beaver and Giebultowicz, 2004). For example, circadian clocks are found to regulate mating rhythm in *Drosophila* (Ikeda, 1976; Sakai and Ishida, 2001). Mating in *Drosophila* involves a series of well defined behaviors such as chasing, avoiding, dancing, rejection, and copulation, all of which involve intense locomotor activity by both males and females (Hall, 1994). In an elegant study by Fujii and coworkers (Fujii et al. 2007) studied such activity and found that while solitary, sex starved, virgin males and females are day active, heterosexual couples display intense nocturnal activity which involves close proximity interactions between males and females. Unlike same sex (male-male or female-female) couples who are day active and night inactive, heterosexual (male-female) couples are active during the night through to the day and were least active only for a few hours in the evening.

In *Drosophila*, virgin females are usually receptive to males, however, following mating they undergo drastic changes in physiology, which alters their response to male partners, and start rejecting them (Kubli, 2003). This switch in female behavior coincides with egg laying and is believed to be due to accessory gland secretions from successful

males that were transferred to the females during mating (Gillott, 2003; Yapici et al., 2008). Among the cocktail of molecules in the male accessory gland proteins, a 36 amino acid peptide which is known to bind to sex peptide receptor expressed in the female reproductive tract and central nervous system plays a key role in such post mating behavioural changes in *Drosophila* females (Kubli, 2003; Yapici et al., 2008).

Post-mating changes in behavior have also been reported in hymenopterans such as ants, wasps, and honeybees (McCluskey, 1967; Sharma, 2004a; Julian and Gronenberg, 2002; Richard et al., 2007). Unlike ants where mating causes irreversible changes in behavior (McCluskey, 1967), such changes in *D. melanogaster* females are reversible (Kubli, 2003). In honeybees, profiles of certain pheromones and gene expression in the brain changes after mating (Richard et al., 2007). While changes in gene expression is noticed first in the ovaries, those in the brain as seen after some delay (Kocher et al., 2008). In *Drosophila*, male-female couples display night time activity as long as they are housed together (Fujii et al., 2007). To the best of our knowledge long term changes in circadian phenotypes following SSI have never been reported in *Drosophila*. It would therefore be interesting to systematically study the consequence of socio-sexual interactions between males and females on the circadian clocks in flies.

Here I report the results of our study aimed at estimating after-effect of SSI on circadian rhythm phenotypes of in fruit flies *D. melanogaster*. I first maintained males and females in same or opposite sex groups (~30 individuals in each group) under 12:12 hr light/dark (LD) conditions, for a period of either 2, 3, 4, or 5 days, and then separated them to record their locomotor activity behaviour individually under LD and constant dark (DD) conditions. By maintaining flies in groups for different durations I wanted to

find out if changes in circadian rhythm do occur, how many days of SSI are needed to make an appreciable impact. To study the role of circadian clocks in SSI mediated after-effects on activity rhythm under LD cycles, I used flies with loss of function mutation for core clock genes – *period* (*per*^{0}), *timeless* (*tim*^{0}), and *cyc* (*cyc*^{0}), or flies interacting under constant light (LL), or flies with electrically silenced Pigment Dispersing Factor (PDF) neurons (*pdfGAL4/UASdORKC1*) or flies with ablated CRYPTOCHROME (CRY) - positive neurons (*cryGAL4/UASrpr)*. Further, I studied the role of olfaction in SSI mediated after-effects on circadian rhythms. I found significant long lasting after-effects in the circadian locomotor activity rhythm in flies following SSI, which is absent in flies lacking functional circadian clocks, and in flies lacking olfactory ability.

7.2 Materials and Methods

Freshly laid eggs from laboratory reared *Canton-S* (*CS*) populations of *D. melanogaster* were collected in glass vials (95 mm length × 10 mm diameter), and maintained under 12:12 hr LD cycles for the entire period of pre-adult development. Freshly emerged virgin males and females were collected and maintained as same sex group of 30 individuals per vial. After 3-4 days, flies were segregated into three groups (30 individuals in each vial) - male only, female only, and male-female (in 1:1 ratio) groups.

CS flies were maintained under 12:12 hr LD cycles, with light of intensity 80-100 lux during the day and red light of wavelength greater than 650 nm during the night. The flies were maintained under LD cycles in groups of 30 for a period of 2, 3, 4, or 5 days, after which they were separated and the locomotor activity behaviour of males was monitored individually under LD or DD. We had a total of five sets of flies – males who were never exposed to females (controls), males kept with females for two days (2 days

of SSI), three days (3 days of SSI), four days (4 days of SSI), and five days (5 days of SSI). Care was taken to ensure that flies in all treatments were age-matched. Flies were transferred every day at random hours to fresh food vials until the time that they were separated for locomotor activity recording. For recording locomotor activity behaviour, flies were placed individually in locomotor activity tubes (5 mm × 65 mm), with corn food at one end and cotton plug at the other. The tubes were then placed in Drosophila Activity Monitors (DAM), Trikinetics, USA. Data obtained from DAM system was analyzed using CLOCKLAB, Actimetrics, USA (www.actimetrics.com/ ClockLab). First five days of activity data was used for plotting average activity profiles, wherein cumulative activity in one hour bins was normalized by the overall activity of that particular day. We divided data from each group randomly into four subgroups, and the average of these subgroups at each time point was used as replicate for analysis. For analysis of variance (ANOVA) the number of days of SSI, and time points (ZT01-ZT24; where ZT00 refers to time of lights-on in LD cycle) were treated as fixed factors. We found that most differences between experimental and controls flies are seen around lights-on and lights-off. We therefore separately analyzed activity data by taking hourly bins only during light/dark and dark/light transitions – at ZT00, ZT01, ZT12, and ZT13. For ANOVA, the number of days of SSI (controls, 2, 3, 4, and 5 days), and time points (ZT00, ZT01, ZT12 and ZT13) were treated as fixed factors. In addition, we also analyzed total activity during the day and night (excluding activity during 2 hr period flanking lights-on and lights-off transitions) to see if activity levels are altered due to SSI. For ANOVA we have taken cumulative activity during the day and night and the number of days of SSI (controls, 2, 3, 4, and 5 days) as fixed factors. In addition, we estimated

anticipation index relative to lights-on and lights-off using the formula proposed by Stoleru et al. (2004) to study the after-effect of SSI on light/dark anticipatory behaviour.

In the second set of experiments, we followed the same protocol as in the first set, except that in this set activity of only females was analyzed, and flies experienced SSI for a fixed duration of 5 days. Food change was given at random hours every day to avoid larval movement in the tube and physiological effects of eggs in the food medium. While we did record the activity of both males and females, for the purpose of analysis, activity of females alone was taken into account. For ANOVA, social status of females (with or without SSI) and time points (ZT00, ZT01, ZT12 and ZT13) were treated as fixed factors. Cumulative day and night time activity was also analyzed by treating activity and social status (with or without SSI) as fixed factors.

To study the effect of SSI on circadian period, locomotor activity behavior of males was monitored under DD following five days of SSI. The locomotor activity of males maintained under similar set of conditions but in same sex groups was also recorded simultaneously. The activity data thus obtained was analyzed using Lomb Scargle Periodogram of CLOCKLAB, Actimetrics, USA. For ANOVA, we used social status of males (with or without SSI) as fixed factor.

To study the role of circadian clocks in SSI dependent after-effects on locomotor activity rhythm we used flies with loss of function mutation for core clock genes - per^0, cyc^0, and tim^0, or flies with electrically silenced PDF neurons (*pdfGAL4/UASdORKC1*) or flies with ablated CRY-positive neurons (*cryGAL4/UASrpr)*. To study the role of olfaction in SSI related after-effects on activity rhythm we used flies with loss of function mutation for the widely expressed olfactory receptor *Or83b* ($Or83b^0$) or flies with ablated

Or83b neurons (*Or83bGAL4/UASrpr*). In all these studies flies were subjected to 5 days of SSI. For ANOVA we used sex (male or female), social status (with or without SSI), and time points (ZT00, ZT01, ZT12, ZT13) as fixed factors. To further elucidate the role of circadian clocks in SSI related after-effect, we have taken CS flies and subjected them to 5 days of SSI in the presence of LL, wherein wild type flies are known to become arrhythmic, and then separated them for locomotor activity recording.

7.3 Results

7.3.1 ***SSI flies show reduced morning and evening activity peaks:*** To study the long term after-effects of SSI on locomotor activity rhythm we housed 30 flies together under LD cycles, in a sex ratio of 1:1, for 2, 3, 4, or 5 days, following which we recorded locomotor activity of individual males. The males used as controls were also maintained under LD cycles but as same-sex group of 30 individuals per vial. The morning and evening activity peaks of males exposed to SSI is significantly reduced compared to controls (Fig. 1). The evening activity peak takes as few as 3 days of SSI to undergo appreciable reduction ($p < 0.0001$), while reduction in the morning peak is apparent only after 4 days of SSI ($p < 0.01$). After 5 days of SSI, activity of SSI males is higher at ZT20 ($p < 0.05$), and at ZT11 their activity is marginally less than controls ($p = 0.07$), while at ZT01 ($p < 0.005$) and ZT12 ($p < 0.0001$) it is significantly lower than controls. In flies with 5 days of SSI, males are less active than controls at ZT12 ($p < 0.005$) and ZT13 ($p < 0.0001$), and at all other time points activity of SSI males does not differ statistically from controls ($p > 0.05$). The sign of SSI mediated after-effects is first seen in terms of reduction in evening activity peak (ZT13) after 2 days of SSI ($p < 0.05$), which become significantly smaller than controls after 3, 4 or 5 days of SSI ($p < 0.0001$).

Upon visual inspection of the activity profiles we found that activity of experimental and control flies are different mostly around lights-on and lights-off transitions. Hence, we decided to analyze activity data collected around lights-on and lights-off (ZT23-00, ZT00-01, ZT11-12 and ZT12-13) separately. The activity of males at ZT01 ($p < 0.05$) and ZT12 ($p < 0.01$) is significantly lower than controls after 5 days of SSI, while SSI of less than 5 days is not effective in bringing about statistically significant changes in activity ($p > 0.05$). Furthermore, at ZT00 activity of males with and without SSI does not differ ($p > 0.05$), while at ZT13 activity of SSI males was significantly lower than controls after 3 ($p < 0.005$), 4 and 5 ($p < 0.0001$) days of SSI (Figs. 1a, b).

7.3.2 ***SSI has opposite effects on night time activity of males and females:*** We analyzed the fraction of activity during the light and dark phases of LD cycles to assess after-effects of SSI. While day time activity of males does not change with number of days of SSI ($p > 0.05$), their night activity is significantly enhanced after 4 days ($p < 0.01$) or 5 days of SSI ($p < 0.001$) (Fig. 1c). Consequently, there is a gradual decrease in lights-off anticipatory activity with the number of days of SSI, while there is no measurable effect of SSI on anticipation to lights-on (Fig. 1d).Cumulative (12hr) day activity was higher ($p < 0.05$) than cumulative night activity in controls where in other flies groups subjected to SSI there was no significant differences in between two ($p > 0.05$) (Fig. 1e). Since flies respond maximally when they are exposed to 5 days SSI, we decided to use 5 days of SSI for further experiments.

Figure 1

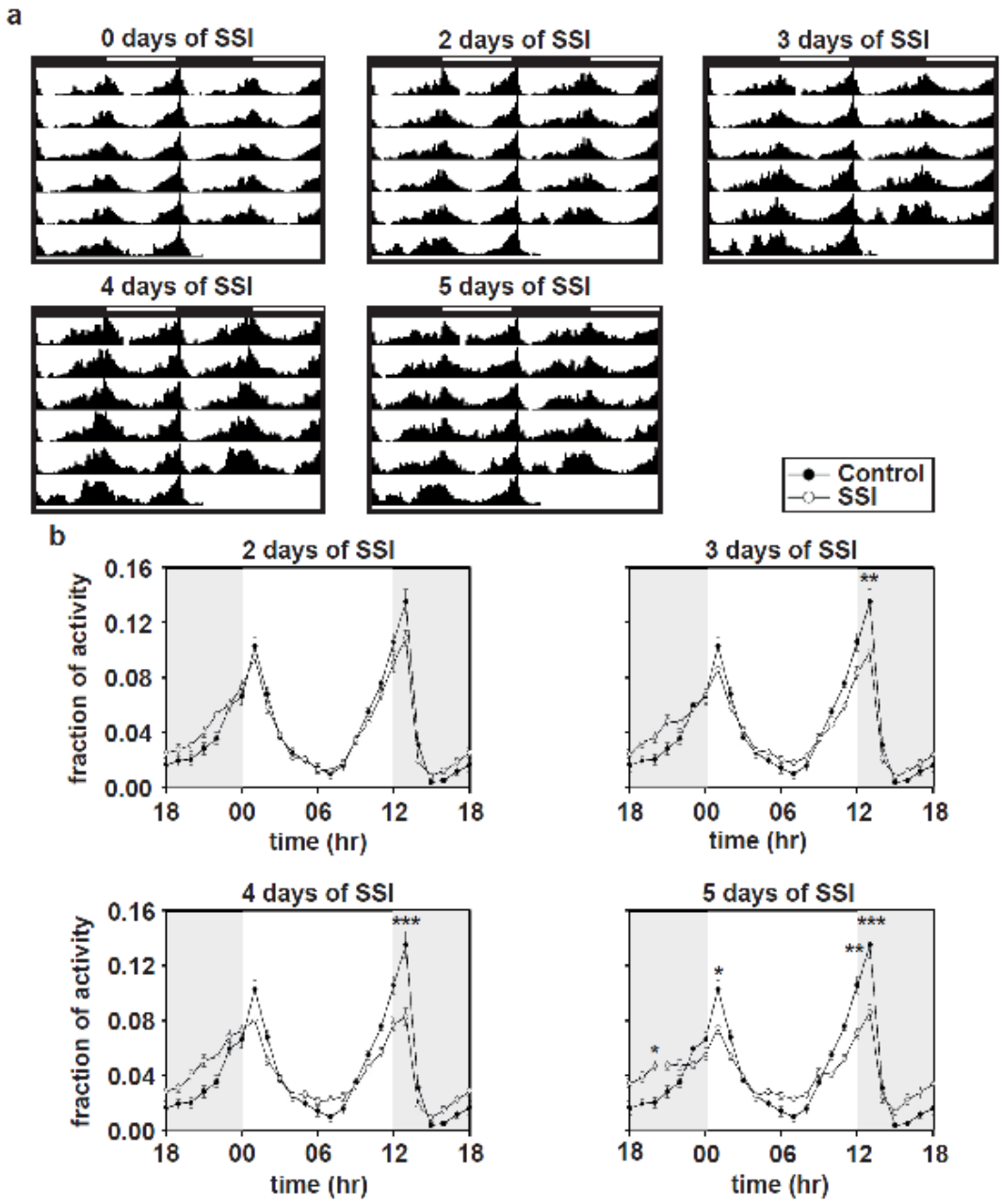


Figure 1 ***SSI results in long lasting after-effects in circadian locomotor activity rhythm.*** (a) Average activity rest graphs (actograms) of males following 0 (control), 2, 3, 4, or 5 days of SSI. White bars above each actogram indicate 12 h of light while black bars indicates 12 h of darkness. Actograms are double plotted for easier visualization. Locomotor activity level (averaged across replicate flies) for each day is shown as black spikes on each line and data from subsequent days is plotted one below the other. Hence day is plotted on the y axis while x axis is time over a period of 48 hours. (b) Activity profiles of males following 2-5 days of SSI shows that after 2 days of SSI activity at ZT13 is reduced compared to controls although the reduction is not statistically significant. After 3 days of SSI activity at ZT13 is significantly reduced compared to controls ($p < 0.005$), and the same is true after 4 days of SSI ($p < 0.0001$). Following 5 days of SSI activity of males decreased significantly compared to controls both after lights-on at ZT01 ($p < 0.05$), and lights-off, ZT12 ($p < 0.01$), and ZT13 ($p < 0.0001$).

Figure 1

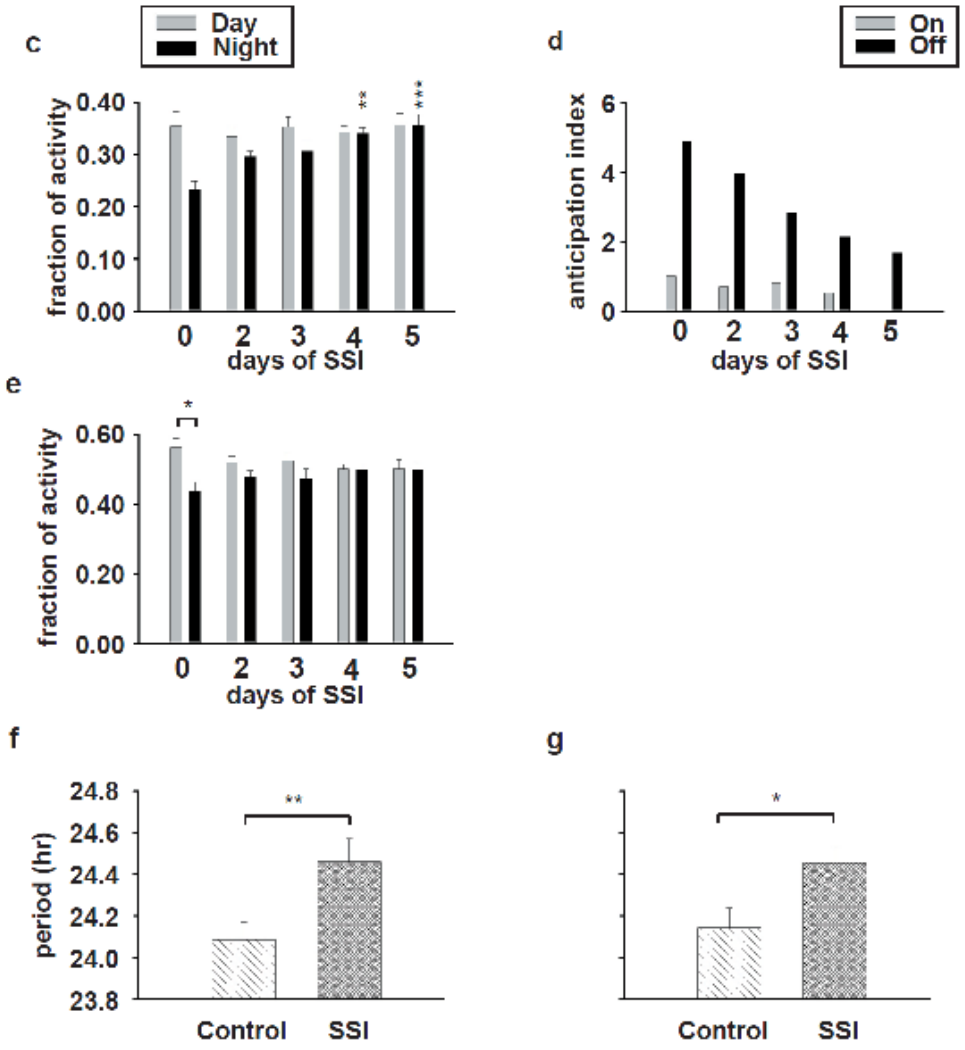


Figure 1 ***SSI results in long lasting after-effects in circadian locomotor activity rhythm.*** (c)Night time activity (10 hour activity excluding those during lights-on and off transition) in males increases as a function of number of days of SSI, and become significantly greater than the controls after 4 ($p > 0.01$) or 5 days of SSI ($p > 0.001$), while their day time activity remained unchanged ($p > 0.05$). (d) Anticipation to lights-off is reduced compared to controls (no SSI) following 2, 3, 4, or 5 days of SSI. (e) In control males, absolute day time activity (inclusive of activity during lights-on and off transition) is significantly greater than night time activity ($p < 0.05$), while in SSI males day and night activity are not different ($p > 0.05$) (f) Circadian period of males is lengthened following 5 days of SSI, recorded either immediately after SSI ($p < 0.01$). (g). This increase in period persists even after being housed in groups with only males under LD for 8 days after SSI ($p < 0.05$).

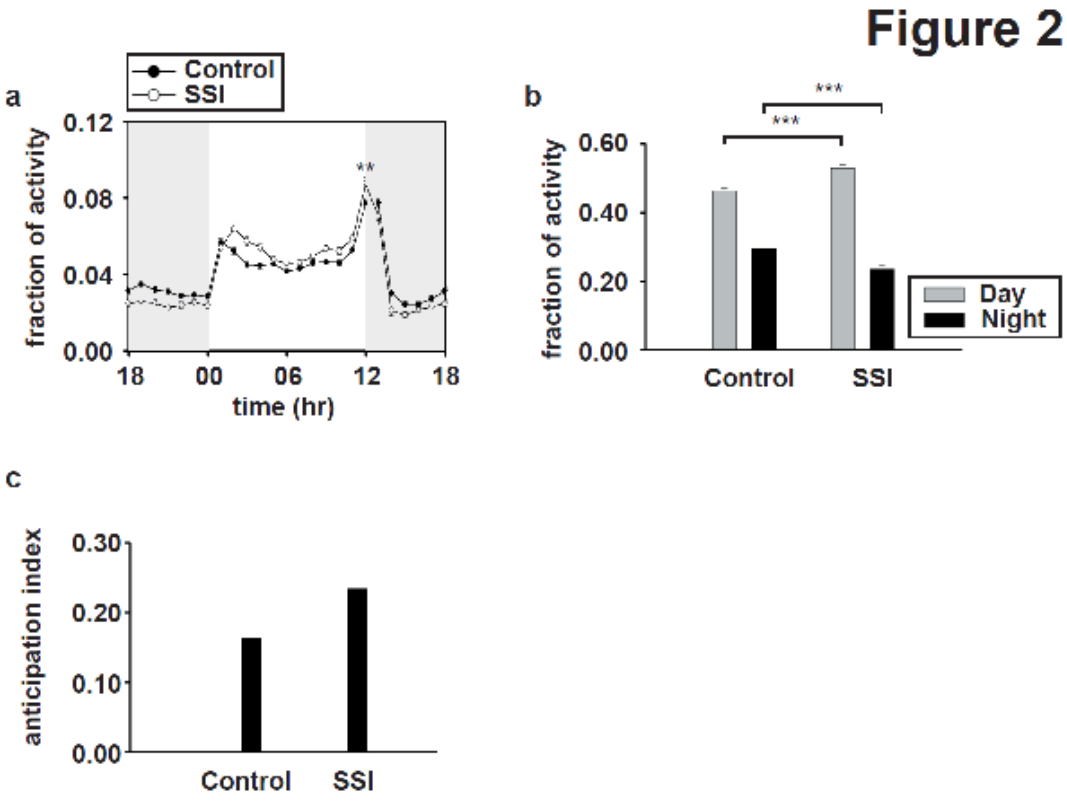


Figure 2 ***SSI results in after-effects in circadian locomotor activity rhythm in females.*** (a) Following 5 days of SSI, activity in females is significantly enhanced at ZT12 compared to controls ($p < 0.01$), and (b) day time activity is higher ($p < 0.0001$), and night time activity lower than controls ($p < 0.0005$). (c). Anticipation to lights-off is increased in females compared to controls.

In a separate experiment we exposed one batch of females to males for 5 days under LD cycles, following which their locomotor activity behaviour was monitored individually. The evening activity peak (ZT12) of females is significantly increased compared to controls ($p < 0.01$), whereas at other time points (ZT00, ZT01, ZT13) their activity does not differ from controls ($p > 0.05$) (Fig. 2a,) associated with increase in anticipation to the lights off (Fig. 2c). SSI females also showed increase in day time activity ($p < 0.0001$) and decrease in night time activity ($p < 0.0005$) (Fig. 2b).

7.3.3 ***SSI causes lengthening of circadian period in males:*** To examine whether SSI has any effect on the endogenous circadian period we exposed males to females for 5 days under LD cycles, after which locomotor activity behaviour of males was recorded individually under DD. The circadian period of activity rhythm of SSI males was significantly longer than controls ($p < 0.01$) (Fig. 1f). In a separate experiment we examined whether lengthening of circadian period could be sustained if SSI is followed by social interactions among members of the same sex before testing them in DD. For this males were first kept together with females under LD cycles for 5 days and then segregated into same sex groups for 7-8 days, following which locomotor activity of males was recorded in DD. We found that SSI males have significantly longer circadian period compared to controls ($p < 0.05$) (Fig. 1g).

7.3.4 ***SSI mediated after-effects are clock dependent:*** To examine the role of circadian clocks in SSI mediated after-effects on activity behaviour we used flies with loss-of-function mutations in core clock genes and exposed them to 5 days of SSI, and then monitored their locomotor activity behaviour under LD cycles. Flies with loss-of-function mutation for *period* (*per*), *timeless* (*tim*), and *cycle* (*cyc*) genes do not show SSI

mediated after-effects, suggesting that functional circadian clocks are necessary for the expression of SSI mediated after-effects in *Drosophila* (Figs. 3a-f).

The activity of per^0 males and females with and without SSI does not differ statistically at any time point tested ($p > 0.05$) (Fig. 3a). The cumulative day and night time activity of flies with and without SSI does not differ ($p > 0.05$) (Fig. 3b). At all the four time points tested activity of cyc^0 males with SSI does not differ statistically from controls ($p > 0.05$) (Fig. 3c). In females, however, activity of flies with SSI is significantly higher than controls at ZT01 only ($p < 0.005$), whereas at all other time points it does not differ from controls ($p > 0.05$) (Fig. 3c). The cumulative day and night time activity of males and females with and without SSI do not differ (Fig. 3d). The activity of tim^0 flies with and without SSI does not differ ($p > 0.05$) (Fig. 3e). The cumulative day and night time activity of tim^0 flies with and without SSI does not differ ($p > 0.05$) (Fig. 3f). These results suggest that functional circadian clock is required for SSI mediated after-effects.

7.3.5 ***Functional clocks are essential for SSI related after-effects:*** Since mutants of core clock genes may be impaired in pathways independent of circadian clock function we used constant light (LL) to disrupt circadian rhythm (Konopka et al., 1989). Flies were exposed to LL as male-only, female-only, and male-female (1:1) groups for 5 days after which their locomotor activity behavior was monitored individually under LD cycles. The activity profiles, day and night time activity of SSI males and females are remarkably similar to controls (Figs. 4a, b). A separate set of males and females were first exposed to 5 days of SSI under LL, and then males were separated and placed

Figure 3

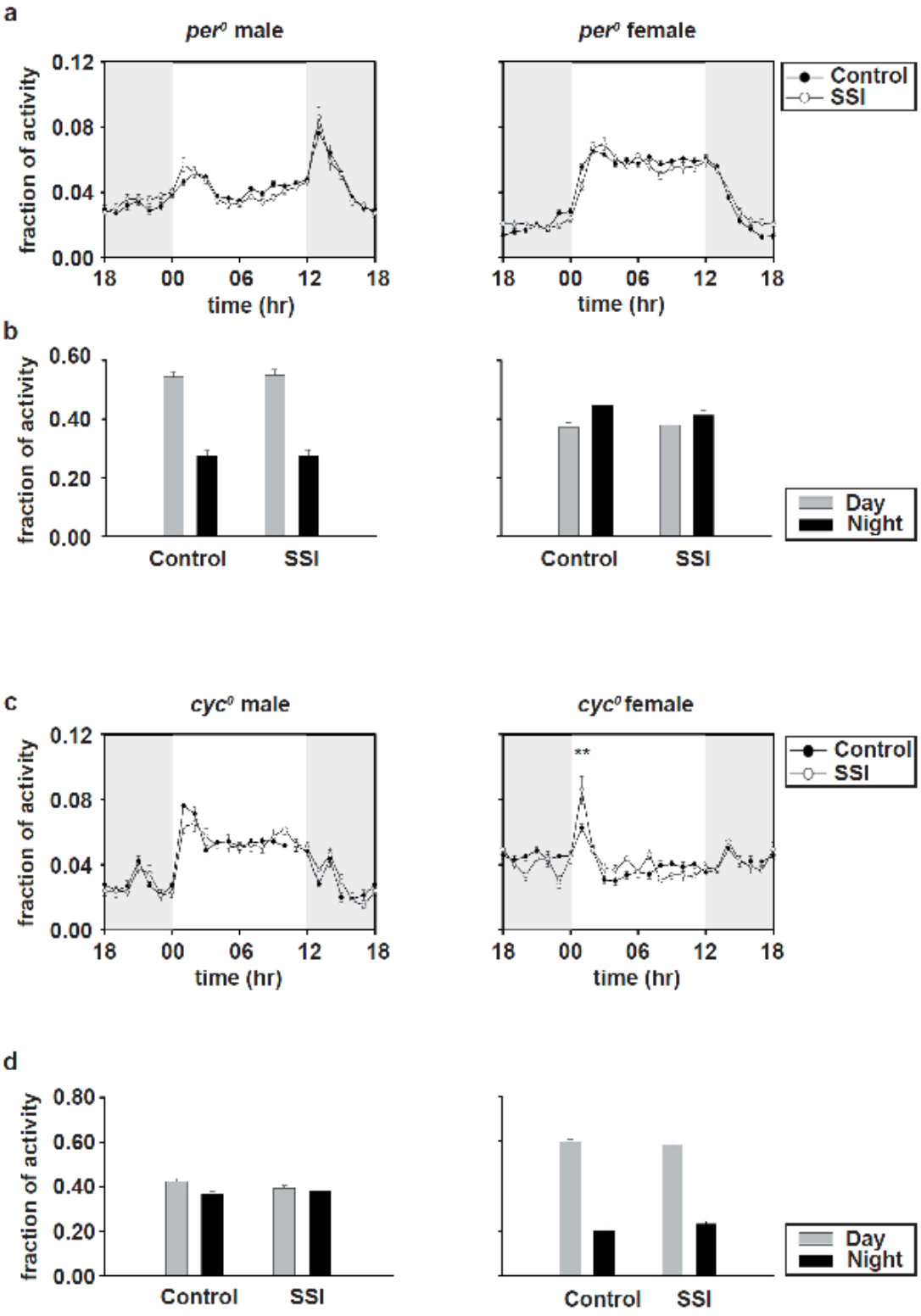

a
per⁰ male
per⁰ female
fraction of activity
0.12
0.08
0.04
0.00
18
00
06
12
18
time (hr)
Control
SSI
b
fraction of activity
0.60
0.40
0.20
0.00
Control
SSI
Day
Night
c
cyc⁰ male
cyc⁰ female
**
d

Figure 3

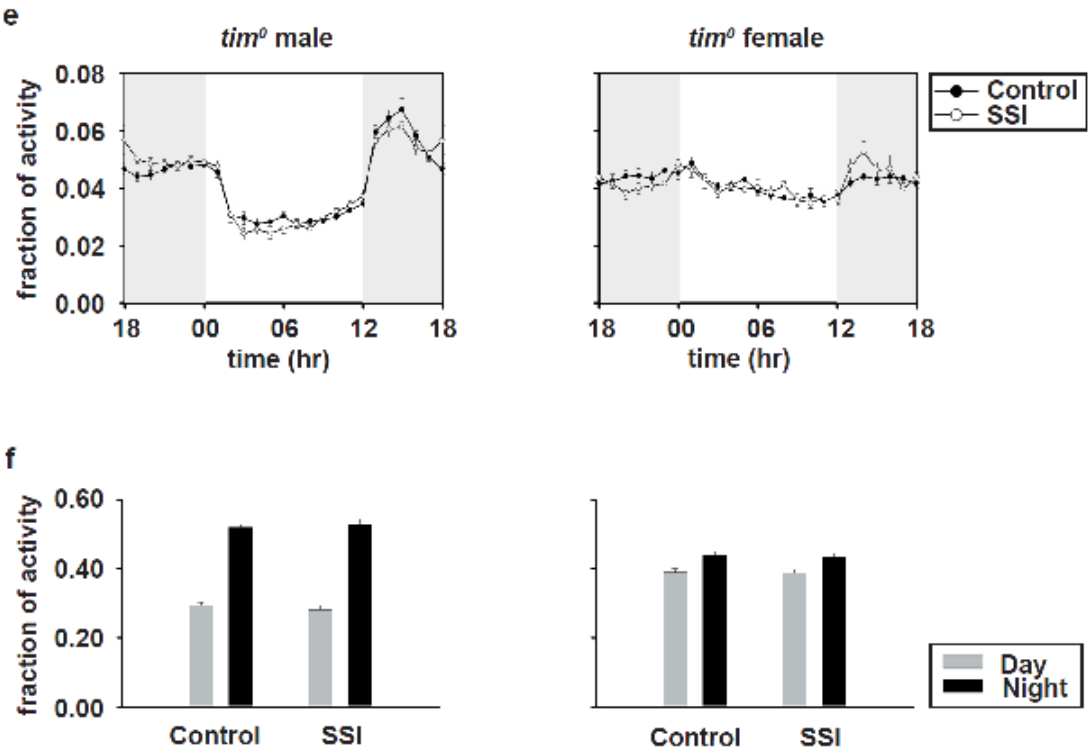


Figure 3 ***Clock mutants do not display SSI related after-effects in circadian phenotypes.*** Activity profile of, *per*^{0} (a), *cyc*^{0} (c) and *tim*^{0} (e) flies with SSI is similar to controls, except for *cyc*^{0} females at ZT01. Cumulative day and night time activity of *per*^{0}, *tim*^{0} and *cyc*^{0} males and females (b, d, f) following SSI is similar to controls.

Figure 4

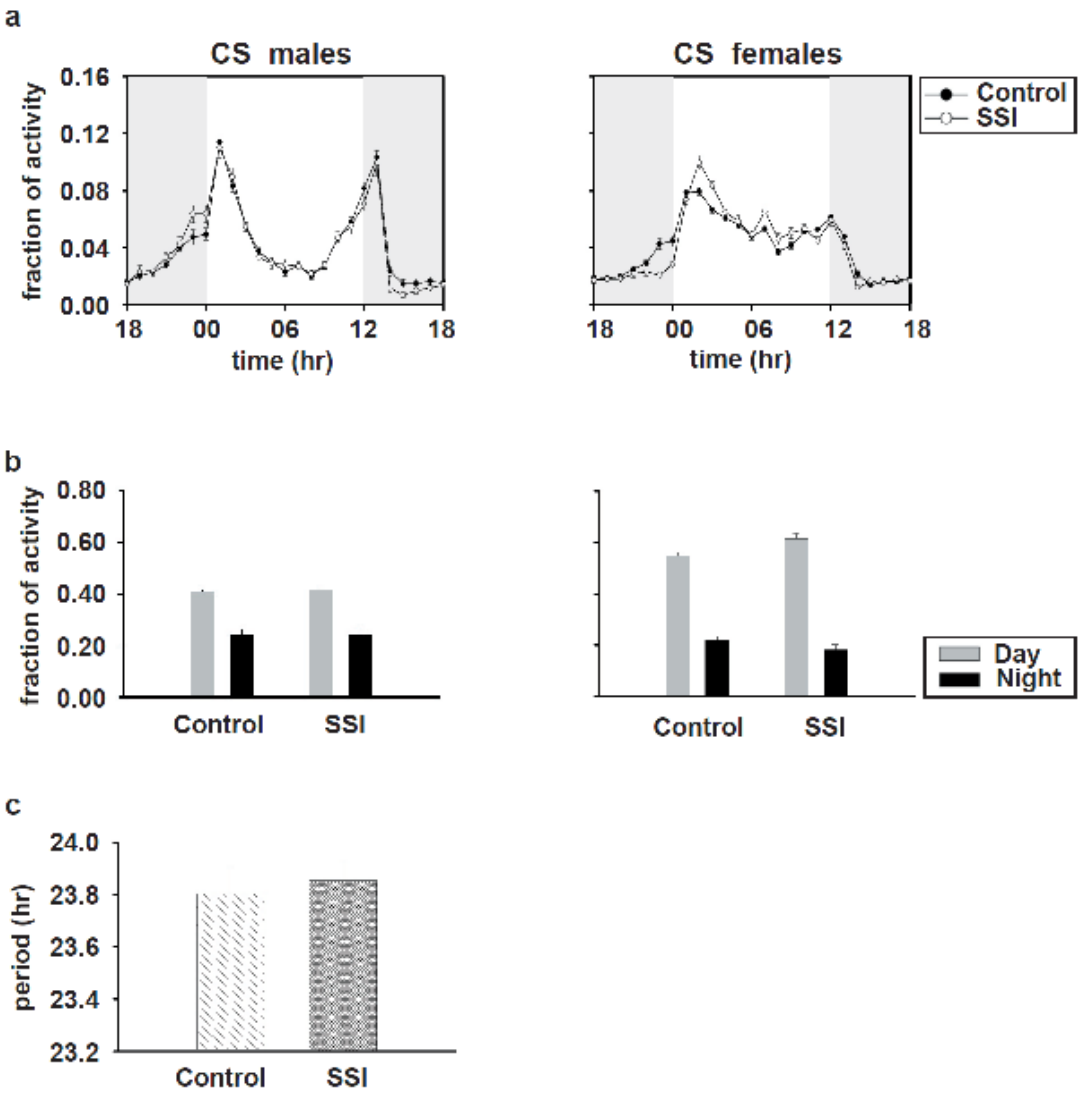


Figure 4 ***Functional circadian clock is necessary for SSI related after-effects.*** Activity profiles show that circadian phenotypes of males and females following SSI under constant light (LL) are similar to controls ($p > 0.05$) (a,). Cumulative activity of males and females (b) following SSI in LL is similar to controls ($p > 0.05$). The circadian period of SSI males is similar to controls ($p = 0.43$) (3c).

individually in DD for monitoring after-effect of SSI on locomotor activity behaviour. The circadian period of SSI males does not differ statistically from controls ($p = 0.43$) (Fig. 4c), suggesting that functional circadian clock is required for SSI mediated after-effects.

7.3.6 ***Flies with electrically silenced PDF-positive clock neurons show partial SSI dependent after-effects:*** To study the role of PDF-positive clock neurons in SSI mediated after-effects on locomotor activity rhythm we used flies with electrically silenced PDF-positive clock neurons. Flies were maintained as male-only, female-only, and male-female (1:1) groups for 5 days, after which their locomotor activity behavior was monitored individually under LD cycles. Males with electrically silenced PDF-positive neurons show significantly reduced evening activity peak after being exposed to females ($p < 0.001$), while their morning activity peak remains unchanged ($p > 0.05$) (Fig. 5a). SSI females, on the other hand did not show any change in activity and their activity profiles are similar to controls ($p > 0.05$) (Fig. 5a). While cumulative day and night time activity of SSI males do not differ from controls ($p > 0.05$; Fig. 5b), those of SSI females are significantly different (Fig. 5b). The cumulative day time activity of females is significantly reduced compared to controls ($p < 0.05$), while night time activity is increased compared to controls ($p < 0.01$) (Fig. 5b). This suggests that PDF-positive neurons mediating the SSI mediated after-effects on the morning activity peak.

7.3.7 ***SSI related after-effects are absent in flies with ablated CRY-positive neurons:*** To study the role of CRY-positive clock neurons in the expression of SSI mediated after-effects on locomotor activity rhythm we used flies in which CRY-positive clock neurons were ablated. Flies were maintained as male-only, female-only, and male-female (1:1)

Figure 5

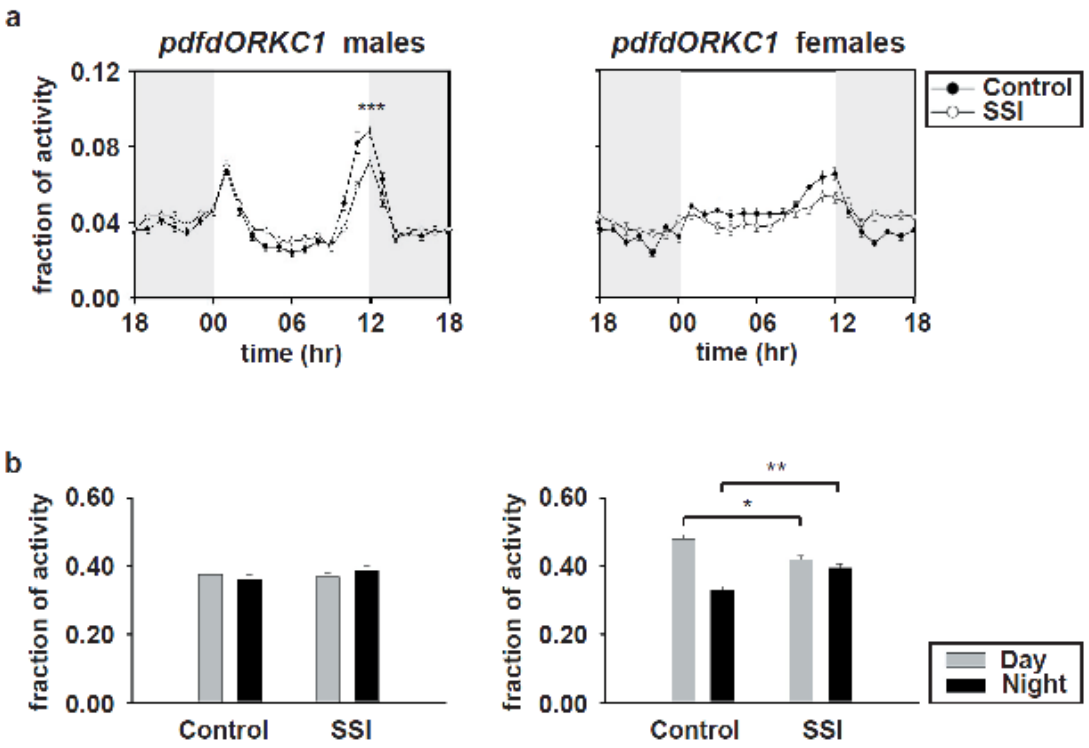


Figure 4 ***PDF-positive neurons are not necessary for evening peak.*** Activity profile of electrically silenced flies (*pdfGAL4/UASDorkC1*) show that activity of males and females (a) with SSI is similar to the controls, except at ZT12, when males with SSI were less activity compared to controls ($p < 0.001$). Cumulative activity of males with SSI is comparable to controls, while in females with SSI activity is significantly higher during night ($p < 0.01$), and lower during day ($p < 0.05$) than controls (b).

Figure 6

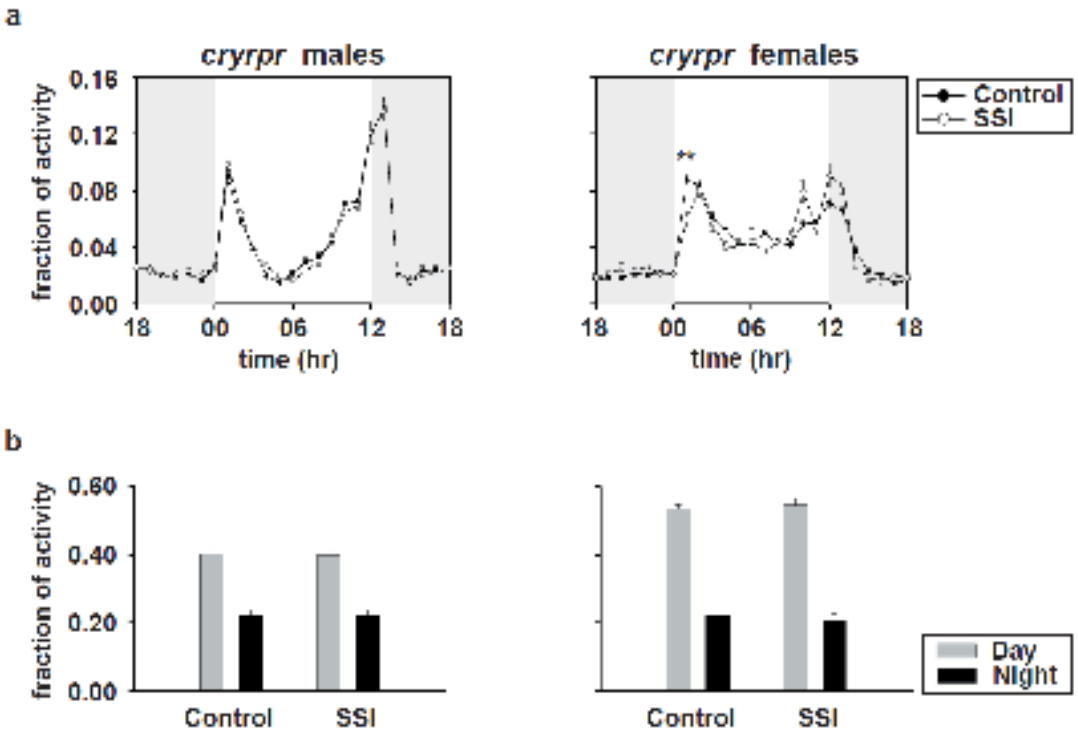


Figure 5 ***Flies lacking CRY-positive neurons do not display SSI related after-effects.*** The activity profiles of *cryGAL4/UASrpr* flies indicating that activity of males and females (a) with SSI is similar to controls ($p > 0.05$), except at ZT01 where females with SSI are less active compared to controls ($p > 0.01$). The day time and night time activity of males and females with SSI is similar to controls ($p > 0.05$) (b).

Figure 7

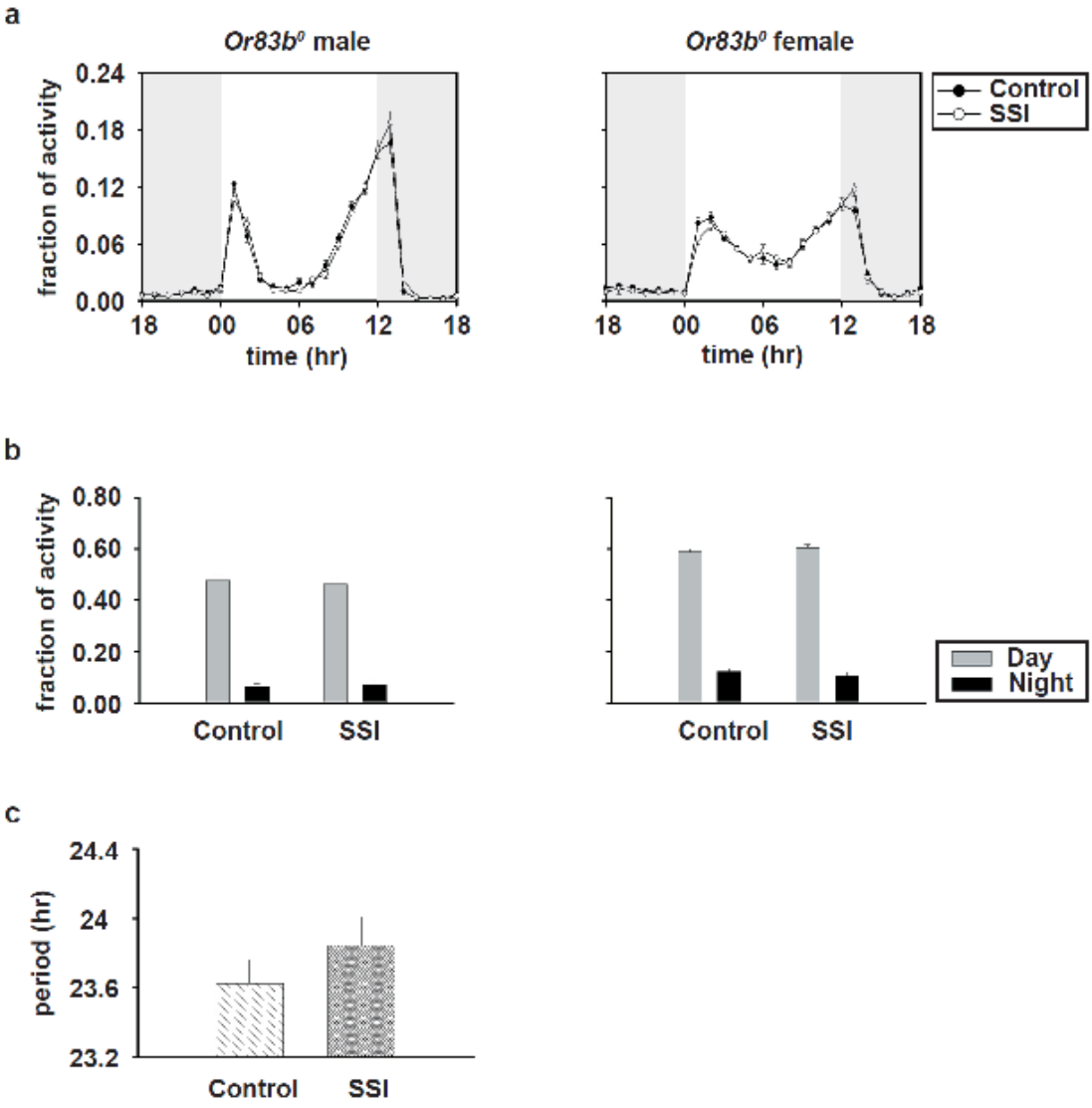


Figure 6 ***SSI is olfaction mediated.*** Activity profile of *Or83b*^{0} flies show that activity of males and females (a) with SSI is similar to controls ($p > 0.05$). The day and night time activity of males and females (b) with SSI is similar to controls ($p > 0.05$). Circadian period of SSI males is similar to control ($p = 0.31$)

groups for 5 days after which their locomotor activity behavior was monitored individually under 12:12 hr LD cycles. The profile of activity, day and night time activity levels of SSI males and females lacking CRY-positive neurons do not differ from controls at most time points ($p > 0.05$) except at ZT01, where SSI females have significantly lower activity compared to controls ($p < 0.01$) (Figs. 6a). Day and night time activity of flies with and without SSI do not differ statistically ($p > 0.05$) (Figs. 6b).

7.3.8 ***SSI related after-effects are olfaction mediated:*** To study the role of olfaction on SSI related long lasting changes in circadian rhythm we examined the effect of SSI in null mutant flies for the gene *Or83b* (*Or83b*^{0}), known to have compromised olfactory ability. In addition, we took flies whose *Or83b* receptor neurons were ablated by expressing apoptotic gene *reaper* (*rpr*) using a widely expressed driver *Or83bGAL4* (*Or83b*$^{-}$). Flies were maintained as male-only, female-only, and male-female (1:1) groups for 5 days after which their locomotor activity behavior was monitored individually under LD cycles. Males and females lacking olfactory ability do not show SSI mediated after-effects (Fig. 7, 8). The activity profile, day and night time activity levels of *Or83b*^{0} and *Or83b*$^{-}$ SSI males and females do not differ from controls ($p > 0.05$) (Figs. 7a,b and 8a,b); however, activity at ZT01 decreased and day time activity of females is increased in the ablated flies ($p < 0.05$). A separate batch of males was subjected to SSI under LD cycles and then transferred to DD for the estimation of circadian period of locomotor activity rhythm. The circadian period of SSI *Or83b*^{0}males does not differ statistically from controls ($p > 0.05$) (Fig. 7c).

Figure 8

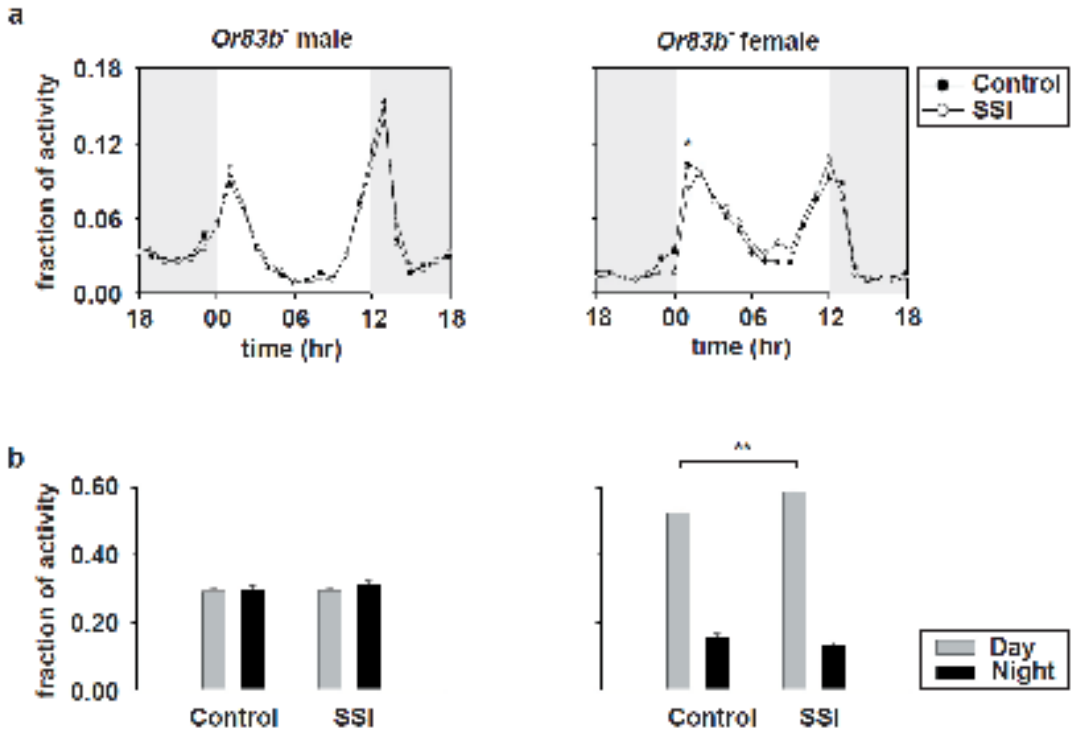


Figure 8 ***SSI is olfaction mediated.*** Activity profiles of *Or83b ablated* (*Or83b*$^{-}$) flies show that activity of SSI males and females (a) is similar to controls ($p > 0.05$), except at ZT01 where activity of females with SSI is lower than controls ($p < 0.05$). The day and night time activity of males with SSI is comparable to controls ($p > 0.05$). The day time activity of SSI females is higher than controls ($p < 0.005$), whereas their night time activity is similar to controls ($p > 0.05$) (b).

7.4 Discussion

Both males and females display after-effects of socio-sexual interactions (SSI); morning and evening activity peaks in males are reduced, and night time activity increased as a consequence of SSI (Fig. 1a,b). In females, evening activity peak and day time activity increases, but their night time activity is decreased (Figs. 2a, b). SSI males show lesser anticipation to lights-off, however, their light-on anticipation remains unaltered. Furthermore in SSI males, circadian period of activity rhythm is lengthened, suggesting that socio-sexual interactions have long lasting after-effects on circadian clocks of *Drosophila*. SSI mediated changes in locomotor activity behaviour is clock dependent, because flies with loss of function mutation in core clock genes (per^o, tim^o, and cyc^o), or wild type flies when made to interact under arrhythmicity inducing LL condition (Konopka et al., 1989), do not show such changes, suggesting that functional circadian clock is necessary for the expression of SSI mediated after-effects. This is further corroborated by the finding that flies with electrically silenced PDF-positive clock neurons, or those lacking CRY-positive clock neurons display partial or no SSI mediated after-effects. Expression of such after-effects requires functional olfactory system because blocking olfactory ability either by loss of function mutation in *Or83b* gene ($Or83b^0$) or by ablating olfactory neurons, completely abolishes SSI dependent after-effect on circadian activity rhythm.

When wild type males and females were allowed to interact in groups for 5 days, flies exhibit altered circadian phenotypes, quite similar to the scenario when males and females are allowed to interact in one-on-one (pair-wise) situation, suggesting that SSI operates in small as well as bigger groups (Fujii et al., 2007; Fujii and Amrein, 2010;

Hamasaka et al., 2010). Changes in circadian phenotype can be seen in as few as 2 days of SSI, suggesting that SSI mediated after-effects are rapid.

Our study is unique in two ways; firstly groups of flies were used which reveals how SSI would occur in nature where normally flies are found in large groups and courtship is not a one-on-one affair. Secondly, we find that after-effects on circadian rhythm persist under both LD and DD conditions for at least 10 days, much after socio-sexual interactions are concluded. This is contradictory to the findings of a previous study where such changes in circadian rhythms were reported to disappear as soon as males and females are separated from each other (Fujii et al., 2007).

Activity and its direct correlate sleep is known to be affected by many factors including the presence of predators (Allison and Cicchetti, 1976). Therefore, SSI mediated decrease in day time activity is likely to help males in escaping predators (Isaac et al., 2009). Increased night time activity would be advantageous in providing enough opportunity for males to court females. Increase in day time activity in females would aid enhanced post-mating food requirement, which in turn is known to be positively correlated to the number of eggs laid (Barnes et al., 2008), and decrease in night time activity would help in nocturnal egg laying behaviour. Changes in female activity profiles and their approach towards males are thought to be due to the transfer of sex peptides by the males during copulation, as shown a study where that RNAi against sex peptide receptors results in virgin like behaviour by females with no change in day time activity after mating (Isaac et al., 2009). Thus, changes in activity of males and females, in opposite directions are likely to help them in different ways.

The role of circadian clocks in SSI mediated changes in circadian rhythms is evident from the fact that flies with loss of function mutation for core clock genes (*per*^{o}, *tim*^{o} and *cyc*^{o}) do not display such after-effects (Fig. 3a-f). Interestingly, even wild type CS males do not display SSI dependent after-effects when allowed to interact with females under LL (Figs. 4a-c), wherein majority of their locomotor activity rhythm and underlying molecular clockwork is abolished. Given that circadian clocks regulate many behaviour related to reproduction (Sakai and Ishida, 2001; Beaver et al., 2002; Beaver et al., 2003; Beaver and Giebultowicz, 2004; Howlader and Sharma, 2006; Fujii et al., 2007), and core clock genes are critical for the release of sperms (Beaver et al., 2002) and for the formation of mature oocytes (Beaver et al., 2003), it is not surprising that SSI dependent after-effects are clock-controlled.

The results of our study suggest that blocking olfactory ability in socially interacting males and females completely eliminates SSI mediated after-effects on circadian rhythm both under entrained (LD) as well as free-running (DD) conditions (Figs. 7,8), suggesting that flies use olfactory signals for communications during SSI. This is consistent with the findings of previous studies which suggests that olfaction mediates effects of social interactions on circadian rhythms in *D. melanogaster* (Levine et al., 2002; Fujii et al., 2007; Krupp et al., 2008).

Most organisms including fruit flies *D. melanogaster* show bimodal locomotor activity pattern under 12:12 hr LD cycles, wherein activity is confined to the two twilights (Dunlap et al., 2004). The morning activity peak is believed to be governed by PDF-positive LNv neurons, and evening activity peak by PDF-negative 5th sLNv and LNd neurons (Grima et al., 2004; Stoleru et al., 2004). In addition, there are a multitude

of peripheral oscillators involved in the regulation of behavioural and physiological responses, which either independently (Tanoue et al., 2004) or in consultation with central pacemakers regulate rhythmic phenomenon (Giebultowicz, 2000; Myers et al., 2003; Xu et al., 2008). In flies with electrically silenced LNv neurons, which is known to cause arrhythmicity in locomotor activity behaviour under DD (Nitabach et al., 2002), SSI affects only the morning activity peak (Fig. 5a), while the evening peak continues to display after-effects characteristic of SSI flies (Figs. 5a, b), which is consistent with the view that LNv neurons have a major influence on the timing of morning activity peak. Interestingly, SSI mediated after-effects are first seen in the evening peak, and then in the morning peak, but only after some delay (Figs. 1b, d), suggesting that it is the evening activity peak which mimics the true state of the circadian oscillator and brings about changes in the morning peak in due course of time. However, flies lacking CRY-positive clock neurons exhibit significantly reduced SSI mediated after-effects (Figs. 6a, b), which suggests that CRY-positive, PDF-negative neurons govern SSI dependent after-effects in *Drosophila*. Since CRY is also expressed in the antennal neurons involved in olfactory communication (Krishnan et al., 2005), ablation of these neurons could also interfere in the process of SSI, and may result in loss of SSI phenotypes. Previously it was shown that social interactions result in decrease in the levels of clock gene transcription in oenocytes and fly head (Krupp et al., 2008), and such interactions occur through pheromones perceived by the olfactory organs. Therefore, it is likely that social signals via olfactory circuit reach CRY-positive neurons, which in turn modify activity profiles either directly or by manipulating PDF-positive neurons via PDF receptors.

In summary, socio-sexual interactions in fruit flies *D. melanogaster* results in long lasting changes in circadian clocks of both males and females. In males, SSI decreases both morning and evening activity peaks, increases night time activity, and lengthens circadian period. In females, it results in increased evening activity peak and day time activity, and decreased night time activity. Functional circadian clocks are necessary for the expression of SSI mediated after-effects because flies with compromised circadian rhythms do not display the phenotype. To the best of my knowledge this is the first report of its kind demonstrating after-affects of socio-sexual interactions on circadian clocks in fruit flies *D. melanogaster*, and the role of functional clocks and olfaction.

Chapter 8

Part 1

Exposure to Light Enhances Pre-Adult Fitness in Ants

8.1.1 Introduction

Circadian clocks maximize performance of a wide range of organisms by scheduling rhythmic behaviours at appropriate time of the day. These clocks help in anticipating rhythmic changes in environments (Pittendrigh, 1993; Wong et al., 1995; DeCoursey et al., 1997), in preparing for events such as migration and reproduction, and in maintaining harmony between behavioural and metabolic cycles (Hurd and Ralph, 1998). Any mismatch between external and internal timings proves to be detrimental for the organism (Pittendrigh and Minis, 1972; Von Saint-Paul and Aschoff, 1978). Malfunctioning circadian clocks have also been shown to have considerable effect on respiration, behavior, and rate of aging (Felkai et al., 1999), and have been shown to cause severe mental disorders and physical discomfort in humans (Healy and Waterhouse, 1990).

While we know a great deal about the structure and function of circadian clocks in a fairly wide range of organisms (Dunlap et al., 2004) , their role in the regulation of life history traits such as pre-adult development time and adult lifespan still remains a mystery. It is generally believed that faster clocks speed-up development and shorten lifespan, and slower clocks slow-down development and lengthen lifespan (Kyriacou et al., 1990; Shimizu et al., 1997; Felkai et al., 1999; Kumar et al., 2006). In fruit fly *Drosophila melanogaster* (Kyriacou et al., 1990; Kumar et al., 2006), melon flies *Bactrocera cucurbitae* (Shimizu et al., 1997), and nematode *Caenorhabditis elegans*

(Wong et al., 1995), circadian clocks have been shown to regulate pre-adult development time.

Environmental light/dark conditions have been shown to have considerable effect on pre-adult development time in *Drosophila*; development is faster in LL, followed by 12:12 hr LD, and then DD (Kyriacou et al., 1990; Saunders, 2002; Paranjpe et al., 2005). Most previous studies (Kyriacou et al., 1990; Sheeba et al., 1999; Paranjpe et al., 2005) on the effect of light regimes on developmental duration have been carried out in insects with relatively short development time, where light-mediated effects are not large enough to draw any meaningful conclusion.

Apart from pre-adult development time, egg-viability is also altered by environmental LD conditions (Saunders, 2002). For example, in the intestinal fluke of waterfowls *Echinostoma caproni*, it has been observed that eggs kept in darkness for up to 56 days do not hatch unless they are exposed to light (Jeyarasasingam et al., 1972), and the frequency of egg-hatching increased with increasing light intensity (Markum and Nollen, 1996). In the maple aphid *Periphyllus californiensis*, eggs hatch much faster under shady sites than under sunny sites, because buds which the aphid feeds upon open earlier in shady areas than sunny areas (Wang, 2006). In katydid *Metrioptera hime*, eggs hatch at higher rate under 12:12 hr LD cycle compared to LL or DD (Tetsuo, 1998). This suggests that the extent and rate of egg-hatching depends upon environmental LD conditions, although the precise nature of such dependency may vary from species to species.

In *Camponotus* ants, pre-adult development time varies from species to species (Hölldobler and Wilson, 1990); under natural conditions it ranges between one to three

months, except in winters when pre-adult development is put on complete hold, particularly in boreal species (species living in Arctic and sub-Antarctic ecosystems). Some species of ants (*Formica polyctena*) also have the ability to raise their nest temperature using heat produced by metabolic activities and by exposing the nests to direct sunlight. This enables them to complete development within five to six weeks and get ready for nuptial flights. The *Camponotus* ants, on the other hand, are not known to use any exclusive thermoregulatory mechanism to regulate development, and therefore their larvae remain inside the nest throughout the winter (Hölldobler and Wilson, 1990). This indicates that *Camponotus* ants use a different strategy to regulate their pre-adult development.

I assayed pre-adult fitness components such as development time and egg-viability, in two dark-dwelling sympatric species of ants (the night active *C. compressus* and day active *C. paria*), under three different light regimes (LL, 12:12 hr LD, and DD), to study the effect of light regimes on fitness, and to evaluate whether developmental response to environmental light/dark conditions is altered in these ants, in the course of resource partitioning and sympatric speciation. The results provide interesting insights into light-dependent regulation of pre-adult fitness in ants.

8.1.2 Materials and Methods

Ground-dwelling ants are genetically programmed to dig deep into the soil to construct safe nests, where their progenies complete development (Hölldobler and Wilson, 1990). The carpenter ant *C. compressus* forms its nest in forests, usually inside decomposed plants, and have evolved mutualism with aphids and coccids (Hölldobler and Wilson, 1990). The *C. compressus* and its sympatric partner *C. paria* undertake mating flights in

the months of April-May-June. After mating they dig deep into the soil to form their nests. The colonies of *C. paria* are found more than one and half feet deep under the soil, while those of *C. compressus* are located further deeper (*SRL and VKS, personal observation*).

Mated queens were collected from the Jawaharlal Nehru Centre for Advanced Scientific Research Bangalore campus (12° 58' N, 77° 40' E), soon after their mating flights, while they were landing on the ground. These ants were immediately transferred into petri plates (diameter × height = 90 mm x 15 mm), and were introduced into LL, 12:12 hr LD and DD regimes. These ants were labeled as CCLL, CCLD, CCDD, CPLL, CPLD, and CPDD, where CC and CP denote the species names, and suffix indicate the assay light regimes. The *C. compressus* queens are black in colour, 1.65 ± 0.07 cm (mean ± SD) long (head to back) and 0.39 ± 0.01 cm wide, while *C. paria* queens have yellowish-greens bands on their abdomen and are slightly smaller in size (length: 1.31 ± 0.02 cm and width: 0.36 ± 0.04 cm).

White fluorescent light of 500 ± 20 lux intensity was used in LL, and during the light phase of LD, and dim red light ($\lambda > 640$ nm) was used under DD and during the dark phase of LD. Humidity (~75%) and temperature was kept constant by keeping ants in temperature controlled incubators (25 ± 1 ^{o}C). Food in the form Bhatkar diet (Bhatkar and Whitcomb 1970) and dilute honey solution was provided *ad libitum*. Food was changed on the same day for all colonies. Observations were made with utmost care to avoid any disturbance to queens and to developing larvae. The numbers of queens and future colonies used in the first set of experiments were CCLL (11), CCLD (18), CCDD (15), CPLL (11), CPLD (8), and CPDD (10). Eggs were counted regularly using

magnifying glass and fine brush until the numbers in a given batch became constant. Percentage viability was calculated as the percentage of eggs that successfully hatched as larvae, while those eggs that disappeared were treated as nonviable (trophic) eggs.

The mean initiation time of 1st, 2nd, and 3rd instar larvae, and of pupal and adult stages was used for the estimation of development time. First, second and third instar larvae of *C. compressus* are 1.05-1.35 mm (min-max), 1.40-2.0 mm, and 2.0-5.0 mm long, and 0.50-0.65 mm, 0.55-0.75 mm, and 0.85-1.40 mm wide, while those of *C. paria* are 1.0-1.30 mm, 1.35-2.0 mm, and 2.0-4.5 mm long, and 0.5-0.6 mm, 0.55-0.60 mm, 0.85-1.30 mm wide. The *C. compressus* pupae are 5.5-7.0 mm long and 2-3 mm wide, while those of *C. paria* are ~5.0 mm long and 1.70-1.90 mm wide [Table 5]. As seen from Figure 3, eggs and pupae have distinct morphology, which made scoring developmental time of these stages easier. The timing of different larval instars was scored based on their sizes as described in Table 5. While there was no overlap between the lengths during three instars, in a few cases breadth did overlap by certain amount. The 2nd and 3rd instar larvae were therefore considered to be in their subsequent stages if on a particular day their measurements were greater than the maximum dimensions prescribed for the subsequent stages, or else they were placed in the respective stages. The numbers of colonies used in these experiments are provided in Tables 2 and 3. Our studies were carried out only on the first batch of eggs because it is this batch that is reared solely by the queens (Hölldobler and Wilson 1990).

8.1.3 Results

We collected mated queens of two sympatric species of *Camponotus* ants (*C. compressus* and *C. paria*) while they were landing on the ground near their nuptial flight zones. These

ants were introduced into three different light/dark regimes (LL, 12:12 hr LD and DD) to study the effects of environmental light/dark conditions on pre-adult fitness components.

8.1.3.1 ***Effect of light/dark regimes on clutch-size and egg-viability:***

8.1.3.1.1 ***Clutch-size:*** Mated queens of each species were segregated into three groups and introduced into LL, LD, and DD, to estimate clutch-size of the first batch of eggs. While exposure to LL does not alter clutch-size in *C. paria*, it causes significant reduction in *C. compresses*. Two-way ANOVA on the clutch-size data showed a significant effect of light regime ($p < 0.005$) and species ($p < 0.0001$), however, light regime × species interaction is statistically not significant ($p = 0.51$) (Table 1 and Fig. 1a). Post-hoc multiple comparisons using Tukey's HSD test revealed that in *C. compressus* clutch-size is significantly smaller in LL than in LD and DD ($p < 0.005$), while those under LD and DD do not differ ($p > 0.05$). In *C. paria*, effect of light regime on clutch-size is statistically not significant ($p > 0.05$), however, even in this species, a trend similar to those observed in *C. compressus* is observed.

8.1.3.1.2 ***Egg-viability:*** Egg-viability is estimated as the number of eggs that successfully hatch as larvae. In both species, egg-viability is significantly enhanced in LL and LD compared to DD (Table 1 and Fig. 1b). ANOVA on the egg-viability data revealed a significant effect of light regime ($p < 0.0001$) and light regime × species interaction ($p < 0.005$), however, the main effect of species is statistically not significant ($p = 0.21$) (Table 1 and Fig. 1b). Post-hoc multiple comparisons using Tukey's HSD test revealed that percentage viability of both species is significantly greater in LL and LD ($p < 0.05$) compared to DD, while those under LL and LD do not differ ($p > 0.05$).

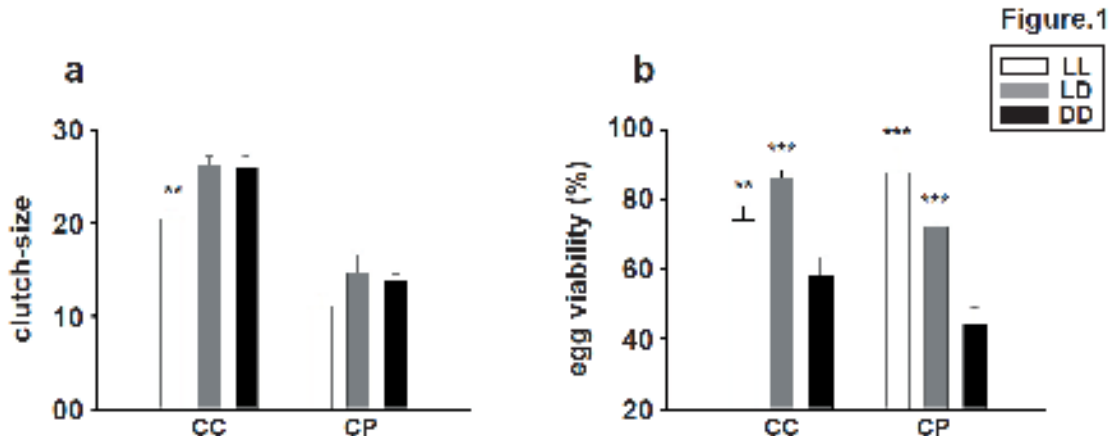


Figure 1 ***Effect of light regimes on clutch-size and egg-viability.*** In *Camponotus compressus* (CC) clutch-size is reduced under LL compared to LD and DD, whereas in *C. paria* (CP) clutch-size does not differ among three light regimes (a). Percentage egg-viability of both species is greater under LL and LD compared to DD (b). Species types are plotted along the abscissa and clutch size and egg viability along the ordinate. For single asterisk ($p < 0.05$), two asterisks ($p < 0.01$) and three asterisks ($p < 0.001$).

Table 1 **ANOVA on clutch size and egg viability of *C. compressus* and *C. paria***

clutch size	***df* effect**	***MS* effect**	***df* error**	***MS* error**	***F***	***p*- level**
Light regime (L)	2	0128.31	67	17.73	07.23	< 0.002
Species (S)	1	1691.92	67	17.73	95.41	< 0.0001
L x S	2	0011.82	67	17.73	00.66	= 0.51

Table 1b

egg viability	***df* effect**	***MS* effect**	***df* error**	***MS* error**	***F***	***p*- level**
Light regime (L)	2	5781.66	67	200.23	28.87	< 0.0001
Species (S)	1	0320.81	67	200.23	01.61	= 0.21
L x S	2	1319.34	67	200.23	06.58	< 0.005

Table.1. Results of two-way ANOVA on the clutch-size and egg-viability data of *C. compressus* and *C. paria*.

8.1.3.2 ***Effect of light/dark regimes on the duration of pre-adult developmental stages:*** For each developmental stage (pre-hatching (L0), 1st instar larvae (L1), 2nd instar larvae (L2), 3rd instar larvae (L3) and pupae (P) stages), time of initiation of the stage was taken into account to estimate development time. In both species, duration of most pre-adult stages are significantly altered by light, albeit by different magnitudes (Tables 2,3 and Fig. 2). In general, development is faster under LL, followed by LD, and then DD, in that order.

8.1.3.2.1 ***Time of initiation of the first instar larvae:*** In both species, development of L1 is faster in LL and LD compared to DD (Tables 2,3). ANOVA on the L1 developmental time data revealed a significant effect of light regime ($p < 0.0001$), while the effects of species ($p > 0.05$) and light regime × species interaction are statistically not significant ($p > 0.05$) (Tables 2-4). Post-hoc multiple comparisons using Tukey's HSD test showed that, in both species, L1 development is significantly faster under LL and LD compared to DD ($p < 0.001$), while those in LL and LD do not differ ($p > 0.05$) (Fig.2a).

8.1.3.2.2 ***Time of initiation of the second instar larvae:*** The trend in L2 development time is similar to those of L1 (Tables 2,3). Results of ANOVA on the L2 development time data revealed a significant effect of light regime ($p < 0.0001$) and species ($p < 0.01$), while the effect of light regime × species interaction is statistically not significant ($p > 0.05$) (Tables 2-4). Post-hoc multiple comparisons showed that L2 larvae take on average lesser time to develop in LL and LD than in DD ($p < 0.0001$), while under LL and LD their development time do not differ ($p > 0.05$) (Fig.2b).

8.1.3.2.3 ***Time of initiation of the third instar larvae:*** The trend in third instar larval development time is similar to those observed for L1 and L2 (Tables 2,3). ANOVA revealed a significant effect of light regime ($p < 0.001$) and species ($p < 0.05$), however, light regime × species interaction is statistically not significant ($p > 0.05$) (Tables 2-4). Turkey's HSD test for post-hoc multiple comparisons revealed that development time of the third instar larvae is significantly reduced under LL and LD compared to DD ($p < 0.001$), while those in LL and LD do not differ ($p > 0.05$) (Fig.2c).

8.1.3.2.4 ***Time of initiation of the pupal stage:*** The trend of light regime effects on pupal development time is similar to those observed for previous stages (Tables 2, 3). ANOVA on the pupal developmental time data revealed a significant effect of light regime ($p < 0.0001$) and light regime × species interaction ($p < 0.0001$), however, the main effect of species is statistically not significant ($p > 0.05$) (Tables 2-4). Multiple comparisons using Tukey's HSD test revealed that in *C. compressus*, pupal development is faster under LL, followed by LD, and then DD ($p < 0.001$). In *C. paria*, pupae develop much faster in LL and LD compared to DD ($p < 0.001$), and developmental rates under LL and LD do not differ ($p > 0.05$) (Fig.2d).

8.1.3.2.5 ***Time of adult emergence:*** The pre-adult (egg to emergence) development time of both species differed significantly under three light regimes; development is faster in LL, followed by LD, and then DD (Tables 2,3). ANOVA on the pre-adult development time data revealed a statistically significant effect of light regime ($p < 0.0001$), while effects of species ($p > 0.05$) and light regime × species interaction are statistically not significant ($p > 0.05$) (Tables 2-4). Post-hoc multiple comparisons using

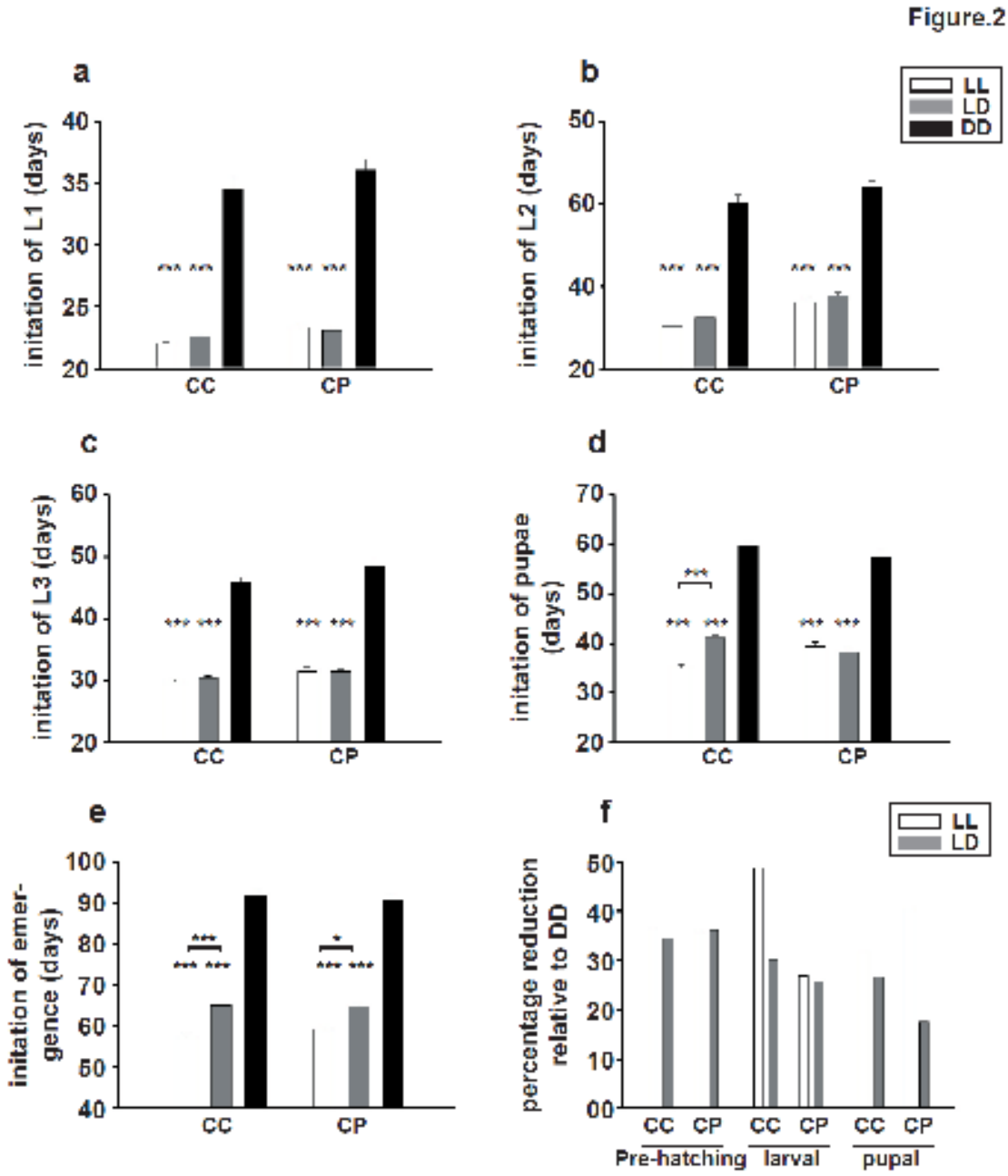


Figure 2 ***Effect of light regimes on the pre-hatching, larval and pupal durations.*** All stages of Pre-adult development are reduced ($p < 0.001$) in LL and LD in comparison to DD (a-e). Pupal stage is reduced in LL compared to the LD ($p < 0.001$) in case of *C. compressus* (d) ; however in both species pre-adult time is reduced in LL ($p < 0.001$) followed by LD ($p < 0.001$ for CC, $p < 0.05$ for CP) in comparison to DD (e). In LL, durations of pre-hatching stages are reduced (relative to DD) by ~36% in both *C. compressus* and *C. paria,* larval stages are reduced by ~ 49% in *C. compressus* and by ~27% in *C. paria*, and pupal stage are reduced by ~32% in *C. compressus* and by ~ 41% in *C. paria.* In LD, durations of pre-hatching stages are reduced (relative to DD) by ~36% in both *C. compressus* and *C. paria,* larval stages are reduced by ~30% in *C. compressus* and by ~26% in *C. paria*, and pupal stage are reduced by ~27% in *C. compressus* and by ~18% in *C. paria* (f). Species types are plotted along the abscissa and percentage reduction of duration of specific developmental stages along the ordinate. Other details same as figure.1.

Table 2

Details of timing of Initiation and duration of differnt developmental stages in *C. compressus*

Regime	no. of colonies	stages	initation of stage (Days) mean ± SD	duration of stage (Days) mean ± SD
LL	11	L0	N/A ± N/A	22.08 ± 0.25
	11	L1	22.08 ± 0.25	03.25 ± 0.91
	11	L2	25.33 ± 0.85	04.50 ± 0.69
	11	L3	29.83 ± 1.21	05.81 ± 1.84
	11	P	35.64 ± 2.53	22.16 ± 1.84
	11	M	57.81 ± 3.41	N/A ± N/A
LD	20	L0	N/A ± N/A	22.61 ± 0.68
	20	L1	22.61 ± 0.68	03.61 ± 0.45
	20	L2	26.23 ± 0.79	04.06 ± 1.34
	19	L3	30.29 ± 1.66	10.81 ± 1.75
	18	P	41.10 ± 2.36	23.84 ± 3.37
	18	M	64.94 ± 3.36	N/A ± N/A
DD	23	L0	N/A ± N/A	34.58 ± 4.39
	23	L1	34.58 ± 4.39	05.68 ± 0.49
	23	L2	40.27 ± 4.28	06.52 ± 1.47
	21	L3	46.79 ± 3.49	11.83 ± 2.08
	18	P	58.62 ± 3.96	32.91 ± 3.58
	18	M	91.53 ± 4.20	N/A ± N/A

Table 2 Details of timing of initiation and duration of different developmental stages in *C. compressus* under three light regimes. The pre-adult stages are indicated as L0 (pre-hatching), L1 (1st instar), L2 (2nd instar), 3rd instar (3rd instar), P (pupal stage) and M (emergence). N/A refers to data not available.

Table 3

Details of timing of Initiation and duration of differnt developmental stages in *C. paria*

Regime	no. of colonies	stages	initation of stage (Days) mean ± SD	duration of stage (Days) mean ± SD
LL	13	L0	N/A ± N/A	23.28 ± 0.72
	13	L1	23.28 ± 0.72	04.80 ± 1.41
	13	L2	28.09 ± 1.78	03.33 ± 1.06
	13	L3	31.42 ± 2.02	06.86 ± 2.17
	11	P	38.29 ± 3.05	19.81 ± 2.16
	11	M	58.11 ± 2.46	N/A ± N/A
LD	20	L0	N/A ± N/A	23.08 ± 0.66
	20	L1	23.08 ± 0.66	05.70 ± 0.56
	20	L2	28.79 ± 0.79	02.62 ± 0.43
	19	L3	31.41 ± 0.97	06.94 ± 1.41
	18	P	38.35 ± 1.24	26.92 ± 1.83
	18	M	65.27 ± 2.08	N/A ± N/A
DD	23	L0	N/A ± N/A	36.09 ± 3.81
	23	L1	36.09 ± 3.81	05.88 ± 0.41
	23	L2	41.97 ± 3.83	06.54 ± 1.47
	21	L3	48.52 ± 4.38	08.08 ± 1.23
	18	P	56.60 ± 4.25	33.22 ± 3.50
	18	M	89.82 ± 3.60	N/A ± N/A

Table 3 Details of timing of initiation and duration of different development stages in *C. paria* under three different light regimes. Other details as in Table 2.

Table 4 ANOVA on developmental time of differnt stages of *C. compressus* and *C. paria*

***1st instar* (L1)**	***df* effect**	***MS* effect**	***df* error**	***MS* error**	***F***	***p*- level**
Light regime (L)	2	1640.51	88	17.73	192.13	< 0.001
Species (S)	1	0020.4	88	17.73	002.39	< 0.13
L x S	2	0000.77	88	17.73	000.20	= 0.81
***2nd instart* (L2)**						
Light regime (L)	2	2198.17	88	8.7	252.50	< 0.0001
Species (S)	1	0065.19	88	8.7	007.48	< 0.01
L x S	2	0004.81	88	8.7	000.55	< 0.58
***3rd instar* (L3)**						
Light regime (L)	2	2889.93	85	8.53	338.73	< 0.0001
Species (S)	1	0016.34	85	8.53	004.23	< 0.05
L x S	2	0013.42	85	8.53	001.57	< 0.21
Pupal stage						
Light regime (L)	2	3029.72	68	8.37	361.61	< 0.0001
Species (S)	1	0003.62	68	8.37	000.43	= 0.51
L x S	2	0094.48	68	8.37	011.03	< 0.0001
emergence						
Light regime (L)	2	6857.40	68	11.82	579.66	< 0.0001
Species (S)	1	0000.97	68	11.82	000.08	= 0.78
L x S	2	0015.98	68	11.82	001.35	= 0.27

Table 4 Results of two-way ANOVA on the duration of different stages of pre-adult development (first instar, second instar, third instar, pupal stage and emergence), estimated from the egg stage.

Table 5

Morphological measurements at differnt developmental stages of *C. compressus* and *C. paria*

Length (L) and Breadth (B) in milimeters						
Species	paramter	Egg (min-max)	Ist instar (min-max)	2nd instar (min-max)	3rd instar (min-max)	pupal (min-max)
C. compressus	Length (L)	1.00-1.20	1.05-1.35	1.40-2.00	200-5.20	05.5-7.00
	Breadth (B)	0.47 0.60	0.60 0.65	0.65 0.75	0.85 1.40	2.00 3.00
C. paria	Length (L)	0.90-1.20	1.00-1.30	1.35-2.00	2.00-1.50	5.00-5.20
	Breadth (B)	0.45 0.57	0.60 1.60	0.65 0.60	0.85 1.30	1.70 1.90

Table 5 Morphological data of ants at different stages of pre-adult development.

Tukey's HSD test suggests that both species develop faster in LL compared to LD ($p < 0.05$) and DD ($p < 0.001$) (Fig.2e).

To gain further insight into the light regime effects on pre-adult development time of specific developmental stages we reanalyzed the data by broadly categorizing pre-adult duration into three stages: pre-hatching, larval and pupal stages, taking duration of the stage in question into account as opposed to total time since the egg stage. This was done to avoid effects of previous stages on the duration of the stage in question. Under LL, duration of pre-hatching stage is reduced by ~36% in *C. compressus* and *C. paria*, larval stage is reduced by ~49% in *C. compressus* and by ~27% in *C. paria*, pupal stage is reduced by ~32% in *C. compressus* and by ~41% in *C. paria* (Fig. 2f, Tables 2, 3). Overall, there is ~37% reduction in *C. compressus* and ~35% in *C. paria*. In LD also, the duration of pre-adult developmental stages are reduced in both species, however, here the reductions are smaller than those seen in LL.

8.1.4 Discussion

The clutch-size of the first batch of eggs in *C. compressus* is significantly reduced under LL and LD compared to DD, while in *C. paria* it does not differ among the three light regimes (Fig.1a). Although, LL inhibits reproductive output in *C. compressus*, it is found to increase egg-viability in both species (Fig1b), which suggests that a sizable proportion of eggs laid in DD are nonviable trophic eggs (Hölldobler and Wilson, 1990). These results are consistent with the findings of a previous study in *Tribolium*, where females are found to lay fewer eggs in LL than in DD (Hawk et al., 1974). Egg-hatching is inhibited under LL, in halibut *Hippoglossus hippoglossus* (Helvik and Walther, 2005), while in intestinal fluke of waterfowls *Echinostoma caproni* it is inhibited in DD (Markum and Nollen, 1996). Apart from the presence and absence of light, even length of the photoperiod have been shown to alter clutch-size; in diamond back moth *Plutella maculipennis*, females lay more eggs when raised under long photoperiod (LD 15:9 hr) than under short photoperiod (LD 9:15 hr) (Saunders, 2002). In leek moth *Acrolepia assectella*, females raised under LD (9:15 hr), LL, DD, are found to have, respectively ~8.3, ~4.1, and ~2.9 eggs in their ovaries, on the second day of emergence (Saunders, 2002). This suggests that light regimes play a crucial role in the modulation of fitness in a wide range of insect species.

In ant species *C. herculeanus*, both worker and reproductive castes are produced around the same time of the year (during late summer or early winter) (Hölldobler and Wilson, 1990), and during peak winter their pre-adult development is suspended completely. In a separate study, we observed that *Camponutus* ants also exhibit seasonal preference in development; the night active *C. compressus* develops faster under shorter (winter) days, while the day active *C. paria* develops faster under longer (summer) days

(Lone et al., 2011). This suggests that pre-adult development in ants is regulated by seasonal timers.

Temporal regulation of development is of prime importance to insects as it controls a large number of critical processes such as cell cycle, growth of tissues, emergence of patterns, formation of organs, and several postembryonic processes (Moss, 2007). Therefore, even small changes in development may lead to catastrophic developmental defects, or may even cause appearance of new phenotypic variants, which may be picked up by selection.(Moss, 2007) The results of our study suggest that exposure to LL and LD regimes speeds-up pre-adult development in two sympatric species of *Camponotus* ants. We also observed that light exerts large effects on the duration of several pre-adult stages (Fig.2), which clearly suggests that light-sensitive processes play a key role in the regulation of pre-adult development in ants. While these results are consistent with the findings of a few previous studies in *Drosophila* (Kyriacou et al., 1990; Sheeba et al., 1999; Paranjpe et al., 2005), it is not clear to what extent circadian timing systems are involved in such developmental regulations.

The rate of pre-adult development in insects is thought to be regulated by multivariate interactions between light/dark cycles, temperature dependent developmental clocks, and temperature-compensated, light and temperature entrainable circadian clocks (Trueman, 1985; Qiu and Hardin, 1996). It is believed that circadian clocks, and/or LD cycles create "developmental gates" which periodically assess developmental states of insects, and if an individual has completed certain developmental state by that time, it is allowed to enter the next stage, or else it is forced to wait until the next gate opens (Qiu and Hardin, 1996). Since circadian rhythms are abolished under LL, such "developmental

gates" would be absent, and individuals would be allowed to enter subsequent stages without any further delay (Qiu and Hardin, 1996). Thus development is expected to be fastest in LL. On the other hand, in LD and DD regimes developmental rates are expected to be primarily determined by the developmental gates. In DD, circadian clocks of *Camponotus* ants free-runs with a mean period greater than 24 hr, and in LD it is 24 hr as clocks of these ants entrain to 12:12 hr LD cycles (Sharma et al., 2004a; Sharma et al., 2004b). Based on the above considerations, pre-adult development in *Camponotus* ants would be faster in LL, followed by LD, and then DD. While it is likely that LD regimes and/or circadian clocks regulate development time, a possible role of social factors can not be entirely ignored. There is growing body of evidence to suggest that social insects have well developed species- and season-specific developmental strategies to meet the challenges arising due to changing environmental conditions and social needs (Toma et al., 2000; Bloch et al., 2001; Sharma et al., 2004b). Such strategies have been reported earlier in honeybees, where nurse bees have the ability to accelerate their development to become foragers in the event that a shortage of foragers arises in the colony (Bloch et al., 2001). It is unlikely that amount of light and/or temperature associated with illuminating dark-adapted ants would cause changes in developmental rates by such large magnitudes, because both species are found to develop significantly faster under 12:12 hr LD cycles than under 14:10 hr or 14:14 hr LD cycles (Lone et al., 2010,2011), although, the amount of light per cycle in 12:12 hr LD cycle is 2 hr shorter than the other two LD regimes.

In an early study, Minis and Pittendrigh (Minis and Pittendrigh, 1968) had shown that pink boll worm moth *Pectinophra gossypiella* develops as embryo faster in LL than in DD. The developmental rates of two species of cutworm *Agrostis* are significantly

different under long and short photoperiods (Saunders, 2002); *A. occulata* develops faster under long days, whereas *A. triangulum* develops slower under the same set of conditions. The results of our present study suggest that exposure to LL and LD reduces the duration of pre-hatching, larval, and pupal stages, in two dark-dwelling sympatric species of ants. The reductions in larval stage under LL and LD are by ~49% and ~30% in *C. compressus* and by ~27% and ~26% in *C. paria*. Similarly, the reductions in pupal stage under LL and LD are by ~32% and ~27% in *C. compressus* and by ~41% and ~18% in *C. paria*. To the best of our knowledge, developmental effects of light/dark regimes, by such large magnitudes, have never been reported earlier in any species of insect.

While exposures to LL and LD regimes is found to speed-up pre-adult development in both day as well as night active species in a similar manner, in the night active species the reductions are more severe during the larval stage, while in the day active species it is more severe during the pupal stage. In a separate study, when we assayed pre-adult development time under three different photoperiods (LD 10:14 hr LD, 12:12 hr LD, and 14:10 hr), we found that while both species develop fastest as pre-adult under 12:12 hr LD cycles, among the asymmetric photoperiods, the night active *C. compressus* develops faster under longer nights (10:14 hr) and the day active *C. paria* develops faster under longer days (14:10 hr) (Lone et al., 2011). This suggests that while the core physiological effects of light are well conserved in both species, in the course of sympatric speciation they have evolved different developmental strategies to deal with changes in their environments.

Developmental plasticity is mediated by signals that cause the expression or repression of several genetic switches (Moss, 2007). The key components of such

switches comprise stage-specific heterochronic genes such as *lin14*, which causes developmental delays in the nematode *C. elegans*. The expression of the gene *lin4*, which binds to the 3' untranslated region of *lin14*, has been shown to prevent developmental delays (Moss, 2007). In addition, the oscillating gene *lin42*, a homolog of *Drosophila period* gene, is shown to regulate the expression of both *lin4* and *lin14*, through yet unknown mechanisms. It is not yet known how light signals modulate expression of the stage-specific heterochronic genes. Alternatively, LD-regime effects could be mediated via darkness, because exposure to darkness is known to slow-down pre-adult development by lowering ecdysteriod levels (Li and White, 2003). It is also known that acute reduction in ecdysteriod level causes complete inhibition of pupation (Hirashima et al., 1998). The steroid hormone ecdysone is known to play a crucial role in molting of larval stages, and it sets the timing of pre-adult stages by binding to nuclear hormone receptors, which in turn regulate the expression of genes that regulate development (Giebultowicz et al., 2008). Furthermore, it is possible that genes involved in numerous signaling pathways, such as notch signaling, insulin signaling and ecdysteroid signaling pathways govern light-dependent developmental regulations in ants (Mensch et al., 2008).

Social insect colonies are faced with numerous challenges arising due to fluctuating seasons, changing colony sizes, modifications in age structure, changes in nutritional requirement associated with colony development, changes in food availability, and predation pressure (Huang and Robinson, 1992; Robinson, 1992; Frisch and Koeniger, 1994; Crailsheim et al., 1996; Moore, 2001). To meet such challenges, social insects seem to have evolved "division of labour" (Bourke and Franks, 1995; Sharma et

al., 2004b), and circadian plasticity (Bloch et al., 2001; Sharma et al., 2004a; Sharma et al., 2004b), to modulate their physiology and behaviour. It is likely that light-mediated developmental plasticity, could be an evolutionary consequence of such ecological and social selection pressures.

In temperate environments where day/night lengths vary drastically throughout the year, photoperiodic signals play a key role in enhancing the fitness of insects (Saunders, 2002). A large number of insect species are believed to use photoperiodic signals to regulate their pre-adult development, an ability that forms a fundamental aspect of insect evolution. This idea is further corroborated by our study, where we show that in two dark-dwelling sympatric species of ants, clutch-size, egg-viability, and pre-adult development time are significantly altered by environmental light/dark conditions. While reproductive fitness of one species is reduced in LL, egg-viability and developmental rates of both species are enhanced considerably. Our study suggests that development plasticity regulated by environmental light/dark conditions may provide an important dimension to aspects related to fitness and overall success of social ants in adapting to diverse spatio-temporal niches.

Chapter 8

Part 2

Photoperiodic Regulation of Development in *Camponotus* Ants

8.2.1 Introduction

Organisms as diverse as insects, reptiles, birds, and mammals use seasonal changes in day length, temperature, rainfall, and food to time critical life processes such as development, metamorphosis, foraging, hibernation, migration, and reproduction (Bowen et al., 1984; Monecke et al., 2006). Seasonal timers make use of day length and direction of its changes (increasing/decreasing) to track time of the year (Saunders, 2002). This involves photoreception, measurement of critical day and/or night lengths, storage of such information in a timer, and finally eliciting a response. In insects, the basic nature of such timers varies from one species to another; e.g., in some species it is an hourglass, while in others it is a circadian clock (Saunders, 2002). It is believed that seasonal timers in insects comprise of two basic subsystems, one that measures short nights or long days, and the other that keeps track of long nights or short days (Vaz Nunes and Saunders, 1999). Signals from both the modes are integrated and processed in the timer, which eventually determines the relative dominance of one photophase over the other. Interesting in butterfly *Pieris brassicae*, short night/long day is temperature compensated, while long night/short day mode is highly temperature sensitive (Spieth et al., 2004). On the other hand in flesh fly *Sarcophaga argyrostoma* (Saunders, 1971), vetch aphid *Megoura viciae* (Hardie, 1990), and in bean aphid *Aphis fabae* (Vaz Nunes and Hardie, 1999), long night/short day is temperature compensated whereas short night/long day is temperature sensitive.

Seasonal timers are of utmost importance to social insects, especially for the production of sexual morphs, initiation of nuptial flights, and for the formation of new colonies (Tauber et al., 1986). Such timers regulate growth, development, and

reproduction in many insect species including ants (Hölldobler and Wilson, 1990; Saunders, 2002). It has also been observed that some species of ants use both circadian and seasonal timers to schedule their mating flights at a specific time of the day, and year (Haddow et al., 1966; Baldridge et al., 1980). For example, males from sixteen species of army ants *Neivamymex* undertake mating flights at different time of the year (Baldridge et al., 1980). Males of eight sympatric species of African *Dorylus* ants fly out for mating flights at different time of the day (Haddow et al., 1966); the *D. moestus* males go out at sunset, *D. burmeisteri* males at sunrise, and males from other species at different times during the night. These studies suggest that mating behaviour in some ant species is temporally regulated.

Photoperiods serve as a key determinant of latitudinal clines for the life history of several insect and mite species, and are known to play an important role in critical decision making processes involved in growth, development, diapause, metamorphosis, and reproduction (Vaz Nunes, 1998; Saunders, 2002). Besides day lengths, pre-adult development time in ants is also highly sensitive to other environmental factors such as temperature, and nutrition (Kipyatkov and Lopatina, 1997; Lone and Sharma, 2008; Lone et al., 2010). Many insect species develop at different rates under short and long day lengths; some develop faster under short days, while others in long days (Saunders, 2002). The pine processionary moth *Thaumetopoea pityocampa*, (Santos et al., 2007), mosquito *Aëdes triseriatus* (Vinogradova, 1967), diamond black moth *Plutella maculipennis* (Atwal, 1955), and flesh fly *Sarcophaga argyrostoma* (Denlinger, 1972; Saunders, 1972, 1976) are a few examples of insects that develop faster under long days. Insects that develop faster under short days include migratory grasshopper *Melanoplus*

sanguinipes (Dean, 1982), and potato beetle *Leptinotarsa decemlineata* (Doležal et al., 2007). Further, sympatric species of some insects develop at different rates under short and long day lengths (Saunders, 2002). For example, larvae of cutworm *Agrostis occulata* develops faster under long days, whereas its sympatric partner *A. triangulum* develops faster under short days (Danilevskii, 1965).

The pre-adult developmental durations of carpenter ants ranges between one to three months, except in winters when their development is completely stopped (Hölldobler and Wilson, 1990). During early summer, some ant species raise their nest temperatures using heat produced by their metabolic activities, and/or by exposing their brood to sunlight (Hölldobler and Wilson, 1990). This enables them to complete pre-adult development rapidly to be ready for their nuptial flights. The *Camponotus* ants on the other hand are not known to use any exclusive thermoregulatory mechanisms to regulate pre-adult development, and rely mostly on light (Lone and Sharma, 2008; Lone et al., 2010). Development time in these ants is plastic; exposure to constant light speeds up pre-adult development in *Camponotus compressus* by ~35 days, and in *Camponotus paria* by ~31 days (relative to their respective development time under constant darkness, Lone and Sharma, 2008). In a more recent study we have shown that both these species develop as pre-adult fastest under 12:12 hr light/dark (LD) cycles (*T24* - 12:12 hr) compared to short (*T20* - 10:10 hr) or long (*T28* - 14:14 hr) LD cycles (Lone et al., 2010). Given that such boreal species of ants do not experience seasonal changes in photophase, at least not during their early life stages, it would be interesting to study if as pre-adults they can differentiate between short and long day lengths.

8.2.2 Materials and Methods

The carpenter ant *Camponotus* usually forms its nest inside forests, in decomposed plants, and has evolved mutualism with aphids and coccids (Hölldobler and Wilson, 1990). *C. paria* forms its nests more than one and half feet deep under the soil, while *C. compressus* in even deeper locations (*Shahnaz Rahman Lone and Vijay Kumar Sharma, personal observation*). The reproductives (virgin males and queens) undertake mating flights in the months of April-May-June after the first rains following a prolonged dry season. Prior to the nuptial flights workers carry out soil from the nest to make way for relatively larger males and queens. The virgin males and queens fly out of their nests, and assemble at the mating site on the evening of the first rainy day. After mating males die and queens start digging into the soil to form fresh nests. It is important to note here that under natural conditions these two species of ants are not known to be exposed to light during the entire period of their pre-adult development. Furthermore, as adults they do not experience large changes in day length because in Bangalore (12° 58′ N, 77° 40′ E) twilights last only for ~50 min, and seasonal variations in day length is also quite small (~2 hr). However, these ants do experience seasonal changes in high and low temperature, dry and wet conditions, and rich and poor vegetation.

For the present study, mated *Camponotus* (*C. paria* and *C. compressus*) queens were collected from the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore Campus, soon after their nuptial flights. They were immediately transferred into petri plates (diameter × height = 90 mm × 15 mm), and introduced into three different LD cycles comprising photophases 10 hr, 12 hr, and 14 hr long, created using two temperature controlled incubators, placed for additional stability inside an

environmentally controlled room with temperature (25.0 ± 1 ^{o}C) and relative humidity (~75%) maintained constant. For creating different LD regimes, two incubators - one (*Inc-I*) constantly illuminated by fluorescent light of 500 ± 20 lux intensity, and the other (*Inc-II*) by dim red light ($\lambda > 650$ nm) were used. Temperature inside the incubators was monitored at every 6 hour interval until the completion of experiment, and was found to be fairly stable (*Inc-I*: 24.97 ± 0.49 ^{o}C; mean ± SD, and *Inc-II*: 24.98 ± 0.49 ^{o}C). Colonies were moved at specific time of the day, from light to dark and dark to light incubators to create different LD cycles (10:14 hr, 12:12 hr and 14:10 hr). For example, to create 10:14 hr LD cycles, ants were first kept for 10 hr in *Inc-I*, and then transferred to *Inc-II* for the subsequent 14 hr, after which they were transferred back to *Inc-I*. This was continued until the end of experiment. A similar protocol was followed to create LD 12:12 hr and LD 14:10 hr. The pre-adult development time of ants was assayed simultaneously under three LD regimes to rule out any microclimatic effect on developmental time. Food in the form of Bhatkar diet (Bhatkar and Whitcomb, 1970), and 10% dilute honey solution was provided *ad libitum*. Incidentally *Camponotus* queens were seen to eat rarely in the laboratory colonies, and no obvious rhythm in feeding was observed.

All our studies were conducted on the first batch of eggs, laid by the foundress queen because it is this batch which is reared solely by the queens (Hölldobler and Wilson, 1990). This is the only generation which does not represent the standard reference of development for different castes in the colony. This particular generation of eggs was chosen for our study to avoid any influence of confounding factors such as those underlying social structure. For estimating developmental rates of different castes

it would be imperative to use well established colonies with matured queens and workers, and a colony size that is representative of the natural colony (Brian, 1978). The overall number of *C. compressus* and *C. paria* (prefix CC and CP respectively) brood used in our study was 48 - CCLD10:14 (from 12 independent colonies), 34 - CCLD12:12 (from 8 independent colonies), 30 - CCLD14:10 (from 7 independent colonies), 15 - CPLD10:14 (from 5 independent colonies), 16 - CPLD12:12 (from 5 independent colonies), and 42 - CPLD14:10 (from 12 independent colonies). The "n" is total number of individuals pooled across all colonies. For example, $n = 48$ in CCLD10:14 implies that 48 individuals from 12 colonies, in roughly equal numbers, were used.

The 'larval development time' was estimated by calculating duration between egg stage and formation of pupae, 'pupal developmental time' as the duration between formation of pupae and adult emergence, and 'pre-adult development time' as the duration from egg stage to adult emergence, which includes all post-embryonic developmental stages. The method of detection of different stages and estimation of developmental durations are described in details in Lone and Sharma, (2008). The eggs, larvae and pupae also have distinct morphology and structure, which made scoring individuals in these stages easy. For pupae, when curved mobile larvae become cylindrical, immobile, and larger in size we consider the brood to be in pupal stage. Pupal stage in *C. compressus* (5-7.0 mm long and 2-3 mm wide) and *C. paria* (~5.0 mm long and 1.70-1.90 mm wide) is well defined, the pupa is initially white in color, and turns brown with time. These morphological, structural, and functional details were used as reference for the estimation of the developmental stages of the brood. Each colony was monitored regularly every 6 hours for eggs, larvae, and pupae and their numbers

recorded. At any given point of time there were some eggs, larvae in first, second, and third instars, and some pupae, and freshly emerged adults. These numbers were then multiplied by the total time taken by individual(s) to reach that state in order to estimate the duration of that particular stage. Individual development times were used as variables for 2-way analysis of variance (ANOVA) in which species and photophase were treated as fixed factors. Post-hoc multiple comparisons among mean development time under different photophases were done using Fisher's LSD test. All analyses were implemented on STATISTICA^{TM} for Windows Release 5.0 B (StatSoft, 1995).

8.2.3 Results

8.2.3.1 ***Pre-adult (egg-to-adult) developmental time:*** Development of both species is fastest under 12:12 hr LD cycles. However, among the asymmetric LD cycles, pre-adult development of night active species (*C. compressus*) is faster under short days (10:14 hr), while those of day active species (*C. paria*) is faster under long days (14:10 hr) (Fig. 1c; Table 1, 2). ANOVA on the pre-adult development time data revealed a statistically significant effect of photophase, and species × photophase interaction, however, effect of species is statistically not significant (Table 1). Post-hoc multiple comparisons using LSD test revealed that pre-adult development time of *C. compressus* is shortest under LD 12:12 hr, followed by LD 10:14 hr ($p < 0.001$), and then LD 14:10 hr ($p < 0.0001$). The pre-adult development time of *C. paria* is shortest under LD 12:12 hr, followed by LD 14:10 hr ($p < 0.0001$), and then LD 10:14 hr ($p < 0.0001$). Among the two asymmetric LD cycles, development time of *C. compressus* is significantly shorter under LD 10:14 hr compared to LD 14:10 hr ($p < 0.05$), while *C. paria* it is significantly shorter under LD 14:10 hr compared to LD 10:14 hr ($p < 0.001$) (Fig. 1c).

8.2.3.2 ***Larval duration (egg-to-pupal formation):*** The larval duration of both species is shortest under 12:12 hr LD cycles. However, among the asymmetric LD cycles, larval duration of the night active species is significantly shorter under short days (10:14 hr), while that of the day active species does not differ between short and long days (Fig. 1a; Table 1, 2). ANOVA on the larval duration data revealed a statistically significant effect of photophase, species, and species × photophase interaction (Table 1). Post-hoc multiple comparisons using LSD test revealed that *C. compressus* pupates earliest under LD 12:12 hr, followed by LD 10:14 hr ($p < 0.05$), and then LD 14:10 hr ($p < 0.0001$). The larval duration of *C. paria* is shortest under LD 12:12 hr compared to LD 10:14 hr and LD 14:10 hr ($p < 0.0001$). However, among the two asymmetric LD cycles, *C. compressus* pupates earlier under LD 10:14 hr than in LD 14:10 hr ($p < 0.02$). In *C. paria* larval duration does not differ statistically between the two asymmetric LD cycles ($p = 0.10$) (Fig. 1a).

8.2.3.3 ***Pupal duration (pupal formation-to-adult emergence):*** To examine whether day lengths alter the duration of pupal stage, we analyzed pupal duration data separately, and we found the that mean pupal duration of *C. compressus* remains unchanged under three different photophases. However, in *C. paria* the pupal duration is significantly reduced under long days compared to short and normal days (Fig. 1b, Table 1, b). ANOVA on the pupal duration data showed a statistically significant effect of species, and species × photophase interaction, however, effect of photophase is statistically not significant (Table 1). Post-hoc multiple comparisons using LSD test revealed that in *C. compressus* pupal duration does not differ statistically under the three photophases, while

in *C. paria* it is significantly reduced under long days compared to short ($p < 0.05$) and 12:12 hr LD cycles ($p < 0.01$) (Fig. 1b).

8.2.4 Discussion

Two sympatric species of *Camponotus* ants develop fastest as pre-adults under 12:12 hr LD cycles compared to short (LD 10:14 hr) and long (LD 14:10 hr) photophases. Faster development under 12:12 hr LD cycles is not entirely surprising in these ants because they live and develop under tropical conditions where for most part of the year, day length is around 12 hr, with a maximum seasonal variation of not more than 2 hr. These results are consistent with the findings of our earlier study, where we showed that both species develop as pre-adult significantly faster under (*T24* - LD 12:12 hr) compared to (*T20* - LD 10:10 hr) and (*T28* - LD 14:14 hr). The two species of *Camponotus* ants – *C. paria* and *C. compressus*, normally develop in dark underground colonies wherein the only time cue present is in the form of food and brood care. It is therefore unlikely that they have developed an elaborate strategy to develop optimally under 12:12 hr LD cycles. However, it is possible that they have evolved the ability to use day length as an indicator of other abiotic and biotic factors.

The *Camponotus* ants exhibit species-specific day length-mediated differences in development time - day active species (*C. paria*) develops faster under long days (14:10 hr), while night active species (*C. compressus*) develops faster under short days (10:14 hr) (Fig. 1c). This suggests that while *Camponotus* ants have evolved the ability to develop faster under 12:12 hr LD cycles, they also exhibit species-specific differences in

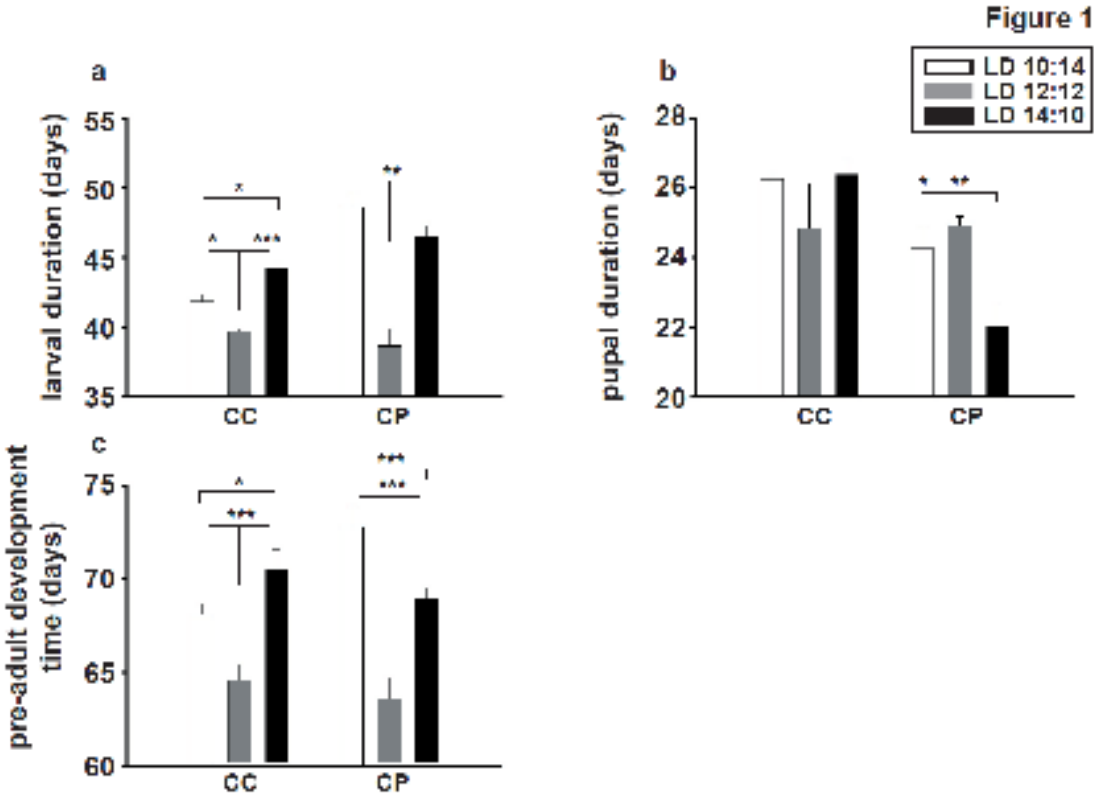


Figure 1 Effect of day lengths (LD 10:14 hr, LD 12:12 hr and LD 14:10 hr) on the pre-adult *(from egg stage to adult emergence)*, larval *(from egg stage to formation of pupae)* and pupal *(from formation of pupae to adult emergence) development time.* The pre-adult development time of day active species (*Camponotus paria*) is significantly shorter under long days (14:10 hr), while those of night active species (*Camponotus compressus*) is significantly shorter under short days (10:14 hr). The effect of photophases on the pre-adult development time in both the species is mediated through larval stages, however, in *C. paria* even the pupal stage is sensitive to photophase. Species type is plotted along the abscissa and larval (a) and pupal (b) durations in days along the ordinate. Error bars are standard error means around means. CC and CP refers to *C. compressus* and *C. paria*, respectively. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 1 Results of ANOVA on the pre-adult, larval and pupal duration data

Pre-adult duration	*df* effect	*MS* effect	*df* error	*MS* error	*F*	*p*- level
Species (S)	1	11459.5	178	11875	00.97	= 0.33
Photoperiod (P)	2	326865.1	178	11875	27.50	< 0.0001
S x P	2	83738.2	178	11875	07.05	< 0.001
Larval duration						
Species (S)	1	153251.4	178	9909.2	15.5	< 0.0001
Photoperiod (P)	2	341715.2	178	9909.2	34.5	< 0.0001
S x P	2	095898.8	178	9909.2	9.68	< 0.0001
Pupal duration						
Species (S)	1	93545.1	178	8352.8	11.10	< 0.001
Photoperiod (P)	2	08912.4	178	8352.8	01.07	= 0.350
S x P	2	37980.2	178	8352.8	04.55	< 0.210

Table 1 Results ANOVA on the duration of different stages of pre-adult development estimated from the egg stage

Table 2 Mean pre-adult, larval, and pupal durations in two species of *Camponotus* ants

Species	Pre-pupal duration mean ± SD	Pupation time mean ± SD	Pre-adult development time mean ± SD
C. compressus			
LD10:14	41.84 ± 03.10	26.24 ± 01.16	68.08 ± 04.13
LD12:12	39.68 ± 03.78	24.86 ± 07.29	64.54 ± 05.27
LD14:10	44.18 ± 03.66	26.38 ± 02.23	70.56 ± 05.62
C. paria			
LD10:14	48.58 ± 04.36	24.25 ± 02.36	72.83 ± 04.15
LD12:12	38.62 ± 04.75	24.93 ± 00.92	63.56 ± 04.70
LD14:10	46.51 ± 07.17	22.03 ± 03.57	68.99 ± 03.24

Table 2 Details of timing of initiation and duration of different developmental stages (in days) of *C. compressus* and *C. paria* under three light regimes.

developmental rates under short and long day lengths. How could two species of ants, otherwise living under tropical conditions, where seasonal variations in day length is small, have evolved differential sensitivity to short and long day lengths? Some insect species have the ability to sense even minor changes in day length; changes as small as 10-15 min can evoke measurable physiological response, and a one hour change is able to completely switch developmental pathways (Saunders, 2002). Although day length does not vary much in the tropics, organisms living in these regions do experience marked seasonal changes in temperature, rainfall, and vegetation. Therefore, it is likely that *Camponotus* ants living in these regions have evolved to use day length as indicator of seasonality in temperature, rainfall, and vegetation. Alternately, it is possible that over evolutionary time scales *Camponotus* ants may have emigrated from temperate regions, where photophase-dependent developmental schedule would obviously be adaptive, and they continued to retain this sensitivity although in tropical conditions such traits are purely vestigial (Kipyatkov and Lopatina, 2000). However to the best of our knowledge, there is no evidence that suggests that such emigration has taken place in *Camponotus* ants.

The pre-adult development of both species is speeded up under 12:12 hr LD cycles, whereas in other photophases it is either accelerated or decelerated in a stage, and species-specific manner (Figs. 1). Stage-specific regulation of development is known to be dictated primarily by the availability and quality of nutrients present. It has previously

been shown that food intake, which in ants is known to vary with season, is correlated with the rate of brood development (Brian, 1978). For example, food items collected by the harvester ant *Veromessor pergandei* (Mayr) between July and March consisted of ~68.2% seeds, ~10.3% insect prey, and ~21.5% other items, while between April and June seed content increased and reached ~91.9%, along with insect prey ~0.9%, flowers ~6.7% and other items ~0.5 % (Lees, 1953; Brian, 1978). This suggests that insects living in tropical conditions are likely to have evolved the ability to use photophase as an indicator of seasonal nutrition variations.

Some stages in an organism's life are more sensitive to photophase than others (Spieth et al., 2004). Our study suggests that the sensitive stages for day length-mediated developmental changes in two sympatric species of *Camponotus* ants are different. While larval stage is responsive to photophase in both species, pupal stage is sensitive to photophase only in day active species. This suggests that in *Camponotus* ants apart from response to day length, developmental stages sensitive to photophase have also segregated in the course of speciation. It is obvious that photophase will have little or no impact on intrinsic factors underlying larval development because normally during this stage *Camponotus* ants do not expose themselves to light, and consequently there is little scope for selection to use LD cycles as a cue for larval developmental regulation. However, day length is expected to influence feeding and brood care by queens, and/or workers, which could influence larval development. The fact that photophases have significant effect on the duration of larval stage of both *Camponotus* species endorses this view. On the other hand, effect of photophase on pupal development can only be mediated through direct effect of photophase on intrinsic factors regulating development.

The fact that photophase has little or no effect on the duration of pupal stage suggests that the effect of photophase on the pre-adult development time is primarily due to changes in brood care schedules.

Taken together the results of my study suggest that ability to use day length to regulate pre-adult development time is conserved in two sympatric species of tropical carpenter ants - *C. compressus* and *C. paria*. It is likely that day length-mediated modulations in development time are brought about by differences in the schedules of brood care, or by exposure of brood to sunlight and/or moonlight (Hölldobler and Wilson, 1990). Whatever be the case, results of our study suggest that day length-mediated modulation in developmental time is a well-conserved phenomenon even in tropical *Camponotus* ant species which normally develop in deep dark underground nests. These results suggest that tropical species of ants, otherwise living in dark underground nests could serve as an ideal model system for the study of temporal regulation of pre-adult development. These results also provide insight into how circadian and seasonal timing systems are built in *Camponotus* ants. Although our study was performed on the first batch of the eggs when there are no workers available, developmental strategies developed for a fully developed colony may not be very different. Further, our conclusions are based on study on only two tropical species of carpenter ants, and therefore further studies are needed on several day and night active species from temperate as well as tropical climates to confirm this.

Chapter 8

Part 3

Circadian Resonance in *Camponotus* ants

8.3.1 Introduction

One of the primary functions of circadian clocks is to synchronize the behaviour and physiology of an organism to the geophysical environment (Michael et al., 2003), and to provide the organism with a temporal view of the external world (Sharma, 2003a). Circadian clocks are also believed to coordinate cyclic metabolic processes within the internal milieu in order to bring about an internal temporal order (Daan, 1981; Sharma and Joshi, 2002; Sharma, 2003a; Paranjpe and Sharma, 2005). While such proposals still await rigorous empirical support, studies under field (DeCoursey et al., 1997; DeCoursey et al., 2000) as well as laboratory conditions (Pittendrigh and Minis, 1972; Von Saint-Paul and Aschoff, 1978; Klarsfeld and Rouyer, 1998; Wyse et al., 2010) indicate that circadian clocks enhance the mean lifespan of some insect species, possibly by enabling cyclic behavioural and physiological processes to entrain optimally to environmental LD cycles (Sharma, 2003a; Paranjpe and Sharma, 2005).

Circadian clocks have been implicated in the regulation of life history traits such as pre-adult development time and adult lifespan (Kyriacou et al., 1990; Wong et al., 1995; Hurd and Ralph, 1998; Felkai et al., 1999; Sheeba et al., 2000; Emerson et al., 2008; Wyse et al., 2010). For example, in a study on the *period* (*per*) mutants of *Drosophila melanogaster* it was shown that short period allele per^{S} flies ($\tau = 19$ hr) develop faster as pre-adults than wild type controls ($\tau = 24$ hr), whereas long period per^{L} flies ($\tau = 28$ hr) take longer to develop (Kyriacou et al., 1990). In a separate study in *C. elegans*, it was shown that allelic mutants (*e2519*, *qm11* and *qm30*) with longer embryonic cell cycle develop slower, and have lower defecation rates than the wild type strain (Wong et al., 1995). While the durations of all postembryonic stages are

lengthened in all the three mutants, in *qm30*, the overall development time is severely affected, as, it is extended by almost two-fold compared to the wild type strain (Wong et al. 1995). In a separate study in fruit fly *D. melanogaster*, it was shown that wild type flies maintained under short (LD 10:10 hr) and long (LD 14:14 hr) LD cycles show short and long period rhythms of adult emergence, and develop faster and slower, respectively than their counterparts maintained under LD 12:12 hr (Paranjpe et al., 2005). Correlation between circadian periodicity and pre-adult development time has also been reported in laboratory selection studies, where fly populations were subjected to selection for faster or slower development, or for morning or evening emergence. In two separate studies in melon flies *Bactrocera cucurbitae* (Shimizu et al., 1997) and fruit flies *D. melanogaster* (Kumar et al., 2006), it was found that flies selected for faster and slower pre-adult development, or for morning and evening adult emergence, show correlated changes in their circadian periodicity.

In insects, inter-specific competition due to ephemeral habitats such as decaying fruits, mushrooms, and dung is a major factor driving their evolution (Krijger et al., 2001). Faster development might provide adult insects with several advantages such as opportunity for early mating and reproduction. In other words flies that emerge early may have more opportunity of reproducing than those emerging later. Therefore, the observations on the links between circadian clocks and pre-adult development time can be taken to suggest that circadian clocks have some adaptive significance, and it is likely that some insect species accrue greater fitness advantage by speeding up their pre-adult development. Indeed, faster development in *Drosophila* species is known to provide greater competitive ability (Krijger et al., 2001), however, conscious selection for faster

development over several generations is found to be associated with trade-offs such as enhanced larval mortality and reduced adult lifespan (Prasad and Joshi, 2003). These trade-offs are inevitable because time is a fundamental aspect of insect development, and each stage of development, from cell division to the formation of phenotypes needs to be timed appropriately by development clocks (Moss, 2007).

In social insects such as ants and honeybees, workers make the supreme sacrifice of forgoing their own reproduction in order to help the queen raise her offspring, and it is believed that this strategy is successful only because the workers also derive some inclusive fitness advantage by rearing their brothers and sisters as opposed to raising their own offspring (Hölldobler and Wilson, 1990). In ants, the first batch of eggs is raised solely by the founder queen, and the queen utilizes her body fat and wing muscles for the energy requirements (Hölldobler and Wilson, 1990). Rearing the first batch of eggs is likely to help the queen in producing workers who could be recruited in future as foragers to bring in resources for the colony (Cassill, 2002). In social insects it is known that colony resources act as a signal for the production of virgin males and females (Brian, 1978), and hence the reproductive fitness of the colony is solely dependent upon the development of workers. In addition, presence of workers reduces time spent by the queen in attending to the brood. For example in *Solenopsis invicta*, time spent by a freshly mated queen in brood care is normally ~65%, which is reduced to ~1% after the adults emerge out of pupae. This may help the queen in laying more eggs, and in taking care of their development. Therefore, in ants faster development of the first batch of eggs is likely to promote success of the colony, and ensure timely presence of workers, which

in turn will ensure the quantity and quality of colony resources (Hölldobler and Wilson 1990).

Many independent studies have provided evidence that circadian clocks provide adaptive advantage to the owners in the state of "circadian resonance", when clock periodicity matches periodicity of the external environmental cycles (Pittendrigh and Minis, 1972; Von Saint-Paul and Aschoff, 1978; Klarsfeld and Rouyer, 1998; Ouyang et al., 1998; Sharma, 2003; Woelfle et al., 2004; Wyse et al., 2010). For example, adult lifespan of fruit flies *D. melanogaster* (Pittendrigh and Minis, 1972), and blow flies *Phormia terraenovae* (Von Saint-Paul and Aschoff, 1978) is significantly enhanced under resonating 24 hr LD cycles compared to non-resonating non-24 hr LD cycles. In a separate study on pitcher-plant mosquito *Wyeomyia smithii*, it was shown that the reproductive output is significantly enhanced under resonating LD cycles compared to non-resonating LD cycles (Emerson et al., 2008). In certain plants too, vegetative growth is known to be maximized under the state of circadian resonance (Highkin and Hanson, 1954). However, it is difficult to conclude that the fitness advantages observed in these studies are due to circadian resonance because the organisms used in these studies already lived under a 24 hr LD cycle, and therefore the fitness advantages under *T*24 may be due to native environment advantage, as it is known that organisms perform better in their native environment than in foreign environments (Leimu and Fischer, 2008). It would therefore be interesting to study fitness consequence of circadian resonance in organisms living under constant conditions.

The tropical *Camponotus* ants *Camponotus compressus* (night active), and *C. paria* (day active), live in dark compartments of deep underground nests, where abiotic

factors such as light, temperature, and humidity are more or less constant (Hölldobler and Wilson, 1990). Yet these ants show considerable level of developmental plasticity, the pre-adult development time of these ants is reduced under LL and LD (relative to DD) by ~38% and ~29%, respectively, in *C. compressus*, and by ~35% and ~28% in *C. paria* (Lone and Sharma, 2008). Furthermore, these ants exhibit photoperiodic preference for pre-adult development; the night active species develops faster under longer nights, while the day active species develops faster in longer days (Lone et al., 2011). These ants are also known to exhibit considerable amount of circadian plasticity; they alter their circadian rhythms to match their roles in the colony (Sharma et al., 2004a-c). For example, nurses perform tasks all around the clock and are found to be arrhythmic; foragers who bring food for the colony are rhythmic, whereas workers that work as soldiers for the colony are arrhythmic. Interestingly, males and virgins are rhythmic, and remain in-phase during the mating season, which is critical for the precise timing of mating flight (Sharma, 2003).

The aim of the present study is to determine the effect of the interaction between circadian timing system and LD cycles on the adaptive fitness, measured in terms of the rate of pre-adult development, in two dark-dwelling sympatric species of *Camponotus* ants. For this we chose to assay pre-adult development time under three different LD cycles - *T*20 (LD 10:10 hr), *T*24 (LD 12:12 hr), and *T*28 (LD 14:14 hr).

8.3.2 Materials and Methods

Ground-dwelling carpenter ants *Camponotus* are genetically pre-programmed to dig deep into the soil to construct safe nests, wherein their progeny complete development (Hölldobler and Wilson, 1990). The colonies of *C. paria* are found more than one and

half feet deep under the soil, while those of *C. compressus* are located deeper (*Shahnaz Rehman Lone and Vijay Kumar Sharma, personal observation*). The *Camponotus* ants usually build their nests in forests; inside decomposed plants, and have evolved mutualism with aphids and coccids (Hölldobler and Wilson, 1990). Mating in these ants is a seasonal event - *C. compressus* and its sympatric partner *C. paria* undertake mating flights in the months of April-May, immediately after the first rains after a prolonged dry season. The workers carry soil from the interiors of the nest outside to make way for relatively bigger virgins and males. After mating, the queen digs deep into the soil to form nests. All these events occur in tandem, which suggests a seasonal life style in *Camponotus* ants.

For the present study, mated queens from two species of *Camponotus* ants (*C. paria* and *C. compressus*) were collected from Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore Campus (12° 58' N, 77° 40' E), soon after their nuptial flights. They were immediately transferred into petri plates (diameter × height = 90 mm x 15 mm), and introduced into three different LD cycles (10:10 hr, 12:12 hr, and 14:14 hr), created in two temperature controlled incubators (25.0 ± 0.5 ^{o}C; mean ± SD), placed additionally inside an environmentally controlled chamber - temperature (~25.0 ^{o}C) and relative humidity (~75%). Temperature inside the incubators was monitored at every six hour interval, throughout the experiment, and was found to be fairly stable. For creating three different photoperiodic regimes, two incubators - one (*Inc-I*) constantly illuminated by fluorescent light of 500 ± 20 lux intensity, and the other (*Inc-II*) by dim red light ($\lambda >$ 650 nm) were used. The mean temperatures of the two incubators were comparable. Colonies were moved at specific time of the day from light to dark and dark to light

incubators, to create different *T* cycles (10:10 hr, 12:12 hr and 14:14 hr). For example, to create 10:10 hr LD cycles (*T*20), replicate ant colonies were first kept for 10 hr in *Inc-I*, and then to *Inc-II* for the subsequent 10 hr, after which they were transferred back to *Inc-I*. This was repeated until the completion of the study. A similar protocol was followed to create other *T* cycles LD 12:12 hr (*T*24) and LD 14:14 hr (*T*28). Food in the form of Bhatkar diet (Bhatkar and Whitcomb, 1970), and 10% dilute honey solution was provided *ad libitum*. Incidentally *Camponotus* queens were found to eat rarely under laboratory colonies, and no obvious rhythm in their feeding pattern was observed.

All our studies were conducted on the first batch of eggs, laid by the foundress queen, because it is this batch, which is reared solely by the queens (Hölldobler and Wilson, 1990). This is the only generation which does not represent the standard reference for development of different castes in an ant colony. This particular generation of eggs were primarily chosen in order to avoid the influence of confounding factors underlying sociality. The number of brood, pooled across different colonies, for the two species *C. compressus* and *C. paria* (prefix CC and CP respectively) were: 33 CCLD10:10 (from 6 colonies), 36 CCLD12:12 (from 6 colonies), 55 CCLD14:14 (from 10 colonies), 21 CPLD10:10 (from 6 colonies), 16 CPLD12:12 (from 5 colonies), and 30 CPLD14:14 (from 9 colonies).

The development time of larval and pupal stages was assayed by counting the number of eggs, larvae, pupae and adults present every six hours. Pupal stage in *C. compressus* (5-7.0 mm long and 2-3 mm wide) and *C. paria* (~5.0 mm long and 1.70-1.90 mm wide) is well defined, the pupae is initially white in color, and with time turns brown. The larval development time was estimated by calculating the duration between

the egg stage and the formation of pupae. Pupal developmental time was estimated as the duration between the formation of pupae and the emergence of adults, and pre-adult development time as the duration from the egg stage to adult emergence. The eggs, larvae and pupae have distinct morphology, which helped us in scoring the individuals in these stages unambiguously.

8.3.3 Results

8.3.3.1 ***Effect of light/dark cycles on the pre-adult development time:*** The pre-adult development time of both species of ants (*C. compressus* and *C. paria*) is faster under *T*24 than in *T*20 and *T*28 (Figure 1c; Tables 1, 2). ANOVA on the pre-adult development time data revealed that the effect of *T* cycle is statistically significant ($F_{2,185} = 31.10$, $p < 0.0001$), whereas the effects of species ($F_{1,185} = 0.56$, $p = 0.45$), and *T* cycle × species interaction ($F_{2,185} = 1.56$, $p = 0.21$) are statistically not significant (Tables 1;2). Post-hoc multiple comparisons using Tukey's test revealed that in *C. compressus* development time under *T*24 is significantly shorter than those in *T*20 ($p < 0.0001$) and *T*28 ($p < 0.005$), and development time under *T*28 is significantly shorter than in *T*20 ($p < 0.001$). The development time of *C. paria* follows more or less a similar pattern, as, they develop as pre-adults faster under *T*24 compared to *T*20 ($p < 0.0001$) or *T*28 ($p < 0.05$), however, in this species the development time under *T*20 and *T*28 did not differ statistically ($p = 0.54$) (Figure 1c).

8.3.3.2 ***Effect of light/dark cycles on the larval duration:*** The larval duration in both the species showed a trend quite similar to that seen for the overall pre-adult development time. Pupation in both the species occurs earliest under *T*24, however, in *C. compressus* it occurs more or less simultaneously in *T*28 (Figure 1a; Tables 1; 2). ANOVA on the

larval duration data revealed that the effects of *T* cycle ($F_{2,185} = 47.58$, $p < 0.0001$), and *T* cycle × species interaction ($F_{2,185} = 11.84$, $p < 0.0001$) are statistically significant, whereas the effect of species ($F_{1,185} = 0.07$, $p = 0.77$) is statistically not significant (Tables 1 and 2). Post-hoc multiple comparisons showed that in *C. compressus* the larval duration under *T*24 and *T*28 is significant shorter than in *T*20 ($p < 0.0001$), and those under *T*24 and *T*28 did not differ statistically. In *C. paria*, larval duration under *T*24 is significantly shorter than those in *T*20 and *T*28 ($p < 0.0001$), while those under *T*20 and *T*28 do not differ statistically ($p = 0.81$) (Figure 1a). .

8.3.3.3 ***Effect of light/dark cycles on the pupal duration:*** ANOVA on the pupal duration data revealed that the effects of species ($F_{1,185} = 42.96$, $p < 0.0001$), *T* cycles ($F_{2,185} = 6.64$, $p < 0.001$), and *T* cycles × species interaction ($F_{2,185} = 14.90$, $p < 0.0001$) are statistically significant (Figure 1b; Tables 1;2). Post-hoc multiple comparisons revealed that, as pupae, *C. compressus* develops faster under *T24* compared to *T20* and *T28* ($p < 0.0001$), whereas pupal durations under *T20* and *T28* did not differ statistically ($p = 0.75$). In *C. paria,* the pupal duration did not differ statistically among the three *T* cycles ($p > 0.05$) (Figure 1b). .

8.3.4 Discussion

Two sympatric species of *Camponotus* ants develop as pre-adults faster under *T*24 compared to two non-24 hr LD cycles *T*20 and *T*28. Under *T*24, *C. compressus* develop faster by ~10 days and ~4 days relative to *T*20 and *T*28 respectively, while *C. paria*

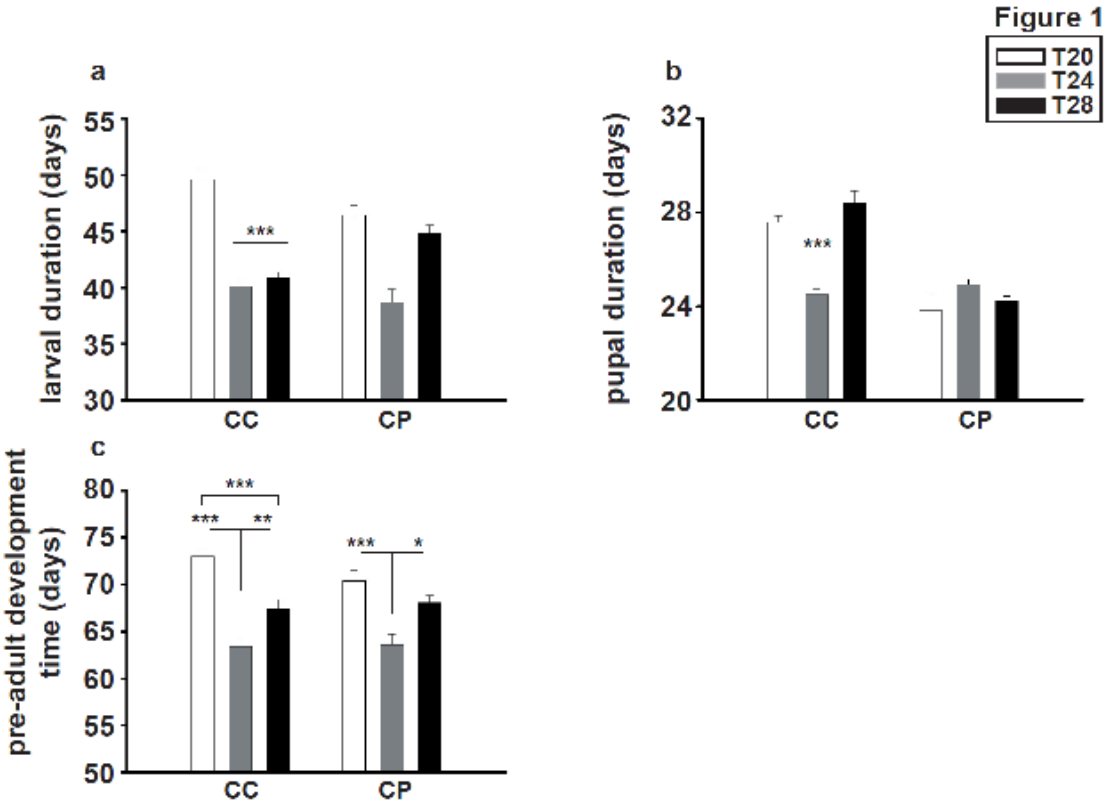


Figure 1 ***Pre-adult (egg-to-adult) time and larval (egg-to pupae) and pupal (larval-to-adult) durations under T20, T24, T28.*** In *C. compressus* (CC), the duration of larval stage is shorter in *T*24 and *T28* compared to that in *T20*, while in *C. paria* (CP), the larval duration is shorter in *T*24 than those in *T28* and *T20* (**a**). In *C. compressus* pupal duration is shorter in T24 compared to *T*20 and *T*28, whereas in *C. paria* pupal duration does not differ under the three LD cycles (b). In *C. compressus* (CC) and in *C. paria* (CP), development time in *T*24 is shorter than those in *T20* and *T28* (c). Error bars are standard error means around means. CC and CP refers to *C. compressus* and *C. paria*, respectively. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 1 Results of ANOVA on the pre-adult, larval and pupal duration data

Pre-adult duration	*df* effect	*MS* effect	*df* error	*MS* error	*F*	*p*-level
Species (S)	1	7052	185	12,599	00.56	= 0.45
T cycle (T)	2	391,835	185	12,599	31.10	< 0.0001
S x T	2	19,688	185	12,599	01.56	= 0.21
Larval duration						
Species (S)	1	865	185	11.032	00.07	= 0.77
T cycle (T)	2	525,005	185	11.032	47.58	< 0.0001
S x T	2	130,670	185	11,032	11.84	< 0.0001
Pupal duration						
Species (S)	1	121,830	185	2835	42.96	< 0.0001
T cycle (T)	2	018,832	185	2835	06.64	< 0.001
S x T	2	042,263	185	2835	14.90	< 0.0001

Table 1 Results ANOVA on the duration of different stages of pre-adult development estimated from the egg stage

Table 2 Mean pre-adult, larval, and pupal durations in two species of *Camponotus* ants

Species	Pre-pupal duration (days) mean ± SD	Pupation time (days) mean ± SD	Pre-adult development (days) mean ± SD
C. compressus			
T 20	49.54 ± 05.65	27.55 ± 01.26	73.02 ± 02.86
T 24	40.08 ± 04.12	24.50 ± 01.35	63.39 ± 04.46
T 28	40.83 ± 04.00	28.42 ± 02.98	67.45 ± 05.70
C. punia			
LD10:14	46.40 ± 04.01	23.80 ± 03.45	70.39 ± 04.95
LD12:12	38.62 ± 04.75	24.93 ± 00.92	63.56 ± 04.70
LD14:10	44.65 ± 03.70	24.25 ± 00.67	68.11 ± 03.67

Table 2 Details of timing of initiation and duration of different developmental stages (in days) of *C. compressus* and *C. paria* under three light regimes.

develop faster by ~5 and ~7 days compared to *T*20 and *T*28 respectively (Figure 1c; Tables 1; 2). While both species show similar trends in their larval developmental duration (Figure 1a), as the overall pre-adult development time, the trend of pupal development time is quite different (except for *C. compressus* under *T*28; Figure 1b). Pupal durations in *C. compressus* is shorter under *T*24 than in *T*20 and *T*28, however, in *C. paria* it remains unchanged in the three *T* cycles, suggesting that circadian resonance-mediated effects on development time is primarily due to developmental changes during the larval stages, however, in *C. compressus* it is also due to changes in the pupal duration. Faster development would imply higher adaptive advantage, which suggests that *Camponotus* ants gain greater fitness advantage by developing faster under *T*24. *Camponotus* ants normally develop in dark underground colonies wherein the only time cue present is in the form of brood care. It is therefore unlikely that these ants would have evolved to develop optimally under 12:12 hr LD cycles. Therefore, the most parsimonious explanation for faster development in *T*24 would be that these ants are in a state of circadian resonance wherein their circadian clocks are in perfect coordination between external LD cycles, and several metabolic and behavioural cycles. The results of our study are consistent with those from an earlier study on flesh fly *Sarcophaga argyrostoma*, which exhibits robust pupal ecolsion rhythm, and when subjected to

resonating *T* cycles (*T*24 or *T*48) develop faster than non-resonating *T* cycles (*T*36 or *T*60) (Saunders 1976, 2002).

Some stages in the life cycle of insects are more sensitive to environmental LD cycles than others (Spieth et al., 2004). In our study too, the LD cycle effect on development time is predominantly mediated through the reduction in larval duration (Figure 1a), however, in *C. compressus* the duration of pupal stage is also reduced (Figure 1b). The *Camponotus* ants are never exposed to external LD cycles during their pre-adult stages, and therefore these ants are unlikely to use LD cycles as time cue for development. However, under natural conditions behavioral and physiological rhythms of workers are entrained to the external LD cycles (Sharma et al., 2004b), and it is likely that these workers in turn entrain circadian rhythms of brood, and other members who are restricted to the constant conditions of the colony (*Shahnaz Rahman Lone and Vijay Kumar Sharma, unpublished data*). It is therefore expected that caretakers will be more effective in fine-tuning the circadian clocks of brood through social interactions possibly associated with transfer of food, thereby having a greater impact on the developmental rates than the LD cycles. Therefore, the effect of LD cycle on the pre-adult development time in *Camponotus* ants is likely to be mediated through feeding by the queen and/or caretaker workers. It is known from previous studies that feeding cycles can synchronize the circadian clocks of honeybees (Frisch and Aschoff, 1987), house sparrows (Hau and Gwinner, 1992), hamsters, mice, and rats (Mistlberger, 2009). Besides feeding, daily migration of brood, by the caretakers, to more peripheral compartments of the nest, illuminated by light, may also contribute to the LD mediated regulation of pre-adult development (Hölldobler and Wilson, 1990).

It is believed that LD cycles modulate pre-adult development time in insects; shorter LD cycles speed-up development, and longer cycles slow it down, provided the circadian clocks in these insects entrain to the imposed LD cycles (Paranjpe et al., 2005). Based on this proposal the *Camponotus* ants would be expected to develop fastest under *T*20, followed by *T*24, and then in *T*28. In case circadian clocks of the ants do not entrain to non-24 hr LD cycles, and free-run with shorter than 24 hr periodicity, development will be slower under *T*24 compared to *T*20 and *T*28, and if the clocks free-run with longer than 24 hr periodicity, development will be faster under *T*24 than in *T*20 and *T*28. In other words, in case ant clocks entrain to the non-24 hr LD cycles, we expect ants to develop at a rate proportional to the length of the LD cycle, and if they do not, and free-run with circadian periodicity, we expect them to develop faster or slower under *T*24 than *T*20 and *T*28. If entrainment to non-24 hr LD cycles is asymmetric, i.e. the limits of entrainment of the clocks includes only one of the *T* cycle (*T*20 or *T*28), we would expect development to occur at different rates under *T*20 and *T*28. The results of our study indicates that in *C. compressus* and *C. paria* development is slower under both the non-24 hr LD cycles, suggesting that circadian clocks of *Camponotus* ants do not entrain to *T*20 and *T*28 LD cycle, and free-run with longer than 24 hr period. Given that the developmental response to LD cycles is marginally asymmetric in both the species, we speculate that there may be some species-specific differences in the development timer as well as in the circadian clocks.

In *C. elegans*, the developmental timer is governed by mechanisms involving heterchronic genes, and signals that cause the expression or repression of genetic switches (Moss, 2007). The key components of the switch comprise stage-specific

heterochronic genes such as *lin14*, which causes developmental delays in the nematode *C. elegans*. The expression of the gene *lin4*, which binds to the 3' untranslated region of *lin14*, has been shown to prevent developmental delays (Moss, 2007). In addition, the oscillating gene *lin42*, a homolog of *Drosophila per* gene, is shown to regulate the expression of both *lin4* and *lin14*, through yet unknown mechanisms. It is also not known yet how light signals modulate the expression of the stage-specific heterochronic genes. However, what is known is exposure to darkness alters ecdysteriod levels in insects (Li and White, 2003). The steroid hormone ecdysone plays a key role in the molting of larval stages, which is known to set the timing of pre-adult stages by binding to nuclear hormone receptors, and in turn regulate the expression of developmental genes (Giebultowicz et al., 2008). It is therefore possible that genes involved in numerous signaling pathways, such as notch signaling, insulin signaling and ecdysteroid signaling pathways govern light-dependent developmental regulations in ants (Mensch et al., 2008). Further, it is possible that in *Camponotus* ants exposure to non-24 hr LD cycles results in a desynchrony among the light sensitive and clock-controlled cyclic processes, which may alter the expression of heterochronic genes and/or their ability to bind to the target sites resulting in an overall change in development time.

In summary, the result of my study suggest that two sympatric species of *Camponotus* ants develop as pre-adults faster under *T*24 compared to *T*20 or *T*28 LD cycles. We speculate that under 24 hr LD cycles *Camponotus* are in a state of circadian resonance, wherein their clock periodicity matches that of the LD cycle, to bring about a perfect coordination among environmental LD cycles, and metabolic and behavioral cycles. This enhances the fitness of the ants as they speed up their pre-adult

development. These results suggest that *Camponotus* ants have the ability to respond preferentially to 24 hr LD cycle, endorsing the adaptive importance of circadian clocks. Their brood care behavior appears to play a key role in eliciting such differential response to *T* cycle.

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