Characterizing activity-rest rhythms of three related ant species, and examining the effect of social interaction on sleep in *Drosophila melanogaster*

Thesis submitted in partial fulfillment for the degree of

Master of Science

By

Goirik Gupta

Chronobiology Laboratory
Evolutionary and Organismal Biology Unit
Jawaharlal Nehru Centre for Advanced Scientific Research
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DECLARATION

I hereby declare that the contents presented in this thesis entitled 'Characterizing activity-rest rhythms of three related ant species, and examining the effect of social interaction on sleep in *Drosophila melanogaster*' submitted to Jawaharlal Nehru Centre for Advanced Scientific Research for fulfillment of the Master's degree is to the best of my knowledge and belief entirely my original work carried out under the guidance of Prof. Vijay Kumar Sharma in Chronobiology Laboratory, Evolutionary and Organismal Biology Unit of the Centre.

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

Date: 17/10/2016

Place: Bangalore. [Goirik Gupta]

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Professor and Chairman

Date: 17/10/2016

CERTIFICATE

This is to certify that the work described in the thesis entitled 'Characterizing activity-rest rhythms of three related ant species, and examining the effect of social interaction on sleep in *Drosophila melanogaster*' is the result of investigations undertaken by Mr. Goirik Gupta under my supervision in the Evolutionary and Organismal Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064 (India), 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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Table of contents

Chapter 1

Introduction

Introduction:

The rotation of the earth about its axis and its revolution around the sun brings about daily and annual cycles in geophysical processes such as light and temperature. Almost all organisms on earth excluding those that live under constant aperiodic conditions (such as organisms living in caves, subterranean habitats and deep seas) need to be able to cope with these changes, and are therefore believed to have evolved mechanisms to synchronize their behavioural, physiological and biochemical processes to the periodically changing environment with the help of endogenous time-keeping mechanisms or clocks (Vaze & Sharma, 2013).

The time-keeping mechanism or clock that has a period close to 24-h is called a circadian clock (*circa*=almost, *diem*=day). Although daily rhythms were observed in various processes in organisms, they were initially considered to be direct responses to the daily changes in environmental variables. However, studies carried out as early as 1729 by Jacques de Mairan (de Mairan, 1729) on the heliotrope plant *(Mimosa pudica)* indicated that these rhythms were, in fact, endogenously generated, as they persisted under constant conditions. In later years, many rhythmic behaviours, in a wide variety of organisms were shown to be endogenous and self-sustained (Pittendrigh, 1960). For biological oscillators to be considered as circadian clocks, they need to satisfy certain criteria such as:

1. They should be endogenously generated and should continue to free-run with a period close to 24-h in the absence of periodic time cues or zeitgebers (zeit=time, geber= giver) such as light, temperature, and social interaction.

2. They should be capable of entraining (maintaining a stable and reproducible phase relationship) to various zeitgebers.
3. They should be temperature compensated, i.e. that the organisms actively compensate for changes in period brought about by temperature, thereby preventing significant alteration of period across physiologically tolerable range of temperatures (Pittendrigh, 1960).

Though it is widely agreed that circadian clocks confer some adaptive advantage to organisms, the nature of this advantage is not entirely clear. The adaptive advantage conferred by clocks may be considered to be of two types: extrinsic and intrinsic (Sharma, 2003). Organisms living under periodic conditions synchronize their behavioural, physiological and biochemical processes to the periodically changing environment in order to exploit opportunities and avert risks, thereby providing an 'extrinsic' advantage (Vaze and Sharma, 2013). In contrast, organisms living under aperiodic conditions for many generations such as cave-dwelling millipedes also continue to exhibit circadian rhythms (Koilraj et al., 2000). In such cases, it is hypothesized that possessing functional clocks may probably help in coordinating metabolic processes with one another, therefore providing 'intrinsic' advantage to the organisms (Vaze and Sharma, 2013). Thus, circadian clocks that evolved in periodic environments may continue to be useful in organisms that live in constant conditions.

Influence of zeitgebers on circadian clocks

Light is considered to be the most potent zeitgeber that influences the circadian clock and entrains circadian rhythms. However, there is increasing evidence to suggest that non-photic environmental cues such as temperature in wood ants (Rosengren, 1977; North, 1993), carpenter ants (Roces and

Nünez, 1989) and Drosophila (Matsumoto et al., 1998); food in rats (Damiola et al., 2000) and social interaction in Killifish (Kavaliers, 1980), bats (Marimuthu et al., 1981), sparrows (Menaker and Eskin, 1966; Gwinner et al., 1967), beavers (Bovet and Oertli, 1974), Drosophila (Levine et al., 2002) among others can also influence the circadian clocks. Nonetheless, studies on the effects of such time cues and the mechanisms via which they influence the circadian clock are not as extensive as that on light. Moreover, their interactions with photic cues, as would be expected in the natural environment, are also not adequately documented.

Although social cues do influence circadian clocks even in insects such as *Drosophila,* which are not eusocial, these effects tend to be greater on eusocial insects such as ants or bees. Hence, in this study, we examined effects of social interactions on sleep in fruit flies and conducted some preliminary recording of ant species upon which we intend to do further work on social interactions and their effects on circadian clocks.

Introduction to work on ants

Eusociality and social influences on circadian timing systems of eusocial insects

The social environment of an organism can be defined as the sum of social interactions, cues and signals encountered by an organism including interactions with conspecifics at different stages of life (egg, larvae, pupa and adult) through various signals such as acoustic signals or volatile pheromones. The presence of such social factors may influence the strength, expression and development of circadian rhythms (Eban-Rothschild & Bloch, 2012). In eusocial insects, the social environment

influences every aspect of life including circadian time-keeping. Eusociality is defined by three specific traits (Wilson, 1971):

1. Caring for offspring of the queen by other members of the colony.
2. Division of labor, where the female workers are sterile and are involved in raising the brood of fertile ones (the queen).
3. Overlap of two or more generations.

Studying the role of social cues as zeitgebers in eusocial organisms can be very informative since an entire colony of eusocial insects function as an individual entity due to the extent of division of labor that is seen. This can be compared to the multiple organs residing within a single non-eusocial organism. Just as in a non-eusocial individual, where there are specific organs for perceiving time cues from the environment, and these cues are conveyed to other organs through communication between them, in a colony of eusocial insects, only certain members perceive external time cues (Hölldobler and Wilson, 1990), and these are perhaps conveyed to other members of the colony through social interactions. Therefore, eusocial organisms serve as ideal model systems to study the role of social cues as entraining stimuli.

Studies on honeybees (*Apis mellifera*)

Extensive studies have been carried out on honeybees (*Apis mellifera*), to try and understand how social cues influence circadian rhythms. Honeybee colonies have age polyethism (age based division of labour) where, as the workers age post eclosion, their task in the hive keeps changing. The timeline is as follows: between the ages of 0-2 days they are involved in nest cleaning, following

which between the age of 2-11 days they act as nurses and are responsible for feeding and taking care of brood and attending to the queen, this is followed by food storage, receiving nectar, storing nectar and packing pollen from day 11 up to approximately day 20, post which they start foraging (Seeley, 1995).

The earliest studies showed no discernable rhythm in the activity or oxygen consumption of the workers that took care of the brood and queen (Lindauer, 1952; Stussi, 1972), which was further confirmed by studies carried out by Spangler (1972), Crailsheim et al. (1996) and Moore et al. (1998). The older bees on the other hand showed a clear free-running rhythm in constant conditions (Spangler, 1972) and the foragers (i.e., the bees post the age of 20 days) showed clear diurnal rhythm in activity (Crailsheim et al., 1996). This led researchers to believe that the difference in activity profile could be due to the fact that foragers experience variation in light and temperature but nurses do not. However, the nurses appeared to be arrhythmic even under strong light and temperature cycles, whereas foragers showed strong rhythms even in the dark (Moore, 2001; Rubin et al., 2006; Shemesh et al., 2007, 2010).

It was later observed that the mean onset of locomotor activity rhythm occurs around day 7 or 8 (Toma et al., 2000). However, though the young bees did not show any discernable rhythms in activity or oxygen consumption, they both showed circadian oscillations in the brain in *per* mRNA levels in DD, with a peak occurring in the subjective night, with expression levels lower than those seen in forager bees (Toma et al., 2000). Thus, the circadian system of nurses was hypothesized to develop with age but when nurses were shifted to the laboratory, they showed strong circadian

rhythms of activity indicating that their circadian systems are not underdeveloped (Bloch et al., 2001; Shemesh et al., 2007, 2010). Even honeybees that were three days old when separated from the hive showed robust rhythms in activity post transfer to constant conditions in the lab (Eban-Rothschild et al., 2012).

Another possibility which has been considered is that may be *Per* and *Cry* mRNA oscillate in only a few pacemaker cells in the nurse brain, whereas cycling stops in most other cells and restarts once the nurse switches to activities with no direct contact with the brood (reviewed in Eban-Rothschild and Bloch, 2012). This hypothesis is supported by the experimental results where nurse-age bees kept in broodless combs, inside or outside the hive, showed robust circadian rhythms in activity and clock gene expression (Shemesh et al., 2010), thus implicating social cues from conspecifics in modulation of circadian rhythms. Further experiments indicated that volatile odors and not direct contact was involved in the ontogeny of circadian rhythms (Eban-Rothschild et al., 2012). Flagella present on the antennae have been implicated in communication of brood signals as honeybee nurses without flagella showed circadian rhythm in brood care with increased activity during day time (Nagari and Bloch, 2012), but the exact brood signals are yet to be established.

Studies have also been carried out on honeybees where the potential of social cues to bring about entrainment in adult rhythmic individuals has been examined. Forager bees trained to feed at one time were transferred to another hive where the bees were trained to feed at a different time, with the result that all the bees foraged at both times, suggesting the influence of social cues on the phase of foraging rhythm (Medugorac and Lindauer, 1967). Similarly, when groups of 50 worker bees

entrained to light-dark cycles 12 h out of phase with each other were made to interact, they adopted a coordinated oxygen consumption rhythm at a phase halfway between the phases of the original groups (Southwick and Moritz, 1987).

Moritz and Kryger (1994) showed that temperature fluctuations of at least 6°C may function as a zeitgeber, bringing about permanent phase-shifts in the free-running group rhythms. They proposed that social groups of honeybees synchronize individual worker bee rhythms through localized temperature fluctuations. Moritz and Sakofski also observed that the queen played a significant role in the synchronization of group rhythms (Moritz and Sakofski, 1991).

The above-mentioned studies apply a methodical approach to the study of influence of social cues on circadian rhythms and have therefore been successful in unearthing a wealth of information on the subject. On the other hand, it is intriguing to note that, circadian timing systems, and the role of social cues in another eusocial insect, the ant, which along with the termites makes up half the terrestrial biomass (Wilson and Hölldobler, 2005) has never been studied with such rigor.

Studies on Ants

Ants belong to the order Hymenoptera. There is extensive division of labor among ants in a colony as is seen in other eusocial insects. The adults can mainly be divided into two groups: the reproductives, which include the queen and the winged drones, and are responsible for increasing the number of individuals in a colony; and the workers, which are responsible for foraging, protection of nest, and maintenance of the nest. Division of labor among worker ants can either be associated

with age-related polyethism, as is seen in honeybees, or with physical polymorphism (Hölldobler and Wilson, 1990).

Studies carried out in the past have indicated that the circadian system in ants are influenced by light, temperature and social cues as zeitgebers, though to the best of our knowledge there are only a few studies that look at influence of social cues on the circadian system and these have been carried out in our lab. McCluskey, in the 1960's carried out some of the earliest documented studies relating to circadian rhythms in locomotor activity of ants. He characterized the locomotor activity of Argentine ants (*Iridomyrmex humilis*) and harvester ants (*Veromessor andrei*) in alternating light and dark regimes (LD) and also recorded their activity under constant conditions (DD) to determine whether the rhythms were endogenous. He found that the males of both species of ant showed a sharp daily activity peak under LD conditions and the rhythms also persisted under DD (McCluskey, 1963). However, he did not find any such rhythm in females of either species. He also characterized the activity rhythms of males of three other species of ants, the bullet ant (*Paraponera clavata*), red ant (*Solenopsis saevissima*) and a species of carpenter ant (*Camponotus clariothorax*) and observed that though all three ant types displayed peak of activity at different phases during the LD regime, only the bullet ant and red ant showed persistent rhythms under constant conditions (DD; McCluskey, 1965). Circadian rhythms of locomotor activity were also recorded in individual workers of wood ants (*Formica rufa*) under different LD regimes and it was observed that they entrained better under LD18:06 conditions (18 h of light and 6 h of darkness) as opposed to 12:12-h LD (12 h of light and 12 h of darkness) (North, 1987).

Temperature cues also influence circadian rhythms in ants. Studies showed that temperature cycles were able to entrain locomotor activity rhythm in wood ants *Formica polyctena* (Rosengren, 1977), *Formica rufa* (North, 1993), and also entrained brood translocation rhythm in a species of carpenter ants *Camponotus mus* (Roces and Nünez, 1989, 1995). It was also seen that a combination of light and temperature cycles led to the activity rhythm, which was unimodal under separate LD and temperature cycles, to become bimodal. The peak of activity occurred at changes from light to dark and changes in temperature (North, 1993).

A few studies have examined the molecular machinery of the ant circadian clock. One such study was carried out on harvester ants, which show age-dependent polyethism, similar to that seen in honeybees. The workers of the harvester ants perform tasks based on their age and move from the interior to the exterior of the nest, as they grow older (Gordon, 2000). Initial results of the study indicated that harvester ants have task-specific expression of two clock genes, *period* and *cycle* and the endogenous fluctuations in these genes are correlated with daily rhythms of ants (Ingram et al., 2009). Similarly, Ingram et al. (2012), used the fire ant genome (Wurm et al., 2011) to find clock gene orthologs and also looked at their expression patterns. The ant *cryptochrome* (*Cry-m*) is an ortholog of the mammalian-type (*Cry-m*), rather than Drosophila-like protein (*Cry-d*) and also the expression patterns of *period* and *Cry-m* mRNA levels are similar to those of honeybees. These results indicate that the circadian clock of ants has more similarities with honeybees and mammals than to that of *Drosophila*.

Background for present study

Previously, work done in this laboratory focused on two different species of carpenter ants viz., *Camponotus compressus* and *Camponotus paria*. These studies characterized the activity-rest rhythm of different castes of *Camponotus compressus* workers under different light regimes (LD and DD; Sharma et al., 2004a). Results indicated that the locomotor activity of the major workers entrain to laboratory 12:12-h LD cycles and free-run under constant darkness (DD) (Sharma et al., 2004a). The media workers also entrained to the LD cycles with most individuals being active during the dark phase while a few were active during the light phase. The minor workers neither entrained to the LD cycles nor did they free-run in DD (Sharma et al., 2004a). However, the sample size for recording of minor workers was very low due to extremely high mortality of these workers within the first few days of recording and may not represent a complete description of rhythms in minor workers from the colony. Under constant conditions, it was seen that the free-running period of the media workers tend to drastically change post 6-9 days of recording, with some showing period lengthening and some showing shortening, while some others displayed large phase shifts, indicating that they may be functioning as shift-workers inside the colony (Sharma et al., 2004b). The activity pattern of the different castes of ants seemed to vary directly with the work they performed. This was also inferred from another study, which showed that the queen of this species of ants was found to be arrhythmic during the egg-laying period, following which it regained rhythmicity (Sharma et al., 2004c). Thus, these studies indicate that the circadian rhythms of these social insects depend on their respective roles and tasks in the colony (Sharma et al., 2004c).

It has also been seen that circadian clocks control timing of mating flights of the reproductive individuals (queens) of *Camponotus compressus* and *Camponotus paria*, perhaps by maintaining appropriate phase-relationships between the sexual castes, and with the cyclic external environment (Lone et al., 2010). The pre-adult fitness in both these species was also influenced by light such that they developed fastest under constant light (LL) conditions followed by 12:12-h LD cycles and slowest under DD conditions (Lone et al., 2008). The pre-adult viability of both species was also higher under LL and LD compared to DD, suggesting that these dark-dwelling species of carpenter ants may use environmental LD cycles to modulate key life-history traits, such as pre-adult development time and viability (Lone et al., 2008). A study on *Camponotus paria* indicates that cyclic social interaction can serve as a zeitgeber for circadian timing systems in the given species (Lone et al., 2011a). In this study, workers were maintained under opposing LD12:12 regimes, following which one group (hosts) were transferred to DD while the other continued in its LD regime (visitor). Post few days in LD, the host individuals were introduced to visitors for 12-h every day for a number of days and were then transferred to DD where their activity was recorded. The results displayed that cyclic presence of the visitors led to the entrainment of the activity rhythm of the host individuals. Depending upon whether the interactions were pair-wise or between a visitor and a group of host workers and queens, the cues were interpreted differently. In case of one-on-one interaction the hosts interpreted the time of interaction to be their subjective day, whereas in case of group-wise interaction the hosts considered the time to be their subjective night (Lone et al., 2011a).

The various castes of ants may be differentially exposed to external cues and are often exposed to multiple cues at the same time. As mentioned previously, a combination of zeitgebers can have a significantly different effect on the circadian system of ants as opposed to a single zeitgeber in

isolation (North, 1993) and therefore social communication may lead to a somewhat different influence on the circadian timing system, as opposed to when there is only one cycling zeitgeber. Therefore, it is essential to first characterize the circadian activity rhythm under the influence of each of the various zeitgebers such as light, temperature and social interaction individually followed by multiple combinations. Additionally, comparing a particular behaviour across species gives us a better understanding of the characteristics and functional significance of the behaviour.

With this long-term goal in mind I characterized the activity-rest rhythms of different castes from three related species of carpenter ants, *Camponotus sericues, Camponotus paria and Camponotus compressus*. Though the activity-rest rhythm of the different castes of one of the species, *Camponotus compressus* has already been studied previously, the present study was carried out using a different recording system. Furthermore, we could possibly augment previous results, which were affected by high mortality rates or low sample sizes so as to obtain more definitive results.

Introduction to work on *Drosophila*

The second part of my thesis deals with the influence of social cues on sleep in *Drosophila melanogaster*. Though *Drosophila melanogaster* are considered to be solitary or facultatively gregarious, they do show a variety of interactions with conspecifics. These interactions include contact with mates, offsprings or conspecifics, with whom they compete for food and mates, and exchange information regarding ideal food surfaces for oviposition (Battesti et al., 2012). They also

possess circadian systems and sleep machinery homologous to mammalian systems, and their circadian rhythms as well as sleep patterns are known to be affected by the social cues in their environment (Levine et al., 2002; Ganguly-Fitzgerald et al., 2006; Lone et al., 2016). However, there remain gaps in our understanding about how social interactions affect sleep, and whether there is a critical age at which these effects are most profound.

Studies on the effects of social cues on circadian systems in *Drosophila*

A number of studies in recent years have demonstrated the influence of social cues on the circadian system and sleep in *Drosophila melanogaster*. In 2002, Levine et al. showed that fruit flies that transmit as well as receive olfactory cues can influence the circadian phase of locomotor activity rhythm. The authors observed that the circadian phases (peak of activity) of locomotor activity rhythm were more coherent with one another in flies maintained in groups rather than in isolation. They also found that housing arrhythmic mutants such as per^{0} flies along with wild-type flies resulted in lower synchrony among wild-type flies. The effect of social cues on synchrony of circadian rhythms was seen even without the presence of conspecifics but with merely olfactory signals from flies in a separate chamber, suggesting that olfactory cues are the major sensory signals involved in such effects. Though presence-absence (PA) cycles of conspecific visitors could not entrain host fruit flies, they were found to alter the phase synchrony of the host rhythms (Lone et al., 2011b). However, the extent to which phase synchrony is affected depends on the time of interaction. Additionally, it was seen that such effects were independent of the sex of the visitors since both male and female visitors showed similar effects on phase synchrony (Lone et al., 2011b).

In addition to the effects of social cues on synchrony of circadian rhythms between individuals, such cues may alter the circadian activity profiles of flies during the interaction itself. For instance, Fujii et al., (2007) found that male-female pairs showed greater nighttime activity during socio-sexual interaction as compared to solitary flies or same-sex pairs. These results suggest that sexual interaction can have greatly different effects as compared to social interactions with the same sex. The distinct activity pattern in male-female pairs was also found to overlap considerably with close proximity encounters recorded between the heterosexual couples, which suggest that such activity may be due to courtship. Furthermore, it was found that the phase of the male circadian pattern was more important in determining the phase of the close-proximity rhythm when males and females from anti-phasic LD cycles were paired together. Additionally, males without a functional *per* gene did not show such courtship rhythms suggesting the importance of the circadian clock in such rhythms. Further experiments revealed that the presence of olfactory receptors as well as expression of clock genes in the antennae were necessary for courtship (Fujii et al., 2007). Moreover, Hamasaka et al. (2010) found that Neuropeptide F (NPF) negative LNds and DN1 neurons (which are components of neuronal clock circuitry in Drosophila along with another class called the LNv) are necessary for the persistence of the close-proximity rhythm while Fujii and Amrein (2010) showed that the LNvs are essential for the trough in activity of the male driven nocturnal sex drive.

However, when flies were isolated after pairing, they reverted back to their regular activity patterns in light-dark cycles. Hence, effects of socio-sexual interactions appear to be largely restricted to the duration of socio-sexual interaction and do not result in major long-term alterations in activity

patterns of flies. Nevertheless, Lone et al. (2011c) in a separate study found that males show reduced evening activity peak and lengthening of circadian period following such socio-sexual interactions, whereas females exhibited reduction in overall activity levels. In males, functional circadian clocks and olfactory system were found to be essential for the after-effects in circadian rhythms following socio-sexual interactions. On the other hand, the reduction in overall activity levels in females after socio-sexual interaction was seen in clock-less female mutants as well, suggesting that these after-effects are not mediated by the circadian clocks (Lone et al., 2011c).

Hanafusa et al. (2013) studied the influence of socio-sexual interactions on the circadian pacemaker neurons and found that clock protein cycling in DN1 neurons in the male brain was slightly increased by their partners when male and female flies with clocks of different periods (wild type and per^{s} flies) were paired. These results suggest that socio-sexual interactions can modulate the clock in DN1 neurons, which are involved in the regulation of close proximity interactions.

Studies have also shown that pheromone release from oenocytes and its detection by the olfactory system are both regulated by the circadian clock and the social environment of the flies (Krupp et al., 2008). Wild-type flies when housed with per^{0} mutants showed lower phase coherence, and reduction in the overall levels and amplitude of the clock gene oscillations in the oenocyte or the fly head. This change in clock gene expression was also associated with alterations in the temporal

profile of cuticular hydrocarbon secretions, thus suggesting that social environment of the male flies affects both central and peripheral clocks (Krupp et al., 2008).

Sleep, and effects of social interactions on sleep in *Drosophila melanogaster*

Sleep is an evolutionarily conserved biological phenomenon widely observed in higher organisms such as mammals. However, recent studies have also shown the presence of sleep like states in a number of non-mammalian organisms such as fish, worms and insects (reviewed in Eban-Rothschild et al., 2012).

Sleep is commonly defined by three behavioural criteria:

1. A quiescence period, with a species-specific posture/resting place.
2. Elevated response threshold.
3. Sleep rebound, post sleep deprivation (Homeostatic regulation mechanism) (Tobler, 1983).

Studies by Hendricks et al. (2000) provided evidence that suggested that sleep-like state is also seen in *Drosophila.* Fruit flies tend to choose a preferred location and become immobile for periods of up to 157-min at particular times of the day, during which time they are relatively unresponsive to sensory stimuli. This resting behaviour was also observed to be subject to homeostatic and circadian influences. Other similarities between sleep in insects and mammals include increased wakefulness when exposed to caffeine and increased sleep when exposed to anti-histamines (reviewed in Cirelli,

2009). Hence, these results suggest that sleep, or a sleep-like state, does indeed exist in *Drosophila* and that fruit flies can be used as a viable model system to study sleep.

Background for present study

Social interactions are known to have varied effects on several behaviours including circadian rhythms and sleep. For instance, maintaining flies in groups of 30-40 individuals immediately post eclosion or 5-days post eclosion, resulted in an increase in sleep levels post interaction irrespective of the sex of the flies (Ganguly-Fitzgerald et al., 2006). Such an increase in sleep levels post social experience required an intact olfactory system as well as visual system. This increase in sleep was also dependent on the group size (Ganguly-Fitzgerald et al., 2006; Donlea et al., 2009) and was associated with dopamine (neurotransmitter which plays an important role in memory modulation) levels. Abnormal upregulation or downregulation of dopamine levels prevented the induction of sleep in flies that had undergone social interactions. The numbers of synaptic terminals of ventral lateral neurons (LNvs, which are known to be the arousal neurons) were also shown to increase in response to social interaction (Donlea et al., 2009), suggesting a possible mechanism via which social interactions may affect sleep levels.

Similarly, Lone et al. (2016) examined whether pairwise interactions were sufficient to induce increases in sleep. They observed that even pairwise same-sex social interaction in males, for a duration of at least 3-days, was sufficient to bring about an increase in sleep levels post interaction.

Such an increase in sleep levels based on same-sex interactions was not found in females. However, contrary to results from group interactions, the LN_v neurons were deemed not necessary for inducing sleep after pairwise interactions though other processes such as dopamine signaling and olfaction were found to be essential. Hence, it appears that the effects of group-wise interactions and pairwise interactions are broadly similar with minor differences with regards to the sex of the flies and the neurons involved.

Although the study on group-wise interactions examined the effects of social experience just after eclosion as well as 5-days after eclosion, pair-wise interaction study was limited to flies, which were 4-days old. Since, social experience in the first week post eclosion affects the development of the brain (Technau et al., 1984; Balling et al., 1987; Heisenberg et al., 1995) and a number of complex behaviours including sleep (Shaw et al., 2000), it may be interesting to test if similar induction of sleep would be seen after pairwise interactions of freshly emerged flies as that seen in 4-day old flies. Hence, we subjected freshly emerged flies to pairwise social interactions. Additionally in experiments by Lone et al. (2016), the flies that were used for pairwise interactions were maintained in same sex groups for the first 4-days. In order to determine whether keeping them in groups for the first few days post-eclosion has any effect on the sleep levels and patterns observed in same sex male pairs, we also carried out an experiment in which the flies were isolated for the first 4-days of emergence prior to pairwise social interactions. Thus, we examined effects of pairwise interactions at different ages and for different combinations of sexes on sleep in fruit flies and compared them with previous results.

With the above mentioned objectives in mind the present study has been divided into two parts:

1. Characterizing circadian activity-rest rhythm of different castes from three related species of carpenter ants under 12:12-h light-dark cycles (LD) and under constant conditions (DD).
2. Examining the effects of stage specific pair-wise social interaction on sleep in *Drosophila melanogaster*.

Chapter 2

Characterizing activity-rest rhythms of three related ant species

Introduction

Eusocial insects such as bees, wasps, ants and termites show division of labour within their colonies. They can be predominantly divided into reproductives (queen and males) and the workers. The allocation of tasks among the workers may be based on age polyethism, as is seen in honeybees (Seeley, 1995) and harvester ants (Gordon, 2000), or may be based on polymorphism (Hölldobler and Wilson, 1990) as is seen in some species of carpenter ants. Studies on circadian rhythms of different castes have largely examined species that show age related polyethism in task allocation (reviewed in Eban-Rothschild and Bloch, 2012). In these species, the individuals are allocated tasks according to their age. The young or freshly eclosed individuals mostly stay in cavities secluded from direct exposure to zeitgebers such as light and temperature. They perform tasks such as brood care and nest maintenance and are mostly arrhythmic in their activity at this stage (Lindauer, 1952; Stussi, 1972; Spangler, 1972; Crailsheim et al., 1996; Moore et al., 1998). As they age, their activity becomes rhythmic as they start performing tasks such as foraging, which require them to come out of the nests (Spangler, 1972; Crailsheim et al., 1996). Several studies in honeybees (Moore, 2001; Toma et al., 2000; Bloch et al., 2001; Shemesh et al., 2007, 2010; Eban-Rothschild et al., 2012) and on harvester ants (Ingram et al., 2009) have examined the mechanisms involved in the initiation of rhythmicity. Experiments showed that though there is no discernible rhythm in activity and oxygen consumption in the young honeybees, *per* mRNA levels do cycle in the brain, albeit with lower amplitude than that seen in adult bees (Toma et al., 2000). It was subsequently observed that only a few cells in the brain showed oscillations in *per* levels as long as the bees were in contact with the brood (reviewed in Eban-Rothschild and Bloch, 2012). When these individuals were involved in

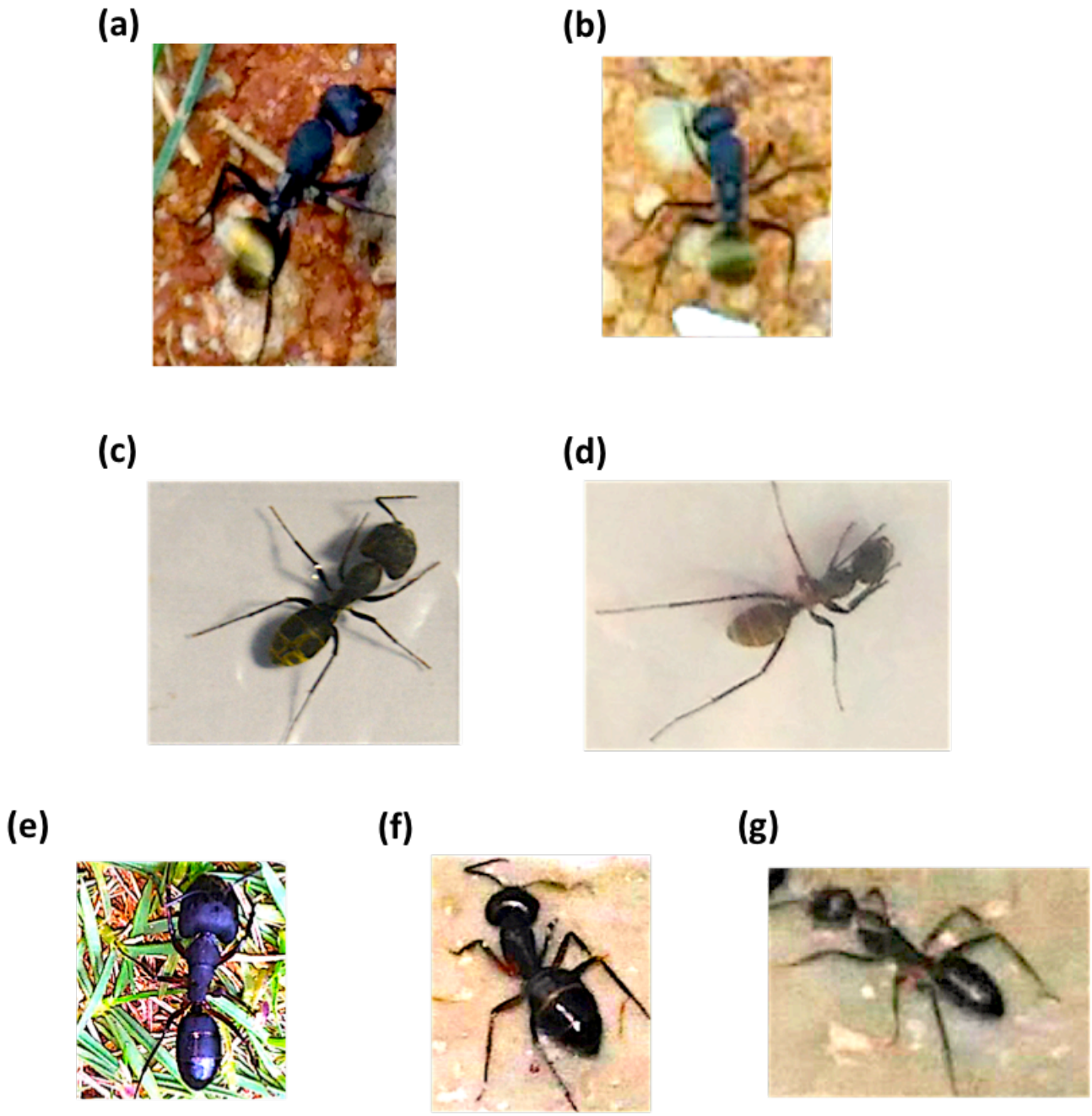

Figure 1. The images represent *Camponotus sericeus* (a) Major (b) Minor worker, *Camponotus paria* (c) Major (d) Minor worker, *Camponotus compressus* (e) Major (f) Media and (g) Minor worker.

work that did not involve direct contact with the brood, the cycling restarted in the other brain cells as well (Shemesh et al., 2010), thus implicating social cues from conspecifics in the modulation of circadian rhythms. Further experiments showed that volatile odours and not direct contact was vital to the onset of circadian rhythmicity (Eban-Rothschild et al., 2012) and also that the flagella present on the antennae played an important role in signal communication from the brood to the young bees or nurses (Nagari and Bloch, 2012).

Circadian rhythmicity in polymorphic ants, in comparison, has not received adequate attention. In such species, the caste of individual worker ants can easily be identified on the basis of their physical attributes, such as body length, and head width (Hölldobler and Wilson, 1990; Sharma et al., 2004a; Mysore et al., 2009). These individuals perform specific tasks right from their emergence on the basis of these physical attributes. The larger individuals, called the major workers, are mostly involved in nest protection and maintenance, whereas the smaller ones i.e., the media and minor workers are involved in brood care, foraging, and nest maintenance (Hölldobler and Wilson, 1990). However, exposure of different castes to various zeitgebers (time cues) has not been studied in detail and not much is known about the circadian rhythms of these individuals. The few studies that have been carried out on a nocturnal species of ants indicate that their circadian rhythms depend on the respective roles that they play in the colony (Sharma et al., 2004a-c). Studies carried out on the nocturnal species *Camponotus compressus* indicate that the major workers, which are primarily responsible for protecting the nest, show rhythms in their locomotor activity with most of their activity restricted to the night (Sharma et al., 2004a). In contrast, the media workers, which are considered to be task generalists, were either nocturnal (75%) or diurnal and show changes

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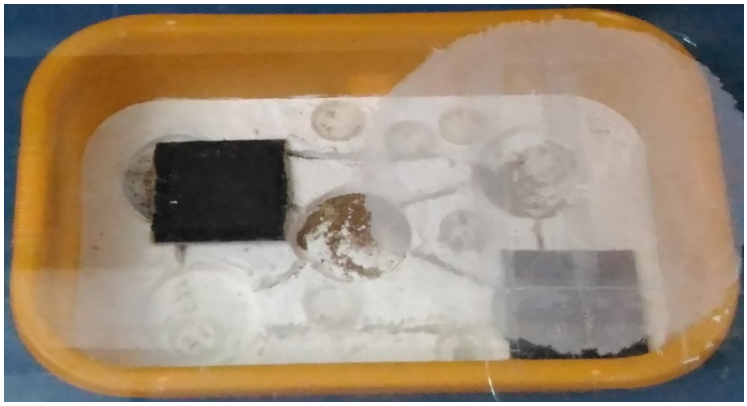

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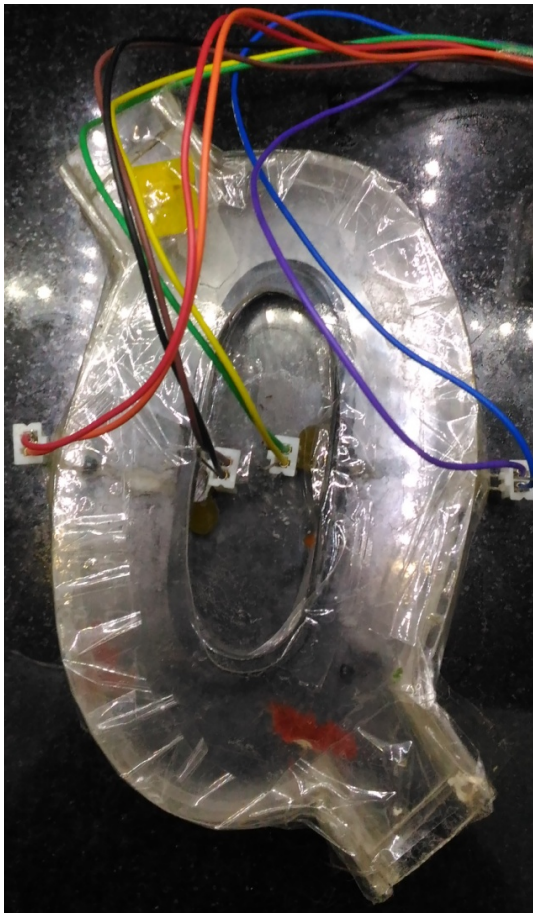

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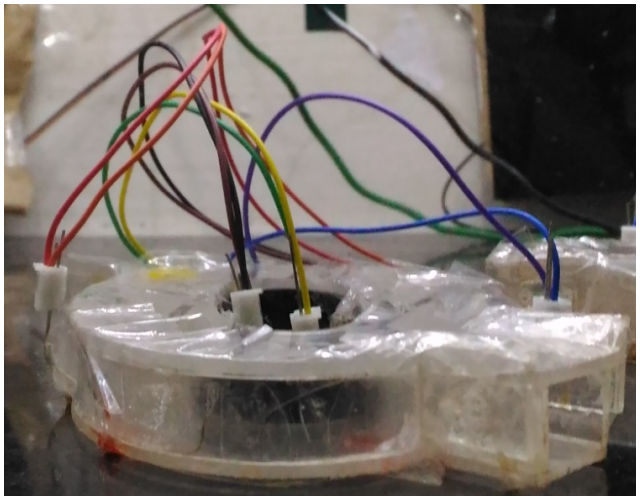

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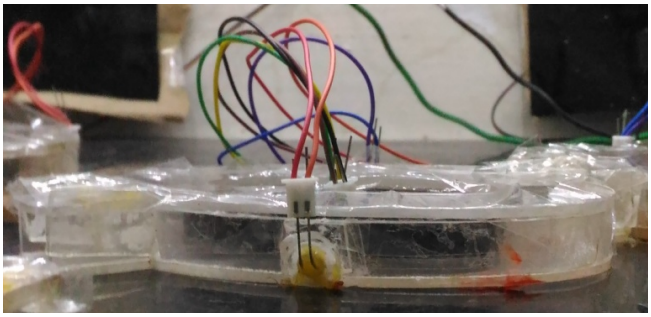

Figure 2. Image (a) depicts a plastic tub containing an artificial nest made of Plaster of Paris in which an ant colony was maintained. (b), (c), (d) represent a racetrack in which activity was recorded for individual ants.

in circadian period under constant conditions (Sharma et al., 2004a, b). The minor workers did not show any rhythm in locomotion under both 12:12-h light-dark (LD) and constant dark (DD) conditions, suggesting that they may be specialized for brood care which needs to be carried out throughout the day and night (Sharma et al., 2004a). Comparing the circadian behaviour of castes from multiple species may help lend insight into patterns of rhythmicity associated with particular tasks. As most of the tasks such as foraging, patrolling, nest maintenance and brood care require locomotor activity we examined the activity-rest rhythm of different castes in three species of carpenter ants: *Camponotus compressus*, *Camponotus sericeus* and *Camponotus paria*.

Each of these three species is found in a similar ecology and has castes of workers performing specific tasks based on their physical attributes. While *Camponotus sericeus* and *Camponotus paria* are known to be diurnal (Mysore et al., 2009; Lone et al., 2011a), *Camponotus compressus* is nocturnal (Sharma et al., 2004a). *Camponotus sericeus* and *Camponotus paria* have two castes based on their polymorphisms viz., major workers, which are larger in size and minor workers are relatively smaller in size. *Camponotus compressus* has three castes viz., major, media and minor workers (Sharma et al., 2004a). As the different castes in each of the species are associated with separate tasks, their exposure to zeitgebers may also vary. For example, it has been seen that the minor workers of *Camponotus compressus*, which are thought to be primarily associated with brood care and probably not exposed to external LD cycles, do not show rhythmicity in activity under LD or DD conditions (Sharma et al., 2004a). This suggests that minors are arrhythmic and that rhythmicity in activity in this caste cannot be induced by LD cycles. However, social and temperature cues may

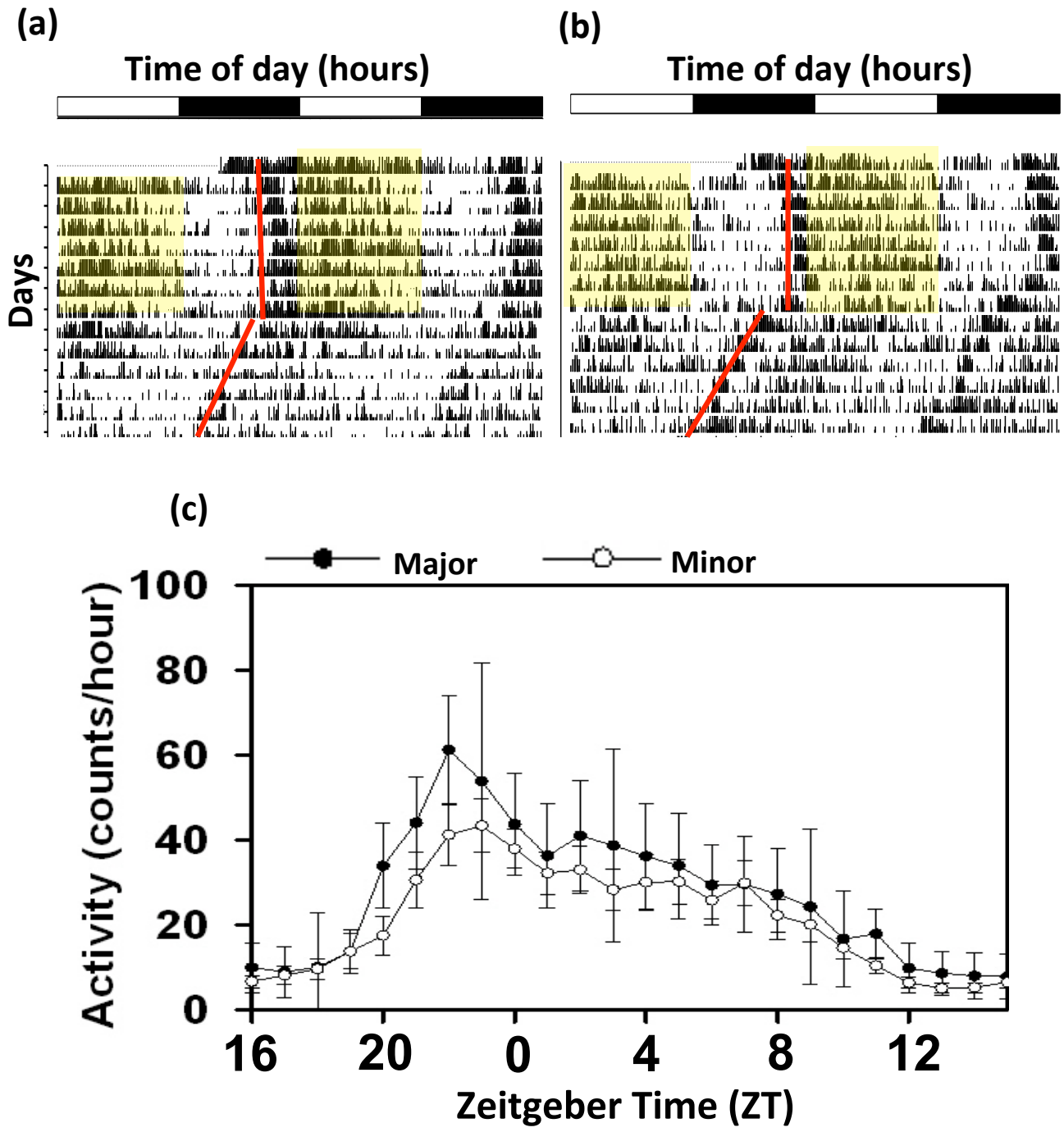

Figure 3. Representative actograms of *Camponotus sericeus* (a) Major workers (b) Minor workers under LD followed by DD regimes. (c) represents activity profiles of major and minor workers under 12:12-h LD. Error bars represent SEM.

yet influence activity profiles of these individuals in their nest environment and remains to be studied. Similarly, the different castes of *Camponotus sericeus* and *Camponotus paria* may show preferential entrainability to different zeitgebers. Hence, as a preliminary exercise to determine the rhythmicity of different castes and their entrainability to light, we recorded locomotor activity of individuals from each of the castes of all three species under DD as well as 12:12-h LD cycles. Further experiments on the effects of social cues may be conducted in future on the basis of results of the present experiments.

Materials and Methods

Ant species used for recording

Camponotus sericeus

Camponotus sericeus is a species of carpenter ants that belongs to the order Hymenoptera and family Formicidae. They can be distinguished from other carpenter ants on the basis of a golden pubescence on their gaster. This species has 2 castes viz., major and minor workers (Fig. 1a, b) which can be distinguished from each other on the basis of morphology (Hölldobler, 1974). The major workers are longer with larger head width compared to minor workers (Keshava et al., 2010). The lengths (head to abdomen) of the minor workers vary from 6-9 mm (*n*=30) whereas that of the major workers varies from 8-12 mm (*n*=22; *personal measurements*).

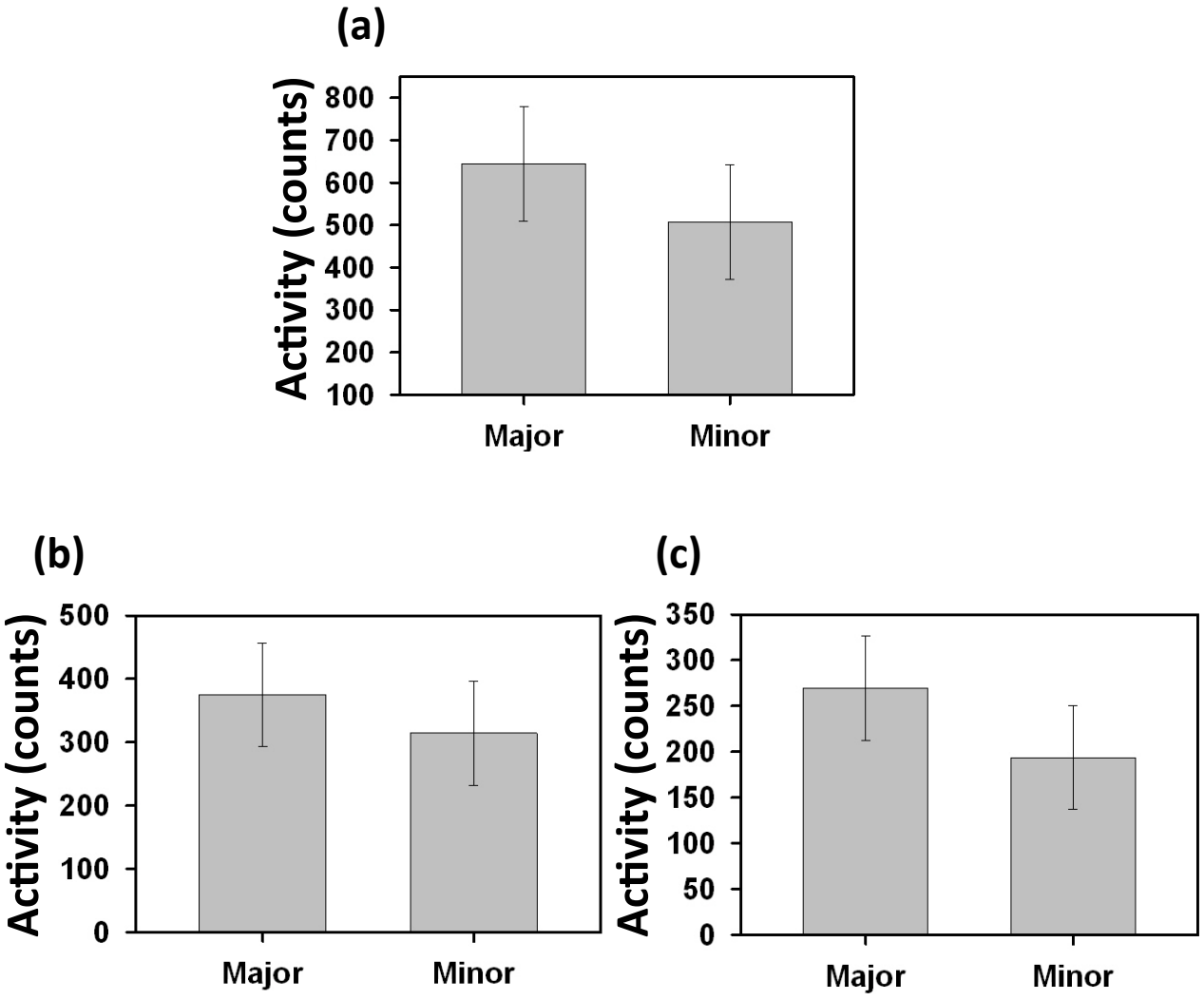

Figure 4. Graphs represent (a) Total activity (b) Day time activity and (c) Night time activity of major and minor workers of *Camponotus sericeus*. Error bars represent 95% CI.

Camponotus paria

Camponotus paria is another species of carpenter ants that also belongs to the order Hymenoptera and family Formicidae (Hölldobler and Wilson, 1990). They can be distinguished from other carpenter ants on the basis of the greyish silver pubescence on their gaster. This species also has 2 castes viz., major and minor workers (Fig. 1c, d) which can be distinguished from each other on the basis of their morphology. Similar to *Camponotus sericeus*, the major workers have larger heads compared to the minor workers (Hölldobler and Wilson, 1990) and are also longer than the minor workers. The length (head to abdomen) of minor workers varies from 5-8 mm (*n*=15) while that of the major workers varies from 8-12 mm (*n*=10).

Camponotus compressus

Camponotus compressus is a species of carpenter ants that belongs to the order Hymenoptera and family Formicidae (Hölldobler and Wilson, 1990). This species has three castes viz., major, media and minor workers (Fig. 1e, f, g) which can be distinguished from each other based on their body lengths (Sharma et al., 2004a). The minor workers range in size from 6-8 mm, the media workers range from 11-16 mm and the major workers range from 14-18 mm (Sharma et al., 2004a). The minor workers are mainly involved in nursing and nest maintenance whereas the media workers are task generalists and take part in foraging, nursing and nest maintenance. The major workers are primarily involved in protection of the nest and nest-mates (Hölldobler and Wilson, 1990).

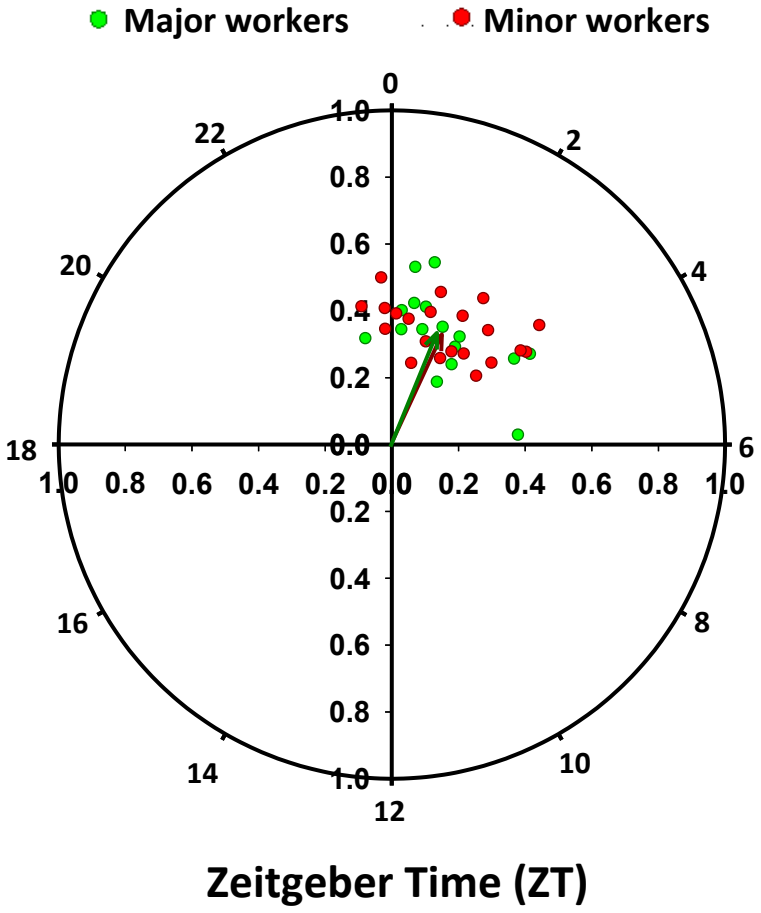

Figure 5. Polar plot depicting phases of entrainment of *Camponotus sericeus* major and minor workers. Each dot represents the mean phase of activity of an individual of a particular caste and the arrows indicate the mean phase of activity of each caste.

Colony collection and maintenance:

Colonies of three species of carpenter ants, *Camponotus sericeus, Camponotus paria* and *Camponotus compressus* were collected from fields within Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru. The colonies were collected by hand into plastic boxes and brought to the lab. These were then transferred to plastic tubs (dimensions 50cm x 30cm x 14cm) with artificial nests made using Plaster of Paris (POP). The surface of the POP has multiple circular indentations, which are connected to each other through deep grooves. The circular indentations were covered with Plexiglas plates wrapped in black paper and filter films in order to simulate the dark cavities present inside actual nests (Fig. 2). A thin coating of petroleum jelly was applied to the sides of the tub and the top of the tub was covered using a transparent Plexiglas sheet with a rectangular hole covered by a mesh to ensure ventilation. The petroleum jelly and the Plexiglas top prevented the ants from escaping from their artificial nests. These artificial nests along with the colonies were placed in a room which was maintained under 12:12-h LD cycles, with a light intensity of 100 lux, with lights coming on at 8 am and switching off at 8 pm. The temperature in the room was maintained at approximately 25 °C. The ants were provided with standard Bhatkar diet (Bhatkar and Whitcomb, 1970), 10% honey solution and water. The media in the nest was replaced every alternate day. Each ant colony was maintained under laboratory 12:12-h LD conditions for a minimum of three days prior to being introduced into the recording setup. The recording setup consisted of racetracks (explained in next section), which were wiped with 70% ethanol and left to dry for a period of 48-h after which they were wiped using tissue paper to remove any trace of alcohol. Prior to loading the ants, one end of the racetrack was sealed using cotton soaked in water.

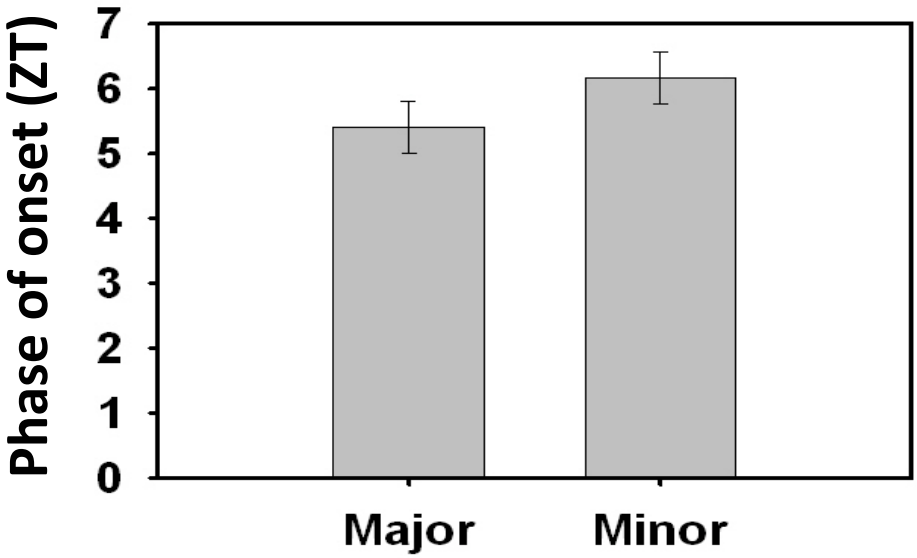

Figure 6. Graph represents phase of onset of activity of the major and minor workers of *Camponotus sericeus.* Error bar represent 95% CI.

Once the ant had been loaded, the other end was also sealed using cotton soaked in 10% honey solution. The ants were then maintained in 12:12-h LD for a minimum of 10 days before being transferred to DD for at least another 8-9 days (16 days in case of *Camponotus compressus*). Water and 10% honey solution was injected into the cotton plugs, using 20 ml syringes, at either end every 2-3 days after ensuring that there was no fungal growth on the cotton. In case of fungal growth, the cotton plugs were carefully replaced with fresh ones. Data from the first 2-days of recording in LD cycles were not considered while carrying out analysis.

Activity recording:

The activity-rest rhythms of the ant were monitored in specially made Plexiglas enclosures, which we refer to as racetracks (Fig. 2b, c, d). The racetracks are oval shaped with openings at two ends where cotton soaked in 10% honey solution or water can be inserted during recording. Each racetrack has two sets of IR emitter-receiver sets to record the activity of ants more efficiently. These IR sensors are connected to a custom made printed circuit board (PCB). When an ant disrupts the IR beam, a signal is sent to the PCB. This signal is amplified by an n-p-n transistor in the PCB and is sent to the recording system where the software records the disruption as an activity count. For the ant activity-rest recording we used the Chronobiology Kit Version 1e, procured from Stanford Software Systems (Santa-Cruz, USA). The '.DAT' files containing these activity counts were obtained from the Stanford Systems Software and then converted into notepad files which can be read by CLOCKLAB in order to carry out further analysis.

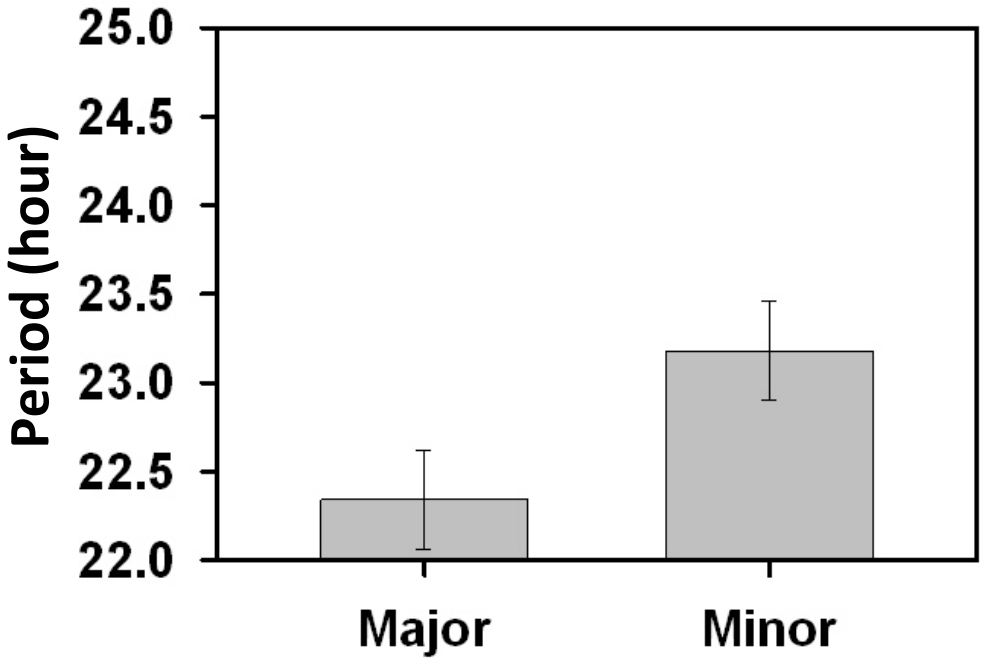

Figure 7. Graph represents period values of major and minor workers of *Camponotus sericeus* measured in DD. The minor workers show a significantly longer period value compared to the major workers. Error bars represent 95% CI.

Statistical analysis

The activity of the individuals belonging to the different castes was recorded in 1-min bins using the software provided by Stanford Software systems, Santa-Cruz, USA. The '.DAT' files obtained were then converted into notepad files and data was summed over 5-min bins. This data was then subjected to analysis using CLOCKLAB where individual animals were classified as rhythmic if they showed a significant periodicity in the Chi-square periodogram. Entrainment of circadian rhythms in 12:12-h LD cycles was verified by extrapolating the phase of activity onset on the first 2-days of DD to the activity onset on the last day of LD cycles and by finding a significant periodicity close to 24 h in the periodogram. For ants that showed such entrained rhythms in LD, we calculated total, day-time and night-time activity over a period of seven days. We performed a two-way ANOVA on the activity profile of these ants with 'caste' and 'time-point' as fixed factors and activity in 1-h bins as dependent variable. We also performed three one-way Analysis of Variance (ANOVAs) using 'caste' as fixed factor and total, daytime and night time activity as the three dependent variables. ANOVA was followed by post-hoc multiple comparisons using Tukey's unequal Honest Significant Difference (HSD).

We also calculated the Centre of Mass (CoM), which represents the mean phase of activity for each individual and further averaged it across all individuals to estimate mean phase of activity for each caste. This CoM was used as a phase marker to help us establish whether the locomotor activity in

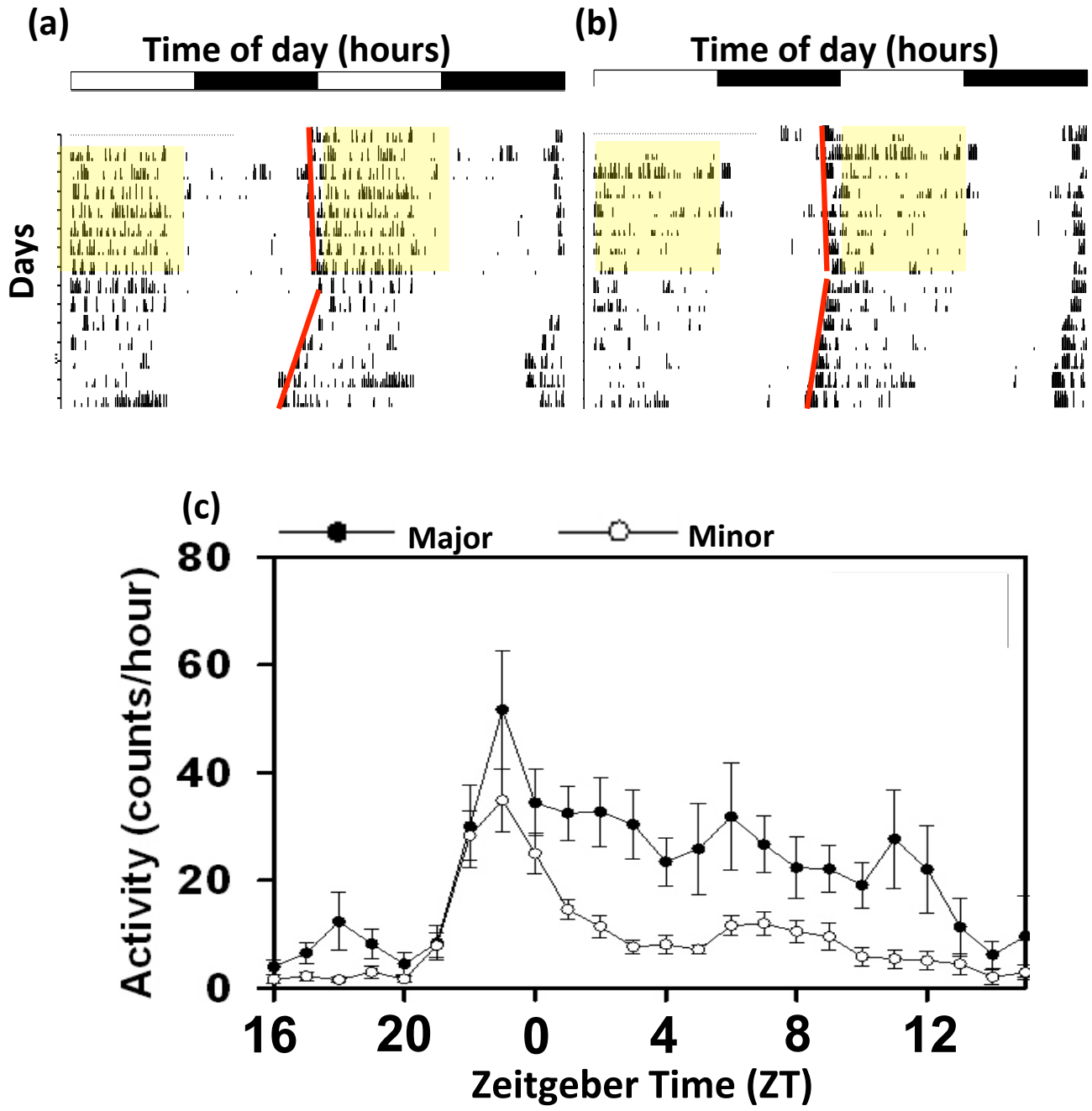

Figure 8. Representative actograms of *Camponotus paria* (a) Major workers (b) Minor workers under LD followed by DD regimes. (c) represents activity profiles of major and minor workers under 12:12-h LD. Error bars represent SEM.

different castes of different species was predominantly nocturnal or diurnal. To calculate CoM we first calculated proportion of activity across each time point per individual. Each time-point (in ZT, where Zeitgeber Time 00 is defined as the time of lights-ON in a 12:12-h light-dark cycle) is converted into degrees using the equation $\varphi_i = (ZT \times 360)/24$. These φ_i were then used to compute the sine and cosine component for each time point. The proportion of activity at each time point is then multiplied with the sine and cosine component of their respective time points. The sum of the products of proportion of activity and sine components (*y*) and the sum of the products of proportion and the cosine components (*x*) were computed. Subsequently, CoM is calculated for each individual using the formula $\varphi = atan\ (y/x)$ as described by Zar, 1999. In order to estimate the CoM of a caste from a given species, the proportion of activity at each time point is averaged across all individuals of a caste.

We obtained period values for each individual from the locomotor activity in DD by carrying out Chi-Square periodogram on eight days of data in DD. The mean period value for each caste was obtained by averaging across all individuals belonging to that particular caste. ANOVA on period values with caste as fixed factor was followed by post-hoc comparisons using Tukey's unequal HSD. All our statistical analyses were implemented on Statistica v5.0 (Statsoft, 1995).

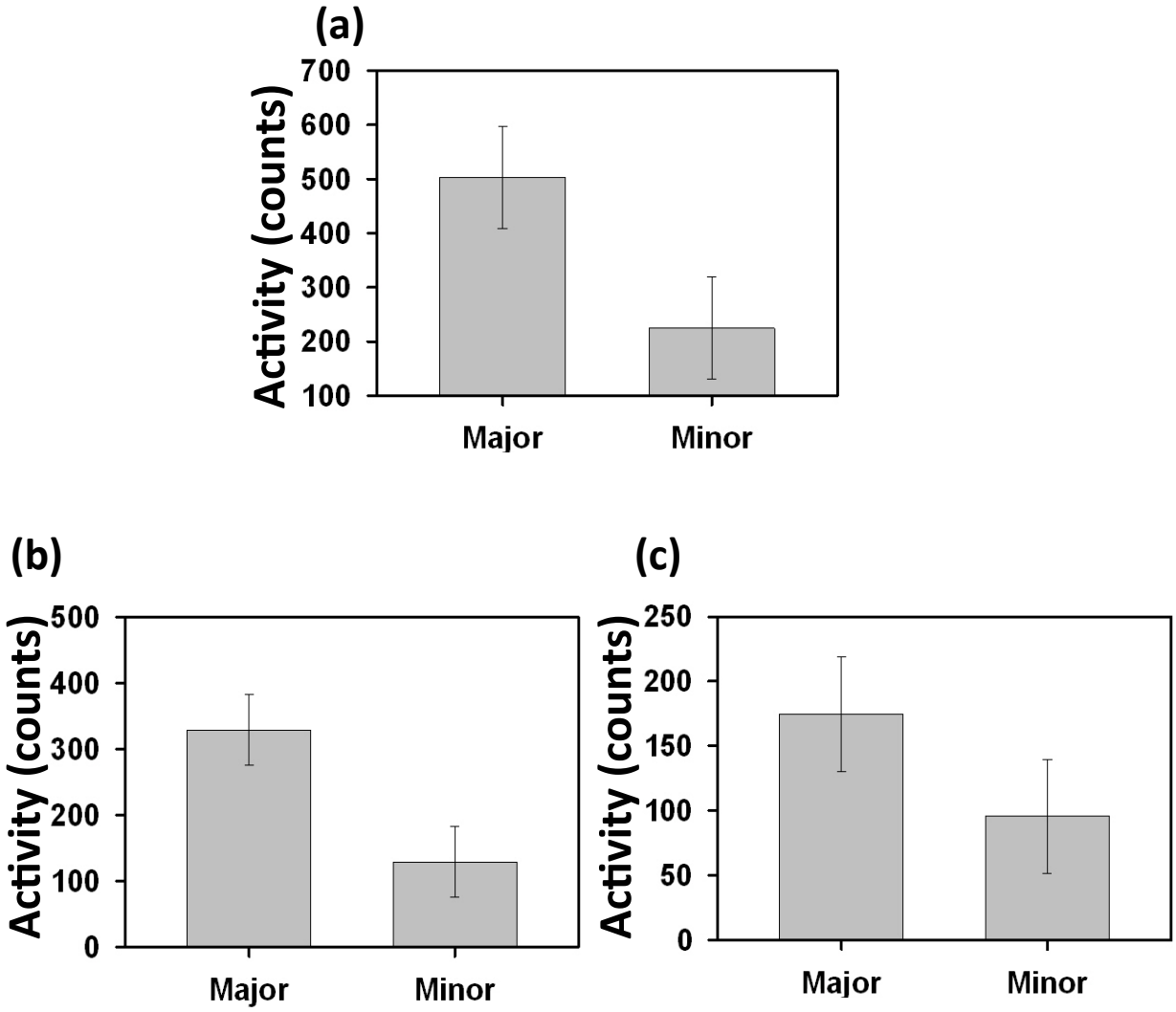

Figure 9. Graphs represent (a) Total activity (b) Day time activity and (c) Night time activity of major and minor workers of *Camponotus paria*. Error bars represent 95% CI.

Results

Camponotus sericeus

Almost all the major and minor caste workers of *Camponotus sericeus* showed entrainment to 12:12-h LD and free-running rhythms under DD conditions (Fig. 3a, b). We also compared the activity profiles of the two castes in 12:12-h LD cycles (Fig. 3c). The locomotor activity of both minor and major workers show a peak close to lights-ON (ZT-00) and is sustained at high levels through most of the day. The levels of activity decrease after lights-OFF (ZT-12) and remain low throughout the dark phase. ANOVA on activity data binned in 1-h intervals with caste and time as fixed factors, showed a statistically significant effect of caste ($F_{1, 816}$ = 14.278, p<0.001) and time ($F_{1, 816}$ = 14.350, p<0.001), but not a statistically significant effect of caste × time interaction ($F_{1, 816}$ =0.502, p=0.976). ANOVA on overall activity levels revealed no significant effect of caste on total ($F_{1, 34}$ = 1.048, p=0.313, Fig. 4a), daytime ($F_{1, 34}$ = 0.557, p=0.46, Fig. 4b) or nighttime ($F_{1, 34}$ = 1.828, p=0.185, Fig. 4c) activity levels. These results suggest that the major (n=15) and minor (n=21) workers exhibit similar entrained activity profiles under 12:12-h LD cycles.

The phase of Centre of Mass (CoM) of major workers was φ_{ZT} = 1.668 with r= 0.363 whereas that of minor workers was φ_{ZT}= 1.701 with r= 0.378, indicating that workers of both castes are predominantly diurnal with most activity concentrated around lights-ON (Fig. 5). We further examined the phases of onset of activity, which appeared to be earlier in major workers (Fig. 6). ANOVA on the phase of onset revealed a significant effect of caste on phase of onset of activity ($F_{1, 34}$= 4.521, p=0.043). However, post-hoc comparisons using Tukey's unequal HSD showed no

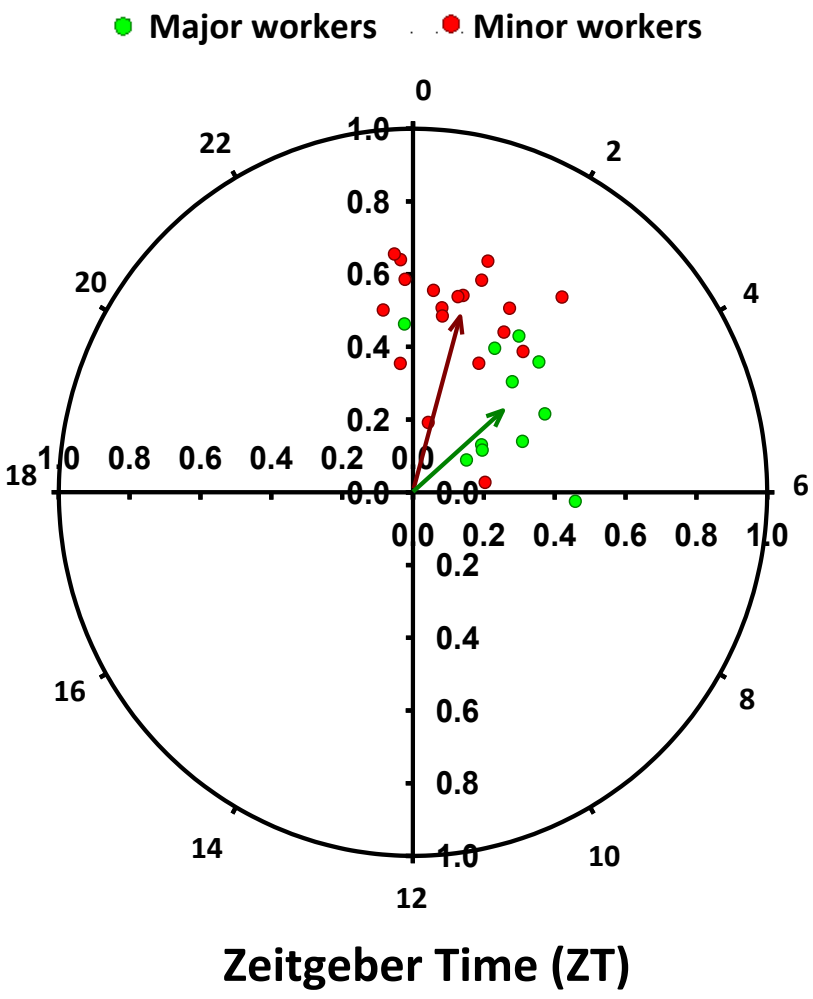

Figure 10. Polar plot depicting phases of entrainment of *Camponotus paria* major and minor workers. Each dot represents the mean phase of activity of an individual of a particular caste and the arrows indicate the mean phase of activity of each caste.

significant difference (p=0.057) in the onset of activity between the two castes. Therefore, the phase of entrainment of activity under 12:12-h LD cycles are similar in both the castes of this species though the major workers appear to show earlier onset of activity.

We calculated the mean free-running period of locomotor activity rhythm of major workers (τ = 22.34; n=10) and minor workers (τ = 23.179; n=11) under DD conditions. ANOVA on the free-running period revealed a significant effect of caste ($F_{1, 19}$=14.31, p=0.0012, Fig. 7). Further, post-hoc multiple comparisons using Tukey's unequal HSD test revealed that minor workers show significantly longer periods as compared to the major workers (p=0.001, Fig. 7).

In summary, the major and minor workers of *Camponotus sericeus* are predominantly diurnal with similar levels of activity throughout the day and night. They also show similar phases of onset of activity as well as CoM. While most major and minor workers are rhythmic under constant dark, the minor workers seem to show a longer period as compared to the major workers.

Camponotus paria

Similar to *Camponotus sericeus,* both major and minor workers of *Camponotus paria* entrained to 12:12-h LD cycles and showed free running rhythms under DD conditions (Fig. 8a, b). We compared the activity profiles of major and minor workers in 12:12-h LD cycles and observed that the locomotor activity levels peak close to lights-ON (Fig. 8c). However, both the peak of activity as well as activity levels during the daytime were considerably lower in minor workers as compared to major workers (Fig. 8c). ANOVA on activity profile with 'caste' and 'time-point' as fixed factors showed a

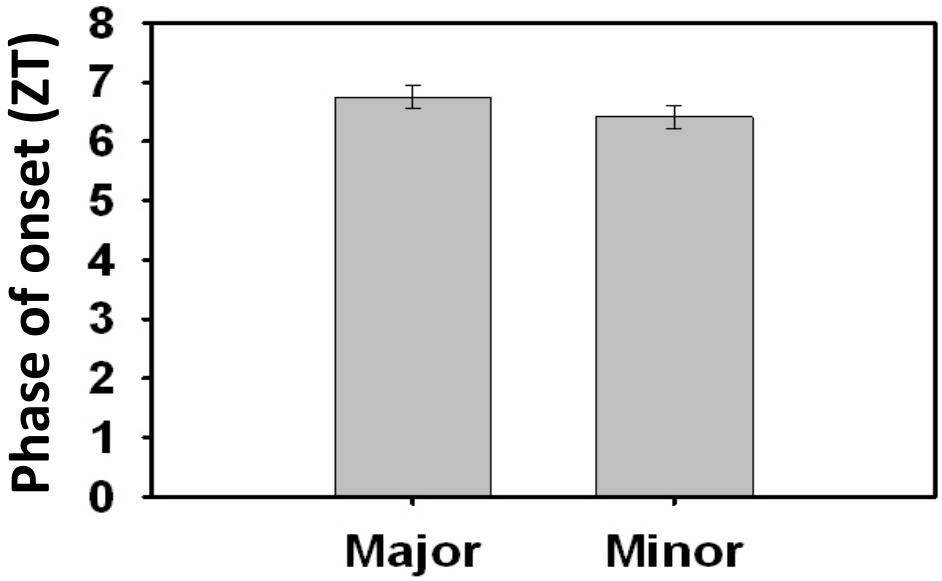

Figure 11. Graph represents phase of onset of activity of the major and minor workers of *Camponotus paria.* Error bar represent 95% CI.

statistically significant effect of caste ($F_{1, 744}$ = 113.010, p=0.001), time-point ($F_{1, 744}$ = 13.8090, p=0.001), and caste × time-point interaction ($F_{1, 744}$ = 0.1.762, p=0.015). Post-hoc multiple comparisons using Tukey's unequal HSD revealed that activity levels of none of the time points were significantly different between major and minor workers. Furthermore, ANOVA revealed a significant effect of caste on total ($F_{1, 31}$=10.299, p=0.003, Fig. 9a) as well as daytime activity levels ($F_{1, 31}$=16.450, p=0.0003, Fig. 9b) but not of nighttime activity levels ($F_{1, 31}$=3.793, p=0.06, Fig. 9c). Post-hoc multiple comparisons showed that both total (p= 0.009) as well as daytime (p=0.001) activity levels were greater in major workers as compared to minor workers. These results suggest that the major (n=11) workers show higher overall levels of activity when compared to the minor (n=22) workers, mainly due to higher daytime activity levels.

The phase of Centre of Mass (CoM) of locomotor activity rhythm of major workers was φ_{ZT} = 3.150 with r = 0.349 whereas that of minor workers was φ_{ZT} = 0.980 with r = 0.490, indicating that workers of both castes are predominantly diurnal with most of their activity observed around lights-ON (Fig. 10). We further looked at phases of onset of locomotor activity of the two castes. ANOVA on phase of onset of activity revealed no significant effect of caste ($F_{1, 31}$= 3.436, p=0.07, Fig. 11) indicating that phase of entrainment of activity under 12:12-h LD cycles are similar in both the castes of this species.

We also calculated the free-running period of locomotor activity of major workers (τ = 22.68; n=8) and minor workers (τ = 23.68; n=17) under DD. ANOVA on the free-running periods revealed a statistically significant effect of caste ($F_{1, 22}$=15.59, p=0.0006, Fig. 12). Post-hoc multiple comparisons using Tukeys' unequal HSD showed that minor workers show longer periods as compared to the

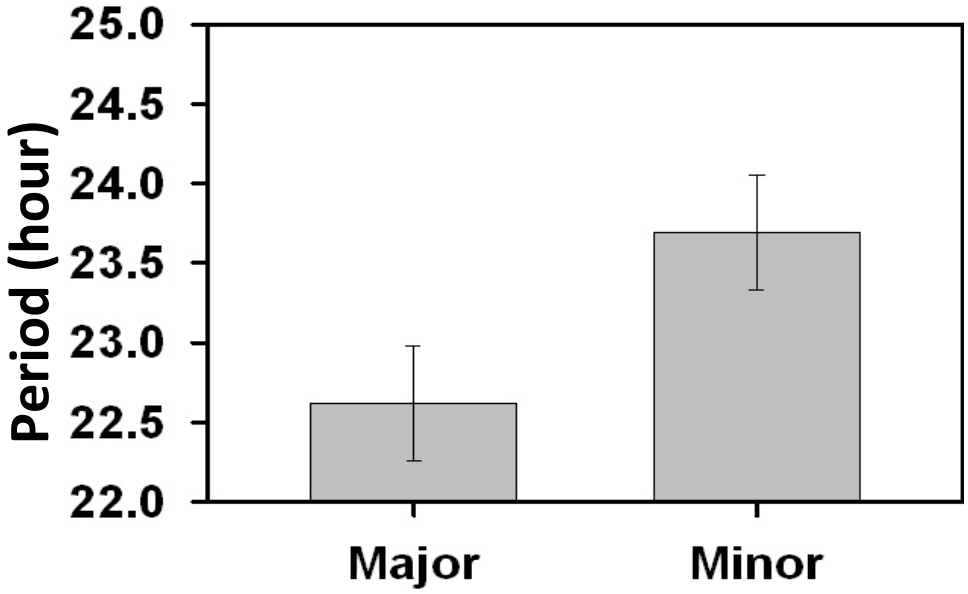

Figure 12. Graph represents period values of major and minor workers of *Camponotus paria* measured in DD. The minor workers show a significantly longer period value compared to the major workers. Error bars represent 95% CI.

major workers (p=0.0001; Fig. 14). Thus, these results suggest that the minor workers of *Camponotus paria* have significantly longer free-running period as compared to major workers.

In summary, we found that both major and minor workers of *Camponotus paria* are predominantly diurnal with similar phases of entrainment. However, the major workers showed greater activity during the daytime compared to the minor workers. Under constant conditions, the minor workers seemed to show longer period rhythms as compared to the major workers.

Camponotus compressus

This species has three castes of workers viz., major, media and minor workers (Hölldobler and Wilson, 1990). The major and the media workers in this species show entrainment to 12:12-h LD cycles and free running rhythms in DD. Although the minor workers showed similar patterns of activity in LD cycles as the other castes, these individuals were arrhythmic in DD suggesting that the activity patterns in LD may be due to masking (Fig. 13 a, b, c). We observed a peak of activity around lights-OFF followed by activity during the dark in all three castes. However, the minor workers appeared to show greater activity during the light phase compared to the media and major workers (Fig. 13d). ANOVA on activity levels with caste and time-point as fixed factors revealed a statistically significant effect of caste ($F_{2, 624}$ = 34.968, p=0.001) and time-point ($F_{2, 624}$ = 9.6430, p=0.001), but not of caste × time interaction ($F_{2, 624}$ = 0.616, p=0.978). We further looked at total, daytime and nighttime activity levels in the three castes. ANOVA on daytime activity levels revealed a statistically significant effect of caste ($F_{2, 26}$=3.910, p=0.032, Fig. 14b). However, post-hoc comparisons using Tukey's unequal HSD showed no significant difference in activity levels in daytime between the three

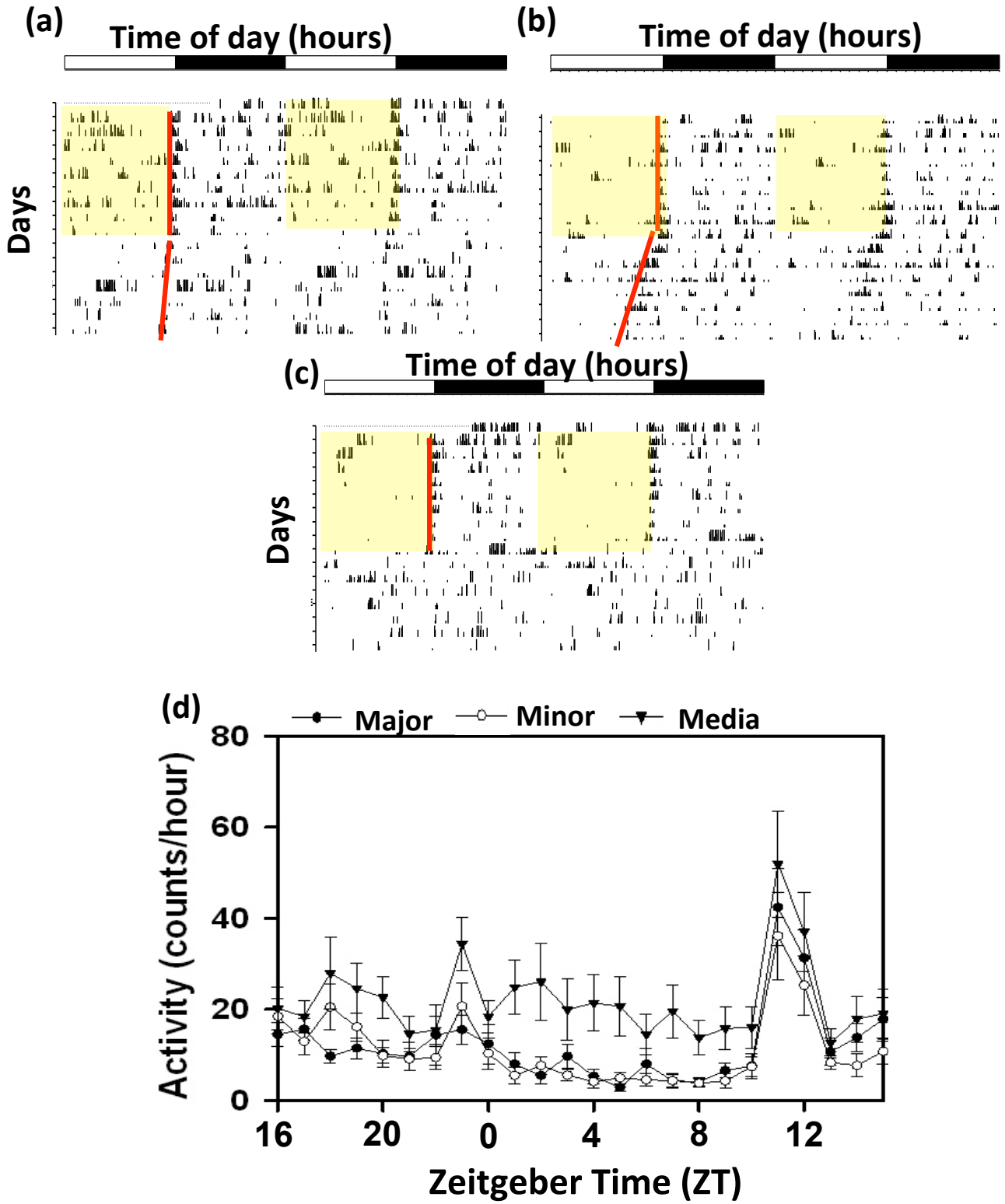

Figure 13. Representative actograms of *Camponotus compressus* (a) Major (b) Media and (c) Minor workers under LD followed by DD regimes. (d) represents the Activity profiles of major, media and minor workers under 12:12-h LD and Error bars represent SEM.

castes (p>0.05). ANOVA also revealed no significant effect of caste on total ($F_{2, 26}$=2.862, p=0.07, Fig. 14a) or nighttime ($F_{2, 26}$=1.63, p=0.213, Fig. 14c) activity levels. These results suggest that the major (n=10), media (n=10) and minor (n=9) workers show similar levels of activity throughout the day. The phase of Centre of Mass (CoM) of activity rhythm of major workers was found to be φ_{ZT}= 14.39 with r= 0.299, whereas that of the media workers was φ_{ZT}= 16.221 with r= 0.266 and that of the CoM of the minor workers was φ_{ZT}= 17.980 with r = 0.08, indicating that workers of all three castes are predominantly nocturnal (Fig. 15). However, there was one media worker that was found to be active predominantly during the light phase. We further examined the phases of onset of activity in the three castes. ANOVA on phase of onset of activity revealed no significant effect of caste ($F_{2, 26}$= 3.111, p=0.06, Fig. 16). Therefore, the phase of entrainment of activity under 12:12-h LD cycles are similar in all the three castes of this species.

Sharma et al. (2004a) had previously observed that the minor workers did not show free-running rhythms in activity under DD similar to what we find in this study. Sharma et al. (2004a, b) had also observed that under DD, the media workers showed changes in period post 6-9 days. In order to determine if such changes in period occurred in our individuals we looked at the periods of individuals in the first eight days and then compared them to their period in the next eight days. Results of ANOVA on period of major workers considering 'number of days post transfer to DD' as a fixed factor and period as a dependent variable showed that there is no significant difference in period ($F_{1, 16}$=0.279, p=0.604) between 1-8 and 8-16 days. Similarly the results of ANOVA on period of media workers considering 'number of days post transfer to DD' as a fixed factor and period as a dependent variable showed that there is no significant difference in period ($F_{1, 12}$=0.081, p=0.780)

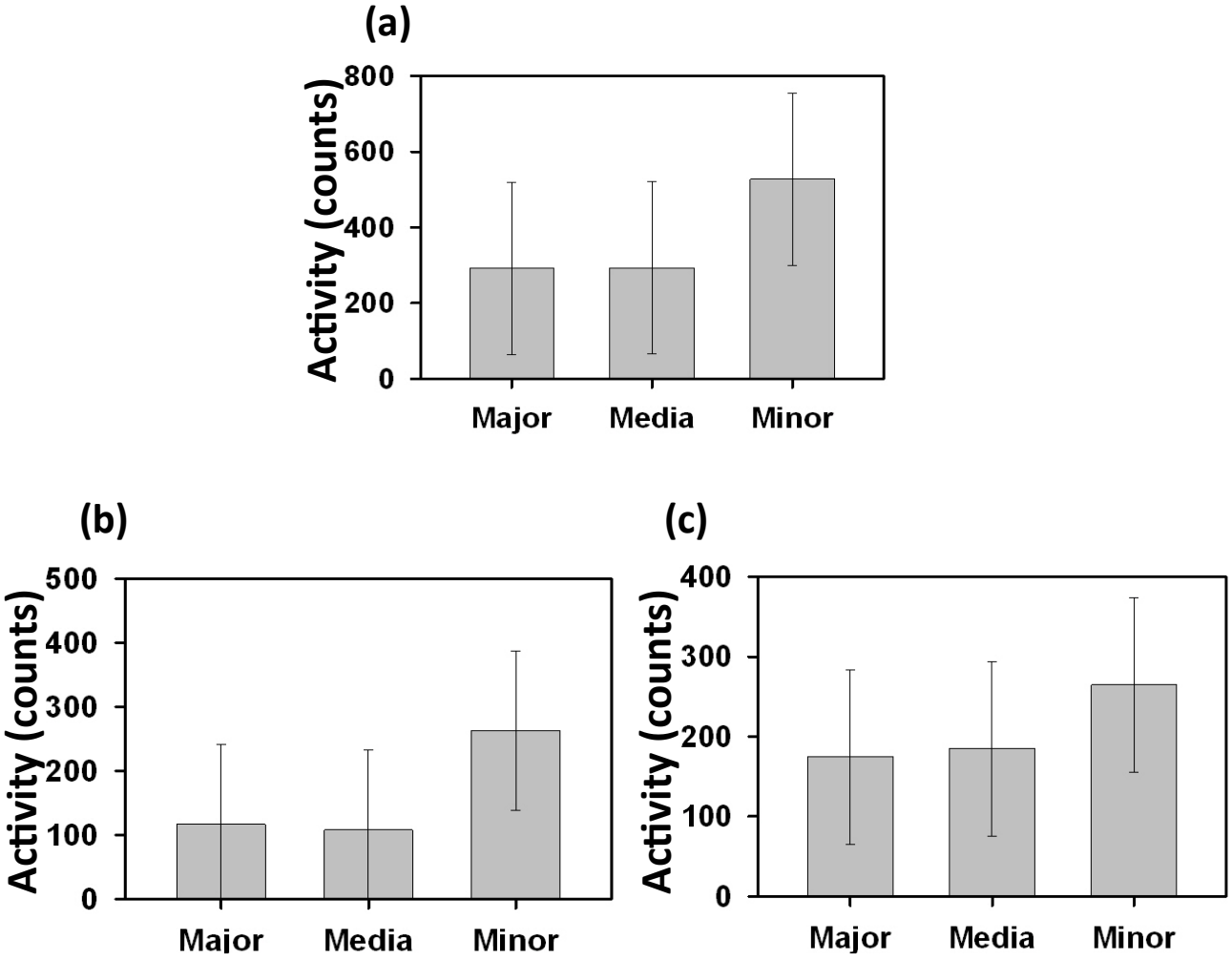

Figure 14. Graphs represent (a) Total activity (b) Day time activity and (c) Night time activity of major, media and minor workers of *Camponotus compressus*. Error bars represent 95% CI.

between 1-8 and 8-16 days. During the first 8 days, the period of locomotor activity rhythm of major workers was $\tau =$ 24.014-h (*n*=9) and that of the media workers was $\tau =$ 24.149 (*n*=8). ANOVA on period values showed no significant effect of caste ($F_{2, 15}$=0.320, *p*=0.581, Fig. 17b). During the second 8 days, the free-running period of major workers was $\tau =$ 24.106-h (*n*=9) and that of the media workers was $\tau =$ 23.929-h (*n*=6). The results of ANOVA on period values showed that there was no significant effect of caste ($F_{2, 15}$=0.430, *p*=0.523, Fig. 17c). Only one major worker showed a change in period (Fig. 18). During the 1st 8-days it had a period value of $\tau =$ 23.537-h but during the 2nd eight days the period lengthened to $\tau =$ 24.287-h.

In summary, the major and the media workers were predominantly nocturnal under 12:12-h LD cycles and show free running rhythms in DD. In contrast, the minor workers are arrhythmic in DD but show nocturnal activity patterns similar to the other castes in LD. The overall levels of activity were similar for all the three castes under 12:12-h LD and no significant differences were seen in the period values of the major and media workers in this species.

Discussion

The results of our experiments reveal that the major and minor workers of *Camponotus sericeus* and *Camponotus paria* show entrained locomotor activity rhythms in 12:12-h LD cycles and free-running rhythms under DD. Both castes of workers in these species are primarily active during the light phase of the LD regime with their peak of activity occurring before lights-ON (Fig. 3c, Fig. 8c). The phase of the CoM of activity also lies in the light phase (Fig. 5, Fig. 10) indicating that these species are

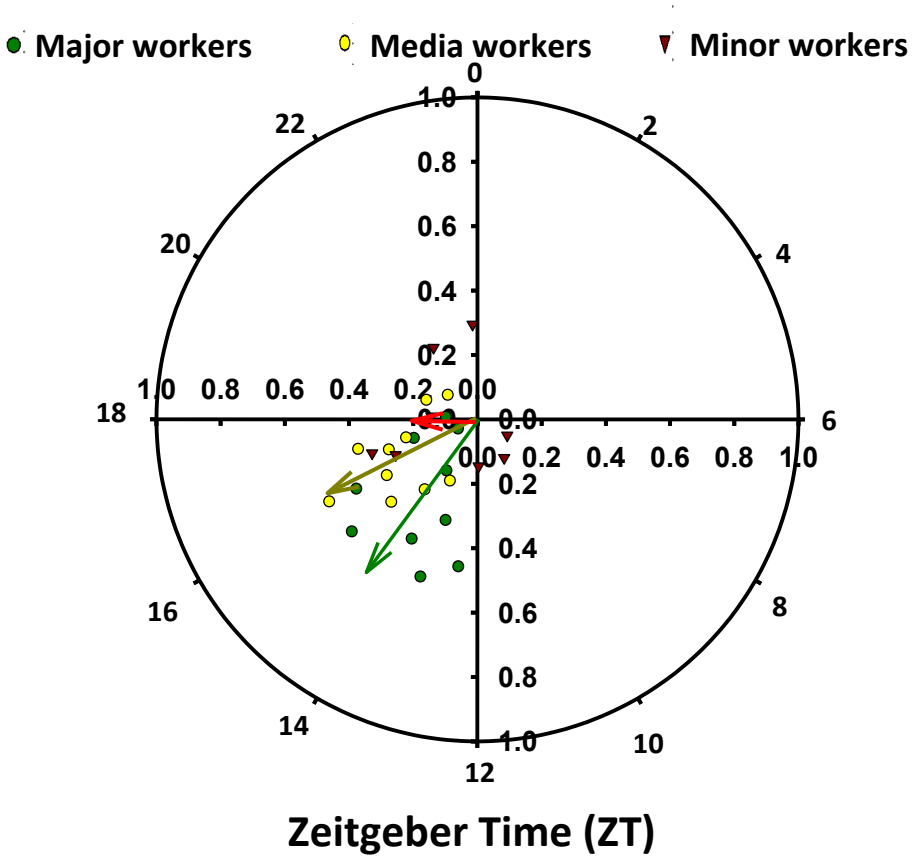

Figure 15. Polar plot depicting phases of entrainment of *Camponotus compressus* major, media and minor workers. Each dot represents the mean phase of activity of an individual of a particular caste and the arrows indicate the mean phase of activity of each caste.

predominantly diurnal. The free-running periods of the minor workers under DD are also significantly greater than that of major workers in both these species (Fig. 7, Fig. 12). The overall levels of activity are similar in both worker castes of *Camponotus sericeus* (Fig. 4a). In contrast, the major workers of *Camponotus paria* show higher activity levels during the daytime as compared to the minor workers (Fig. 9b). This difference in activity levels seen in *Camponotus paria* may be due to functional differentiation of the two castes in this species. The major workers of *Camponotus paria* primarily take part in guarding the nest and are present around the nest entrance all through the day, whereas minor workers are responsible for foraging for food (Hölldobler and Wilson, 1990). It is possible that the minor workers of *Camponotus paria* forage only during dawn where as the minor workers of *Camponotus sericeus* forage throughout the day. Such differences in foraging strategies are consistent with studies on two related species of harvester ants *Pognomyrmex rugosus* and *Messor pergandei,* which occupy the same habitat (Johnson, 1991).

Alternately, we have observed that the minor workers of *Camponotus paria* show a great affinity for moisture. When reared in artificial nests in the lab with cotton balls providing moisture, we observed that all the minor workers congregate on the surface of the cotton balls (*personal observation*). When these cotton balls dried up, these minor workers were found to die rapidly. However, this was not found to be the case for the major workers. Hence, it is possible that the desiccation resistance of the minor workers is lower as compared to that of the major workers, and since daytime temperatures are high and the humidity low, the minor workers show reduced activity in the daytime to avoid desiccation. These results are consistent with the increase in desiccation resistance with body weight in ants (Hood and Tschinkel, 1990) since minor workers are also smaller than major

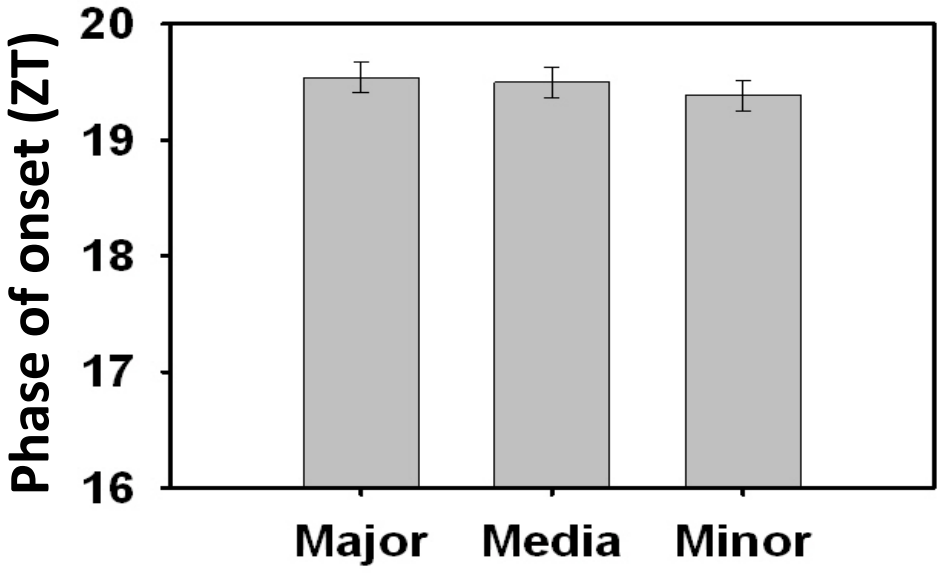

Figure. 16 Graph represents phase of onset of activity of the major, media and minor workers of *Camponotus compressus.* Error bar represent 95% CI

workers. Furthermore, it is known that ant foraging activity shows variability according to heat and desiccation conditions in the local environment (Phillips et al., 1996; Cerdá et al., 1998). Thus, the differences between the major and minor workers could be due to differences in their desiccation resistance. The minor workers of *Camponotus sericeus* on the other hand were not seen to gather on moist cotton balls. This could indicate lower moisture requirements in minor workers of this species and thus, enabling them to remain active and forage throughout the daytime. Thus, minor and major workers of this species show comparable levels of activity during the daytime. This is consistent with the variation in desiccation resistance seen across different ant species (Hood and Tschinkel, 1990). We can further test for desiccation resistance in all the castes of the two species in order to ascertain if differences in desiccation resistance are indeed responsible for the differences seen in their activity levels.

In the case of *Camponotus compressus*, major, media, and minor workers showed rhythmic activity patterns under 12:12-h LD cycles, but only major and media workers showed free running rhythms in DD while the minor workers were arrhythmic (Fig. 13a, b, c). The workers of different castes of this species are primarily active during the dark phase of the LD cycles with their CoM of activity located in the middle of the night (Fig. 15), indicating that this species is predominantly nocturnal. We also see that one of the media workers showed a change in period post 8-days in DD. These results are consistent with observations in an earlier experiment by Sharma at al. (2004a). However, Sharma et al. (2004a) found period changes post 6-9 days in 70% of the media workers, whereas we found it only in one out of 10 media workers. Such differences may be due to differences between colonies

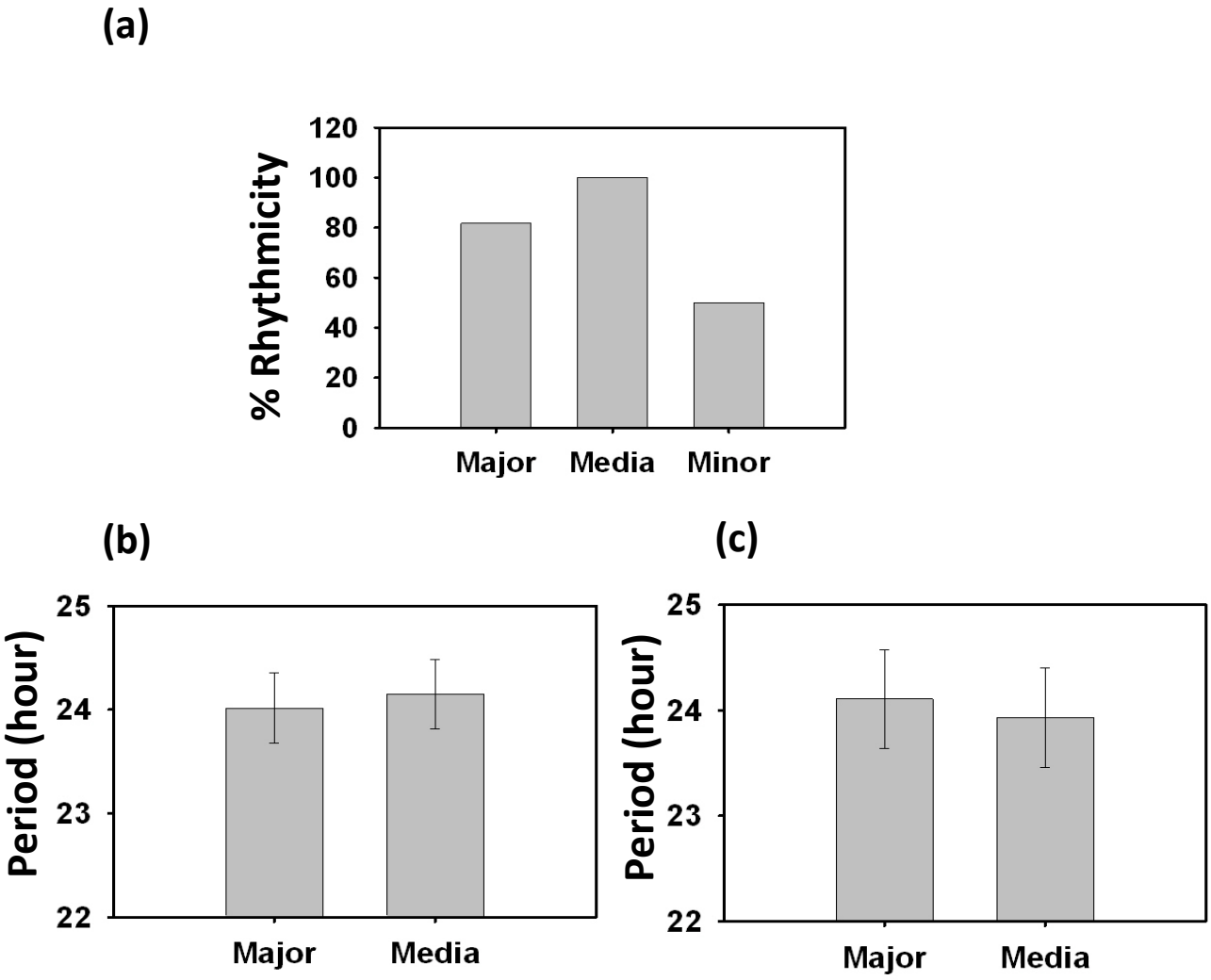

Figure 17. Graphs represent (a) percentage rhythmicity and period values measured in DD for the major and media castes of *Camponotus compressus* during the (b) first eight days and (c) days eight to sixteen. Error bars represent 95% CI.

of the same species or due to lack of conclusive actograms in the samples of media workers that were obtained in this study. Sharma et al. (2004a) had also observed that minor workers did not display any discernible rhythms in LD. However, we did find some masking in the minor workers under LD conditions though these rhythms were noticeably weaker than those seen in major and media workers (Fig. 13c). Nevertheless, these minor workers were arrhythmic in DD similar to the results of Sharma et al. (2004a). Such arrhythmicity may correspond to the functional roles of the minor workers such as brood care and nest maintenance, which is usually underground and within the nest. However, under certain circumstances, they may be capable of synchronizing their activity to external time cues such as light in order to carry out activities such as foraging. Similar instances have been recorded in the stick insect *Carausius morosus* and locust *Schistocerca gregaria* where locomotor activity behaviour was found to be rhythmic under 12:12-h LD cycles but was observed to be arrhythmic when shifted to constant conditions (Page, 1989).

In conclusion, from our current experiment we have been able to establish that major workers of three related species of carpenter ants and the media workers in *Camponotus compressus* show rhythms in activity under 12:12-h LD cycles and also, largely, show free-running rhythms under DD. We have also found that the minor workers of all the three species showed rhythms in activity under LD conditions, but free running rhythms under DD are seen only in the two diurnal species, viz. *Camponotus sericeus* and *Camponotus paria*. We have also established that the major and minor castes of *Camponotus sericeus* and *camponotus paria* are predominantly diurnal whereas all three castes of *Camponotus compressus* are predominantly nocturnal under 12:12-h LD cycles.

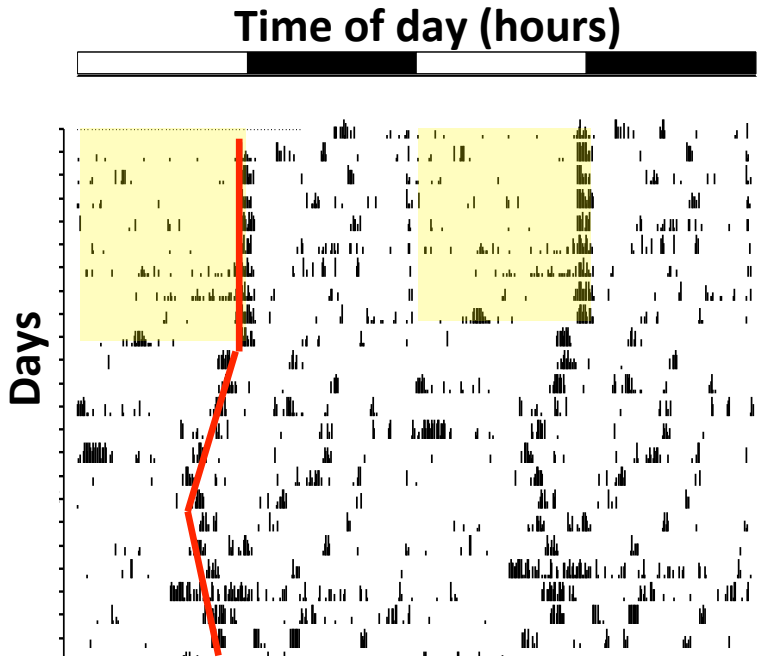

Figure 18. Representative actogram of *Camponotus compressus* major worker depicting change in period in DD from a short period to long period post 7-days.

The results of experiments on three related eusocial insects which show division of labour based on physical polymorphism has helped further our understanding of the effect of such a social organization on the circadian system of such insects under the influence of light as a zeitgeber. However, further experiments with different zeitgebers, such as temperature cycles and social cues and multiple combinations of all the three need to be carried out to get a holistic understanding of the circadian systems in different castes of such ant colonies.

Chapter 3

Examining the effect of pair-wise social interaction on sleep in *Drosophila melanogaster*

Introduction

Sleep is an evolutionarily conserved biological phenomenon that is essential for a number of processes such as neurogenesis (Guzman-marin et al., 2005), neural maintenance (Cirelli et al., 2005; Tononi and Cirelli, 2003, 2006), and learning and memory (Ambrosini and Giuditta, 2001; Heuber et al., 2004; Donlea et al., 2011; Seugnet et al., 2011). While most studies on sleep have focused on mammals, recent studies have shown that invertebrates ranging from nematode worms (Raizen et al., 2008) to *Drosophila* (Hendricks et al., 2000) also show a sleep-like state.

Hendricks et al. (2000) observed that flies chose a specific location and were immobile for long durations during certain times of the day. During these periods of rest, their response to sensory stimuli was low, suggesting an increased arousal threshold similar to sleep. This rest behaviour was also subject to homeostatic and circadian control similar to sleep in mammals. Other similarities with the mammalian system include decreased sleep when exposed to caffeine and increased sleep when exposed to anti-histamines (reviewed in Cirelli, 2009). These results suggest that a sleep-like state is indeed observed in Drosophila.

Fruit flies interact with their conspecifics to compete for mates, food, and to exchange information regarding ideal surface for oviposition (Battesti et al., 2012). Thus, social interactions with conspecifics are an important component of the environment of fruit flies. Since social interactions are known to affect sleep patterns (Matsumoto et al., 1996; Meerlo and Turek, 2001), and fruit flies

display social and sleep behaviours similar to mammalian systems, *Drosophila melanogaster* could be used as a model system to study the influence of social cues on sleep.

A number of recent studies have examined the influence of social cues on sleep in fruit flies (Ganguly-Fitzgerald et al., 2006; Donlea et al., 2009, 2011; Lone et al., 2016). Ganguly-Fitzgerald et al. (2006) observed that freshly eclosed males or females maintained in groups of 30-40 individuals for 5 days showed an increase in daytime sleep post interaction relative to solitary flies. This increase in sleep was not dependent on the sex of the conspecifics but was enhanced by larger group sizes.

On the other hand, Lone et al. (2016) looked at the effects of pair-wise same-sex interactions on sleep levels. Such pair-wise interactions showed a duration-dependent effect such that only pair-wise interactions for 3 or more days induced an increase in daytime sleep levels in males. However, such increases in sleep post social interactions were not seen in females in contrast to the effects of group-wise interactions. Furthermore, while the effects of group-wise social interactions on sleep were mediated by visual cues and synaptic plasticity of the arousal-promoting large ventral lateral neurons (LNvs; Donlea et al., 2009), the increase in sleep after pair-wise interactions in males was not dependent on the LNvs or visual cues (Lone et al., 2016). Hence, despite similarities between group-wise and pair-wise interactions, there are significant differences in the mechanisms of such sleep induction as well as sex-specific differences. Thus, it might be interesting to further examine the similarities and differences between the effects of such interactions.

Additionally, it is known that sleep in *D. melanogaster* is sexually dimorphic, with females sleeping much less during the daytime as compared to males (Huber et al. 2004; Andrene & Shaw 2005). Such differences in daytime sleep may be associated with foraging and searching for oviposition sites in females (Isaac et al., 2009). Females also show differences in responses to socio-sexual interactions as well as mating with multiple partners, suggesting that the effects of social interactions may be sex-specific (Lone et al., 2012; Vartak et al., 2015). Hence, we decided to further examine the differences in the effects of social interactions on sleep between males and females.

Furthermore, certain effects of social interaction may be stronger at an early age (Greenough et al., 1978; Miyamoto et al., 2003). Social interaction in mice during rearing resulted in a higher proportion of cortical synapses compared to those reared in isolation (Greenough et al., 1978). Moreover, rearing in dark during a critical postnatal period has been shown to affect sleep patterns in cat and mice (Miyamoto et al., 2003). Similarly, a number of studies in the past have indicated that social experience in the first week post-eclosion in fruit flies greatly modulates the development of the fly brain (Technau et al., 1984; Balling et al., 1987; Heisenberg et al., 1995) as well as a number of complex behaviours including sleep (Shaw et al., 2000). Technau et al. (1984) and Balling et al. (1987) observed that social environment during the first week post-eclosion affects the turnover in kenyon cell numbers which form a part of the mushroom body (involved in olfaction, learning and memory) in the fly brain. Social interaction during the first week was found to cause an increase in the number of Kenyon cell fibres by 15% as compared to solitarily individuals. However, social experience after the first week of adulthood did not result in such differences. Heisenberg et al. (1995) found that flies maintained in groups during their first week after eclosion showed larger

mushroom bodies compared to solitary individuals. Since the mushroom bodies are known to play a role in regulating sleep in Drosophila (Joiner et al., 2006; Pitman et al., 2006), social interactions in early adult life may affect their sleep patterns as well.

In a separate study, Ganguly-Fitzgerald et al. (2006) looked at the effect of group-wise social interactions on sleep both immediately post-eclosion as well as five days post emergence. However, they did not find any differences between the effects of group-wise interactions at these two ages. In contrast, Lone et al. (2016) looked at effect of pair-wise interactions on flies that were 4-days old and maintained in same sex groups of approximately 30 individuals for the first 4-days prior to carrying out pair-wise interactions. Since previous studies indicate that social interactions at early ages may show different effects than those at a later age, and that group-wise and pair-wise interactions show significant differences in their mechanisms and effects, we carried out an experiment where pair-wise interactions were set-up immediately as well as 4-days post-eclosion. We examined the effects of both same-sex as well as mixed-sex pair-wise interactions on the subsequent sleep levels in both males and females. The results of our study do indicate a stage-specific effect of pair-wise interactions on males though the trends in increase of daytime sleep are similar across both stages of interaction. Additionally, similar to the previous study on pair-wise interaction (Lone et al., 2016), females did not show increases in sleep as a consequence of social interactions. Thus, our study highlights the effects of age as well as sex on the influence of social experience on sleep.

Materials and methods

Canton S (CS) fly lines were obtained from the laboratory of Dr. Sheeba Vasu, Neuro Science Unit (NSU) at Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru. These flies were reared on corn-food medium and collected in a Plexiglas cage till a population size of approximately 300-400 flies was obtained. Subsequently, the cage was provided with a food-plate layered with yeast paste for 2-days in order to increase the fecundity of the flies. After 2-days of yeasting, a fresh plate of corn media upon which flies laid their eggs replaced the yeast plate. These eggs were transferred individually to 7 mm × 65 mm glass tubes with the help of a brush.

Freshly emerged flies from such tubes were identified as male or female and introduced into a fresh tube as solitary individuals or were paired with an individual of same sex or opposite sex. Hence, we had male-male pairs (18 pairs; *n*=36), female-female pairs (18 pairs; *n*=36), male-female pairs (36 pairs, males *n*=36, females *n*=36) and solitary males (*n*=18) and females (*n*=18). These flies were maintained in their respective social regimes for 4-days in the glass tubes. Post 4-days, the flies that had undergone social interaction were separated and transferred into 5 mm × 65 mm glass tubes for recording locomotor activity using Drosophila activity monitors (TriKinetics, Waltham, USA). The set of flies that were kept solitarily were also transferred into 5 mm × 65 mm glass tubes for recording their activity, and served as controls for the flies that had undergone interaction for the first four days. These flies, subjected to pair-wise interaction immediately post eclosion along with their solitary controls will be referred to as stage 1 interaction flies.

Another set of flies were maintained solitarily immediately after eclosion by transferring individually into fresh tubes. These individuals were kept solitary for the first four days before being paired with an individual of the same sex or opposite sex or continued in isolation. Thus, we had male-male (16 pairs; *n*=32), female-female (15 pairs, *n*=30), male-female pairs (27 pairs; males *n*=27, females *n*=27) and isolated males (*n*=27) and females (*n*=27) for this treatment as well. These pairs were maintained in 7 mm × 65 mm glass tubes for social interactions for the next 4-days (days 5-8). Following days 5-8, the sets of flies which underwent interaction and those that were solitary during this period were both transferred into individual 5 mm × 65 mm glass tubes and their locomotor activity was recorded using Drosophila activity monitors (TriKinetics, Waltham, USA). We will henceforth refer to flies that underwent interaction between days 5-8 and their controls as Stage 2 flies.

Data from the recording of activity monitors was collected in 1-min intervals and was then converted into 5-min bins using DAM file scan (TriKinetics, Waltham, USA) and subjected to analysis for sleep. The data from monitor files was pasted onto an excel sheet template which had been previously used by Lone et al., (2016) to estimate number of sleep bouts in 30-min intervals and total sleep during daytime and nighttime. Sleep was estimated as a consolidated period of 5-min or more of continuous inactivity, as has been defined in previous studies (Hendricks et al., 2000; Shaw et al., 2000). Average sleep of flies over the first 3-days after separation was estimated. Total, daytime and nighttime sleep was calculated over a period of three days post-interaction for both the paired as well as solitary individuals. We then performed Analysis of Variance (ANOVA) using 'regime' as fixed factor for each of total, daytime and nighttime sleep. ANOVA was done separately for comparison

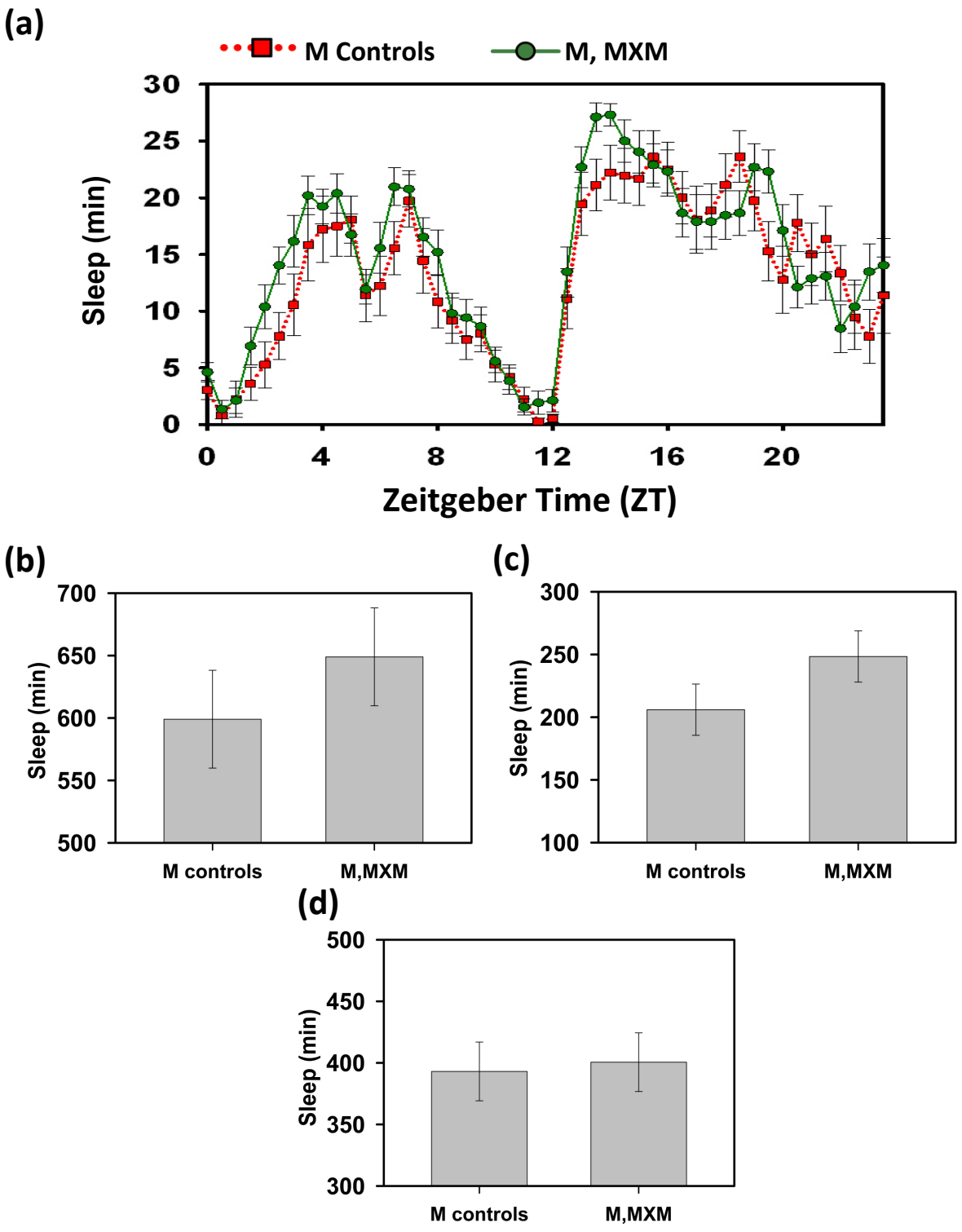

Figure 1. Figure shows a comparison of (a) Sleep profile (b) Total sleep (c) Daytime sleep (d) Nighttime sleep of males that were made to interact with other males immediately post eclosion and their isolated controls (Stage 1 flies). Error bars in (a) represent SEM, in case of (b),(c),(d) represent 95%CI.

between each social regime with their respective solitary controls. ANOVA was followed by post-hoc multiple comparisons using Tukey's unequal Honest Significant Difference (HSD). All our statistical analyses were implemented on Statistica v5.0 (Statsoft, 1995).

Results:

Stage 1 flies

(1.1) Sleep levels of males following pair-wise same sex interactions immediately post-eclosion:

We compared sleep profiles of male flies which were subjected to pair-wise interactions with other males, which were kept isolated immediately post-eclosion (Fig. 1a). Males from both regimes slept during the midday as well as most of the night (Fig. 1a) with males subjected to pair-wise interactions showing marginally greater sleep. However, ANOVA on sleep levels showed no statistically significant effect of 'regime' for total ($F_{1,42}$=1.35; p=0.251; Fig. 1b), daytime ($F_{1,42}$=3.58, p=0.065, Fig. 1c) or nighttime ($F_{1,42}$=0.08, p=0.775, Fig. 1d) sleep levels. These results suggest that sleep levels do not differ between males that have undergone same sex interactions (n=27) and their solitary controls (n=17).

(1.2) Sleep levels of females following pair-wise same sex interactions immediately post-eclosion:

We compared sleep profiles of female flies which were subjected to pair-wise interactions with other females and those of female flies which were kept isolated immediately post eclosion (Fig. 2a). ANOVA on sleep levels in females subjected to same sex pair-wise interactions also revealed no statically significant effect of 'regime' on total ($F_{1,45}$=0.750, p=0.390, Fig. 2b), daytime ($F_{1,45}$=0.251,

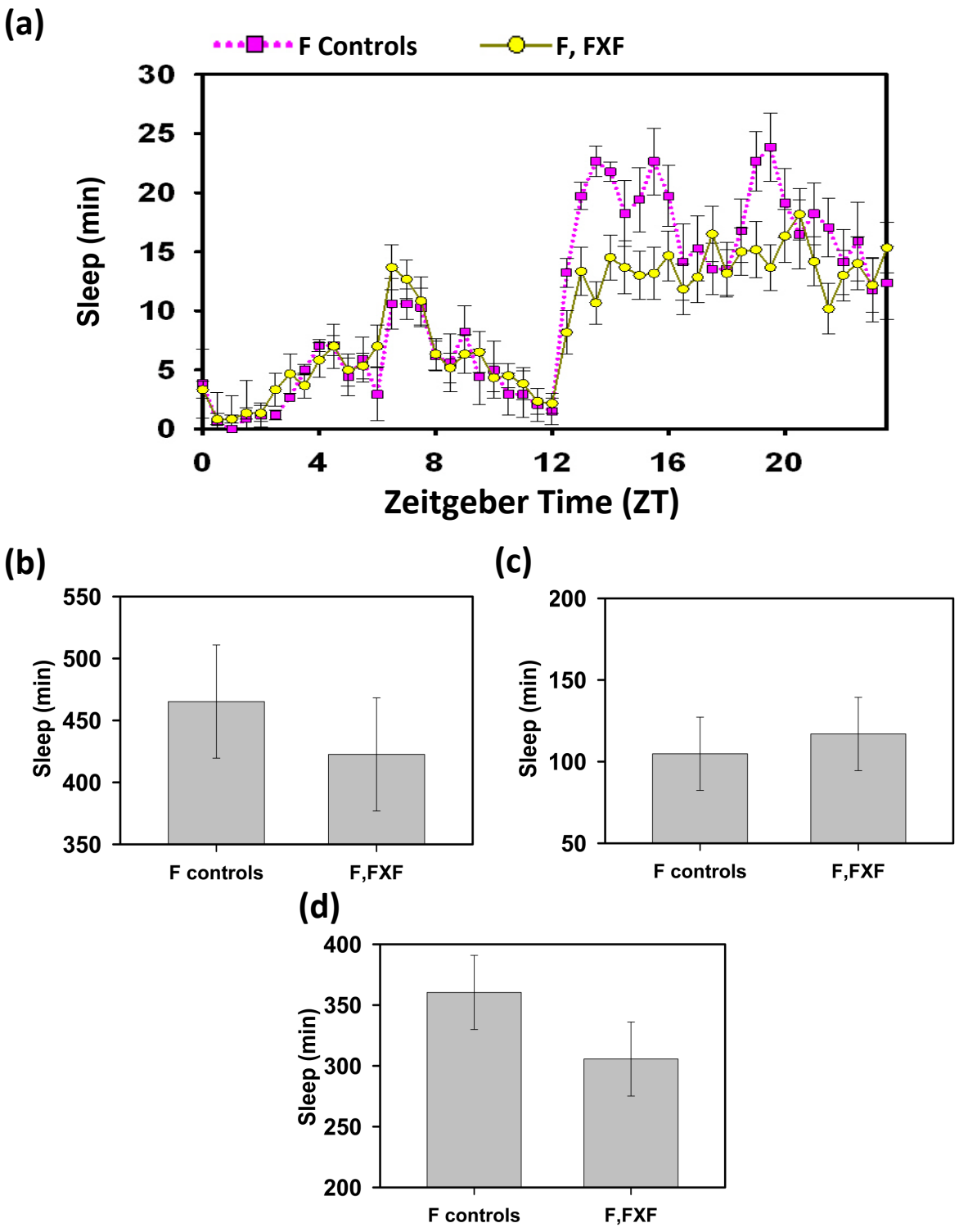

Figure 2. Figure shows a comparison of (a) Sleep profile (b) Total sleep (c) Daytime sleep (d) Nighttime sleep of females that were made to interact with other females immediately post eclosion and their isolated controls (Stage 1 flies). Error bars in (a) represent SEM, in case of (b),(c),(d) represent 95%CI.

p=0.618, Fig. 2c) or nighttime ($F_{1,45}$=2.780, p=0.102, Fig. 2d) sleep levels. Thus, there appears to be no difference in sleep levels between females that had undergone same sex interactions (n=30) and their solitary controls (n=17).

(1.3) Sleep levels of males following pair-wise interactions with females immediately post-eclosion:

We compared the sleep profiles of males that had interacted with females and males that were kept isolated immediately after eclosion (Fig. 3a). Results of ANOVA on sleep levels of males that had undergone interactions with females and that of solitary controls did not reveal any significant effect of 'regime' on total ($F_{1, 39}$=1.091, p=0.302, Fig. 3b), daytime ($F_{1, 39}$=1.451, p=0.235, Fig. 3c) or nighttime ($F_{1, 39}$=0.517, p=0.417, Fig. 3d) sleep levels. Thus, sleep levels do not differ between individuals that had undergone interactions with the opposite sex in males (n=24) and solitary controls (n=17).

(1.4) Sleep levels of females following pair-wise interactions with males immediately post eclosion:

ANOVA on daytime sleep in isolated females and females post-interaction with males showed statistically significant effect of 'regime' ($F_{1, 36}$=4.438, p=0.042, Fig. 4c). However, post-hoc comparison using Tukey's unequal HSD showed no differences between sleep in females subjected to pair-wise interactions and solitary female controls (p=0.052) though females that had interacted with males appeared to sleep more (Fig. 4c). ANOVA also revealed no statistically significant effect of regime on total ($F_{1, 36}$=0.377, p=0.542, Fig. 4b) or nighttime ($F_{1, 36}$=0.255, p=0.616, Fig. 4d) sleep levels. Hence, there are no significant differences in sleep levels between females that had undergone interactions with opposite sex (n=21) and their solitary controls (n=17).

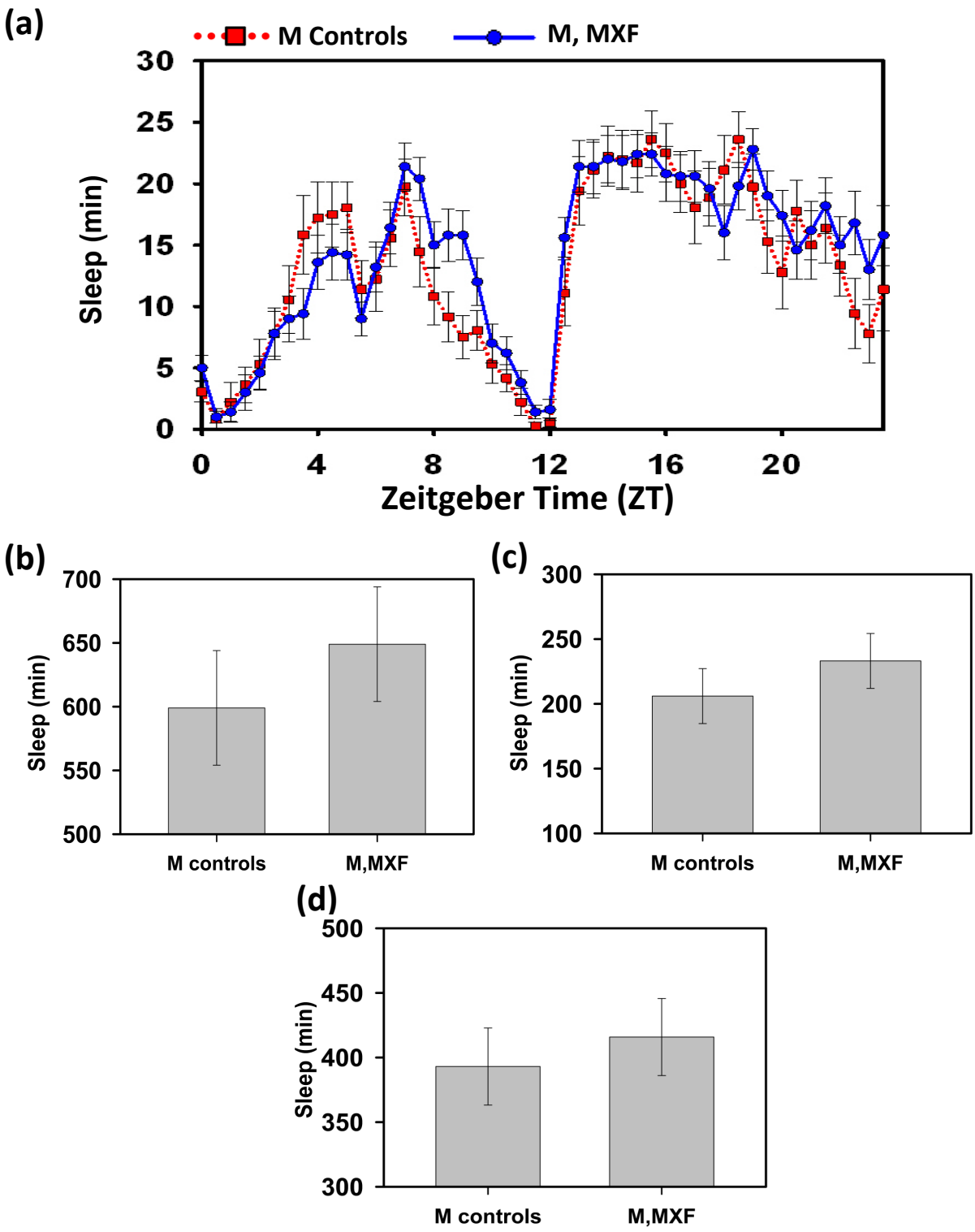

Figure 3. Figure shows a comparison of (a) Sleep profile (b) Total sleep (c) Daytime sleep (d) Nighttime sleep of males that were made to interact with other females immediately post eclosion and their isolated controls (Stage 1 flies). Error bars in (a) represent SEM, in case of (b),(c),(d) represent 95%CI.

Overall, the results from Stage 1 flies indicate that neither same sex nor opposite sex interactions have significant effects on levels of sleep post interactions in either males or females.

Stage 2 flies

(2.1) Sleep levels of males following pair-wise same sex interactions 4-days post eclosion:

We compared the sleep profiles of males that had interacted with same sex individuals four days post eclosion with isolated males that served as controls (Fig. 5a). The results of ANOVA on sleep levels in isolated males and those that had undergone same sex interactions revealed a statistically significant effect of 'regime' on total ($F_{1, 41}$=7.952, p=0.007, Fig. 5b) as well as daytime sleep ($F_{1, 41}$=11.600, p=0.001, Fig. 5c). Post-Hoc comparisons using Tukey's unequal HSD (total sleep: p=0.008; daytime sleep: p= 0.009) showed that males that had undergone same sex interactions in Stage 2 showed significantly greater sleep than their isolated controls (Fig. 5c, d). There is no such effect of 'regime' on nighttime sleep levels ($F_{1, 41}$=3.127, p=0.084, Fig. 5d). The results indicate that males (n=21) in Stage 2 flies show significantly increase in daytime and total sleep levels as compared to their solitary controls (n=22) after same sex interactions four days after eclosion.

(2.2) Sleep levels of females following pair-wise same sex interactions 4-days post eclosion:

We also compared the sleep profile of females of Stage 2 interactions with that of females kept as solitary controls (Fig. 6a). The results of the ANOVA reveal no statistically significant effect of 'regime' on total ($F_{1, 41}$=0.605, p=0.440, Fig. 6b), daytime ($F_{1, 41}$=0.719, p=0.401, Fig. 6c) or nighttime ($F_{1, 41}$=0.282, p=0.598, Fig. 6d) sleep levels suggesting that sleep levels are not different among

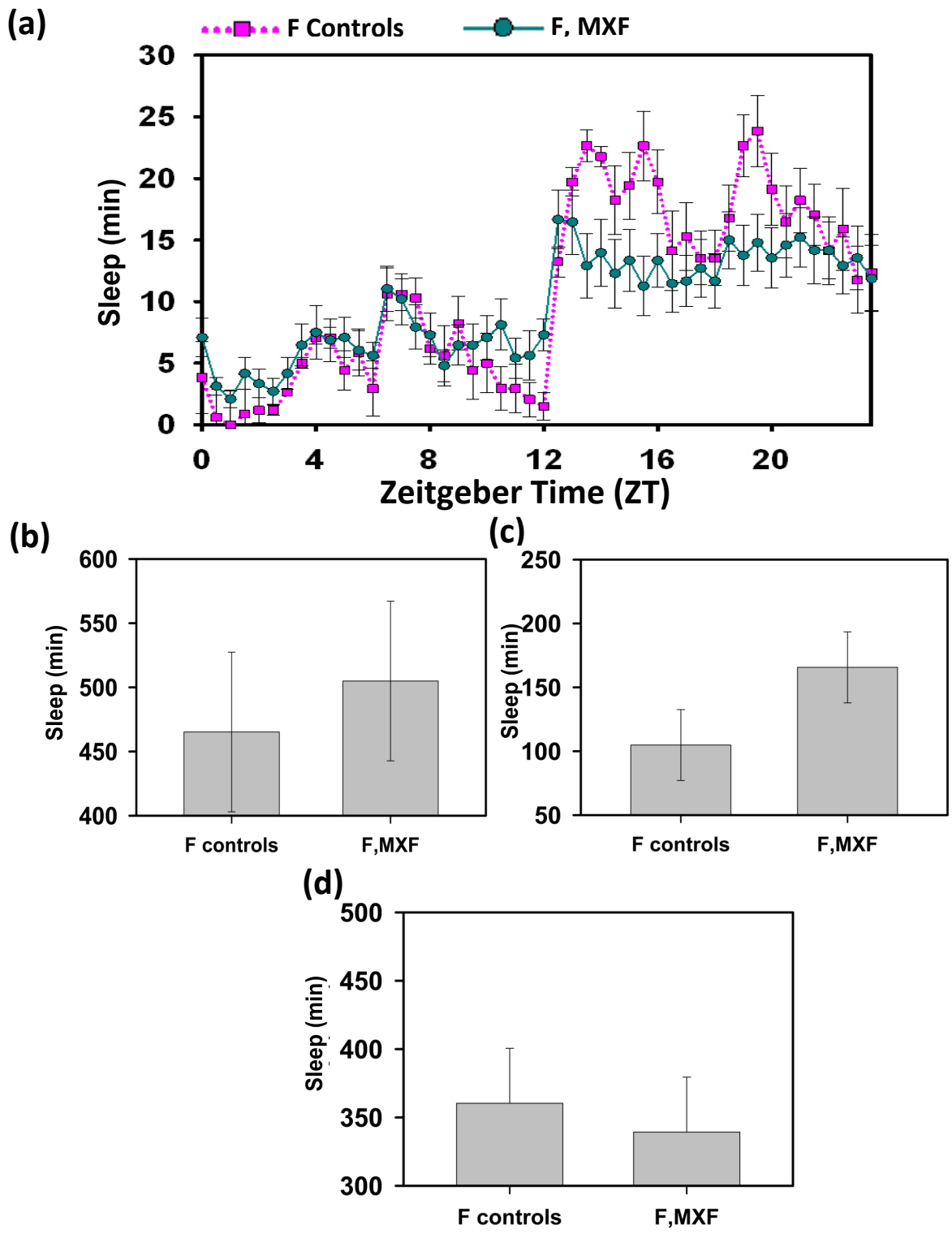

Figure 4. Figure shows a comparison of (a) Sleep profile (b) Total sleep (c) Daytime sleep (d) Nighttime sleep of females that were made to interact with males immediately post eclosion and their isolated controls (Stage 1 flies). Error bars in (a) represent SEM, in case of (b),(c),(d) represent 95%CI.

individuals that had undergone same sex interactions in females (*n*=24) of Stage 2 flies and their solitary controls (*n*=19).

(2.3) Sleep levels of males following pair-wise interactions with females 4 days post eclosion:

Results of ANOVA revealed a significant effect of 'regime' on daytime sleep ($F_{1, 37}$=4.580, *p*=0.038, Fig. 7c) when sleep levels were was analyzed in males post interactions with females as compared to their solitary controls. However, post-hoc comparisons using Tukey's unequal HSD showed no statistically significant difference between sleep levels in males that have interacted with females and their solitary controls (*p*=0.051) though males subjected to interactions with females showed a trend of greater daytime sleep. No significant effect of 'regime' was seen in total ($F_{1, 37}$=1.382, *p*=0.247, Fig. 7b) or nighttime ($F_{1, 37}$=0.135, *p*=0.714, Fig. 7d) sleep levels suggesting no significant differences in sleep levels between individual males of Stage 2 flies that had undergone opposite sex interactions (*n*=17) as compared to their solitary controls (*n*=22).

The results indicate that there is a trend of increase in daytime sleep when males are made to interact with females four days post eclosion though the increase in level is not statistically significant.

(2.4) Sleep levels of females following pairwise interactions with males 4-days post eclosion:

Results of the ANOVA on sleep levels of isolated females and females that interact with males post four days of eclosion revealed no statistically significant effect of 'regime' on total ($F_{1, 38}$=0.688, *p*=0.411, Fig. 8b), daytime ($F_{1, 38}$=3.651, p=0.063, Fig. 8c) or nighttime ($F_{1, 38}$=0.004, *p*=0.946, Fig. 8d)

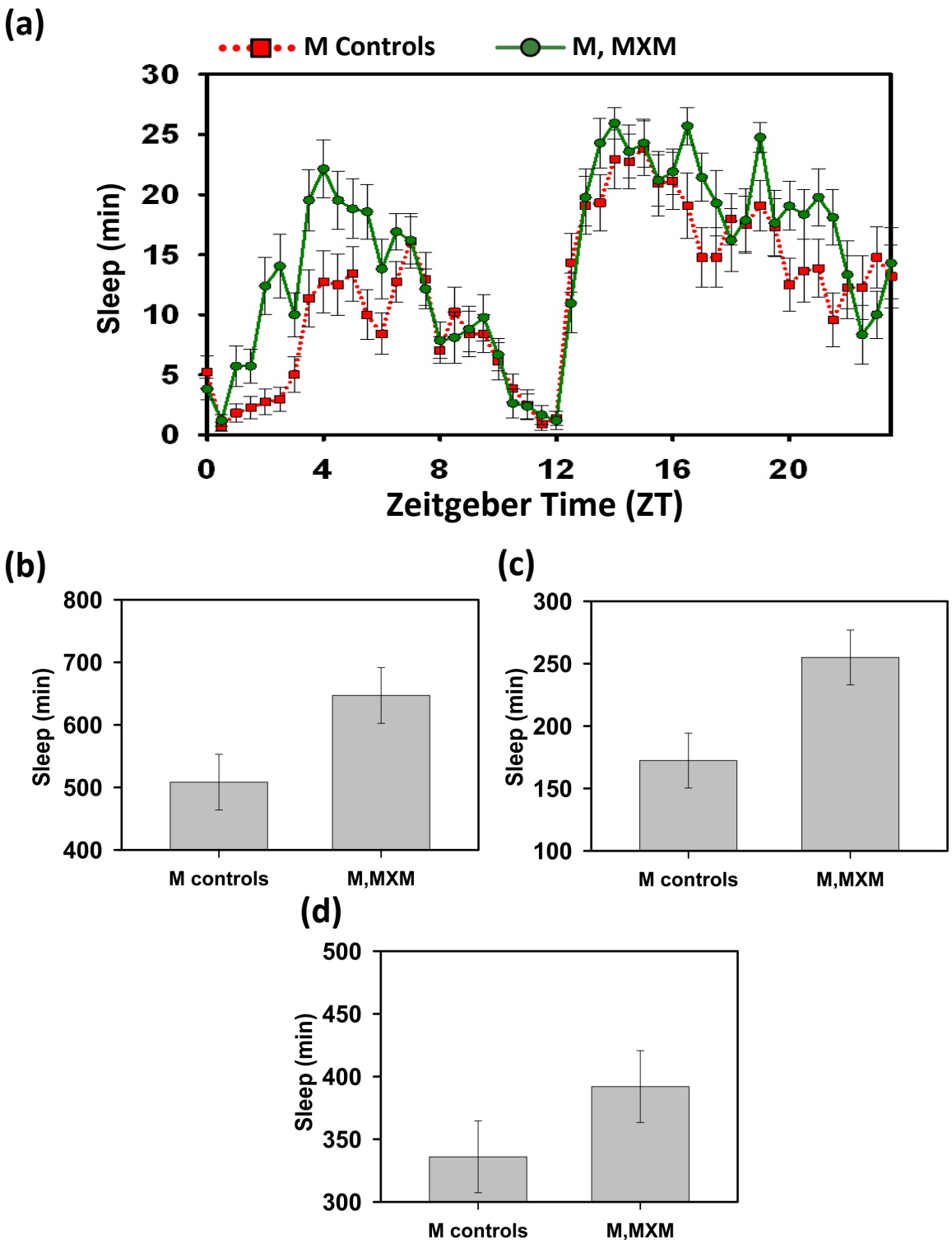

Figure 5. Figure shows a comparison of (a) Sleep profile . (b) Total sleep. (c) Daytime sleep. (d) Nighttime sleep of males that were made to interact with other males 4 days post eclosion and their isolated controls (Stage 2 flies). Error bars in (a) represent SEM, in case of (b),(c),(d) represent 95%CI.

sleep levels suggesting that sleep levels do not differ between females that had interacted with males (*n*=21) and their solitary controls (*n*=19).

Overall, our results indicate that males subjected to same sex pair-wise interactions showed an increase in sleep levels primarily due to increase in daytime sleep. Such an increase in sleep levels is not seen in females that interact with other females. In case of males interacting with females or vice versa there is a trend that indicates that there is an increase in daytime sleep levels, but this increase is not statistically significant.

Discussion:

Lone et al. (2016) looked at pair-wise same sex interactions in males and females and their effect on sleep levels post-interaction. They found an increase in daytime sleep levels post 4-days of same sex interaction in males. The flies that were used for the experiment were four days old. In light of the evidence of importance of the first week post emergence on brain development (Technau et al., 1984; Balling et al., 1987; Heisenberg et al., 1995) and complex behaviours like sleep (Shaw et al., 2000), we carried out similar experiments on the effects of pair-wise social interactions with flies that were freshly eclosed.

Our results indicate that when freshly eclosed males experience sex interactions for a duration of four days (males of Stage 1 flies) there is no significant increase in daytime sleep levels post interaction as compared to their solitary controls (Fig.1c). A possible reason for not seeing significant

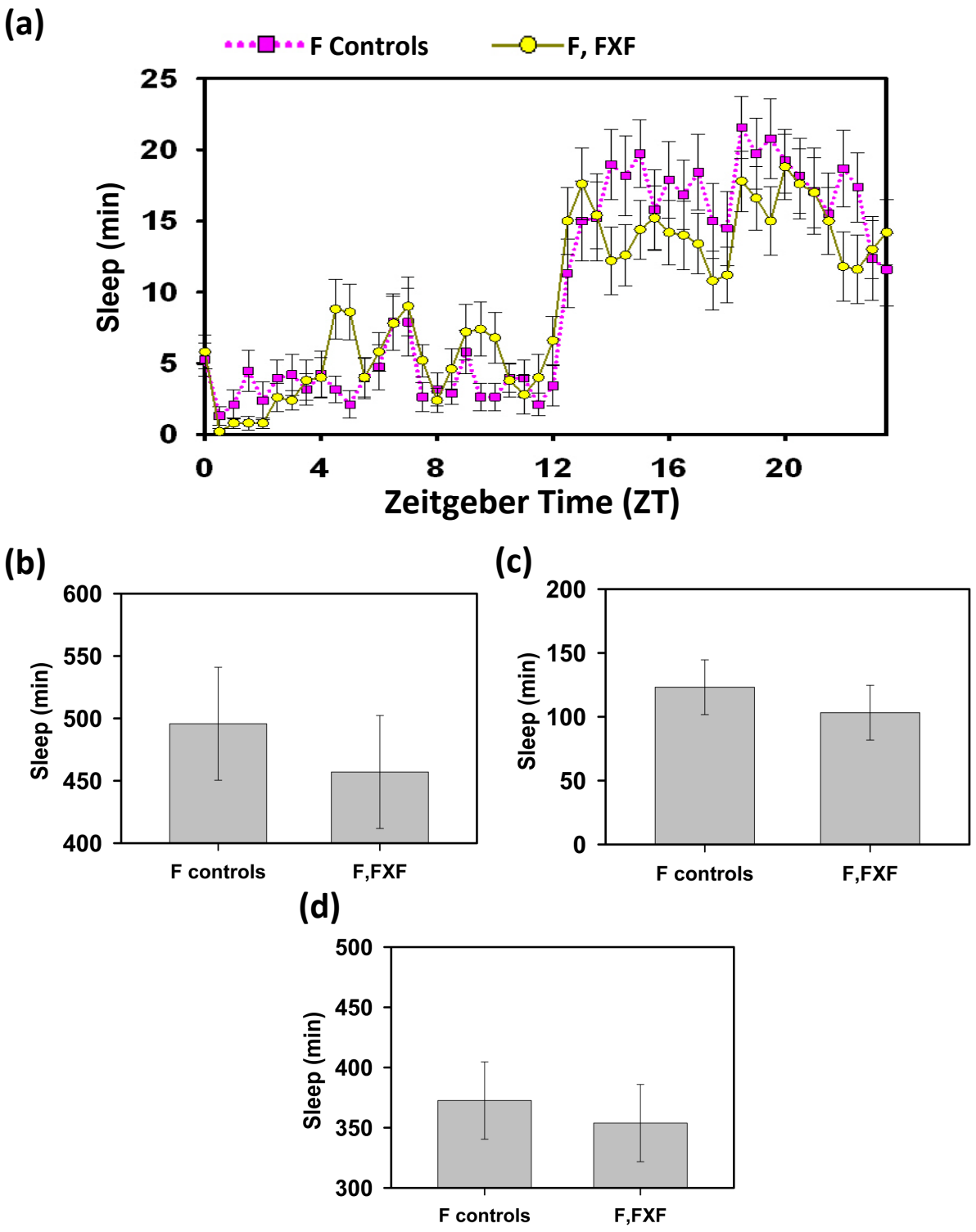

Figure 6. Figure shows a comparison of (a) Sleep profile . (b) Total sleep. (c) Daytime sleep. (d) Nighttime sleep of females that were made to interact with other females 4 days post eclosion and their isolated controls (Stage 2 flies). Error bars in (a) represent SEM, in case of (b),(c),(d) represent 95%CI.

differences in sleep in such males may be due to the high levels of daytime sleep in the solitary individuals during the first few days post-emergence (Shaw et al., 2000; Fig. 1a, b).

Lone et al. (2016) had also maintained flies in groups for the first 4-days following eclosion after which they were subjected to different regimes of pair-wise interactions. We also carried out an experiment to determine if grouping flies for 4-days prior to carrying out pair-wise interactions has an effect on sleep levels as opposed to if they are kept solitary for the first four days by maintaining flies in solitary conditions before subjecting them to pair-wise interactions. We found that males subjected to same sex interactions after four days of isolation post-eclosion (males of Stage 2 flies), also showed a significant increase in daytime sleep levels post interaction (Fig. 5c), which is similar to what is seen by Lone et al. (2016). These results suggest that the increase in sleep levels post same sex interactions is consistently seen regardless of whether flies were maintained in groups or in solitary conditions immediately post eclosion. Thus, the first 4-days post-eclosion do not appear to be a critical period for the effects of pairwise social interaction on sleep levels in adult fruit flies. Although the differences in sleep levels in case of Stage 2 flies is similar to that seen in Lone et al. (2016), the overall levels of total, daytime and nighttime sleep in our experiments were much lower than seen in Lone et al. (2016). These results indicate that the overall levels of sleep may be determined by both early social experiences immediately post eclosion as well as the social interactions experienced just prior to recording.

The results with regard to the females in Stage 1 and Stage 2 flies are similar. Neither of them showed significant differences in levels of sleep post interaction when compared to their respective

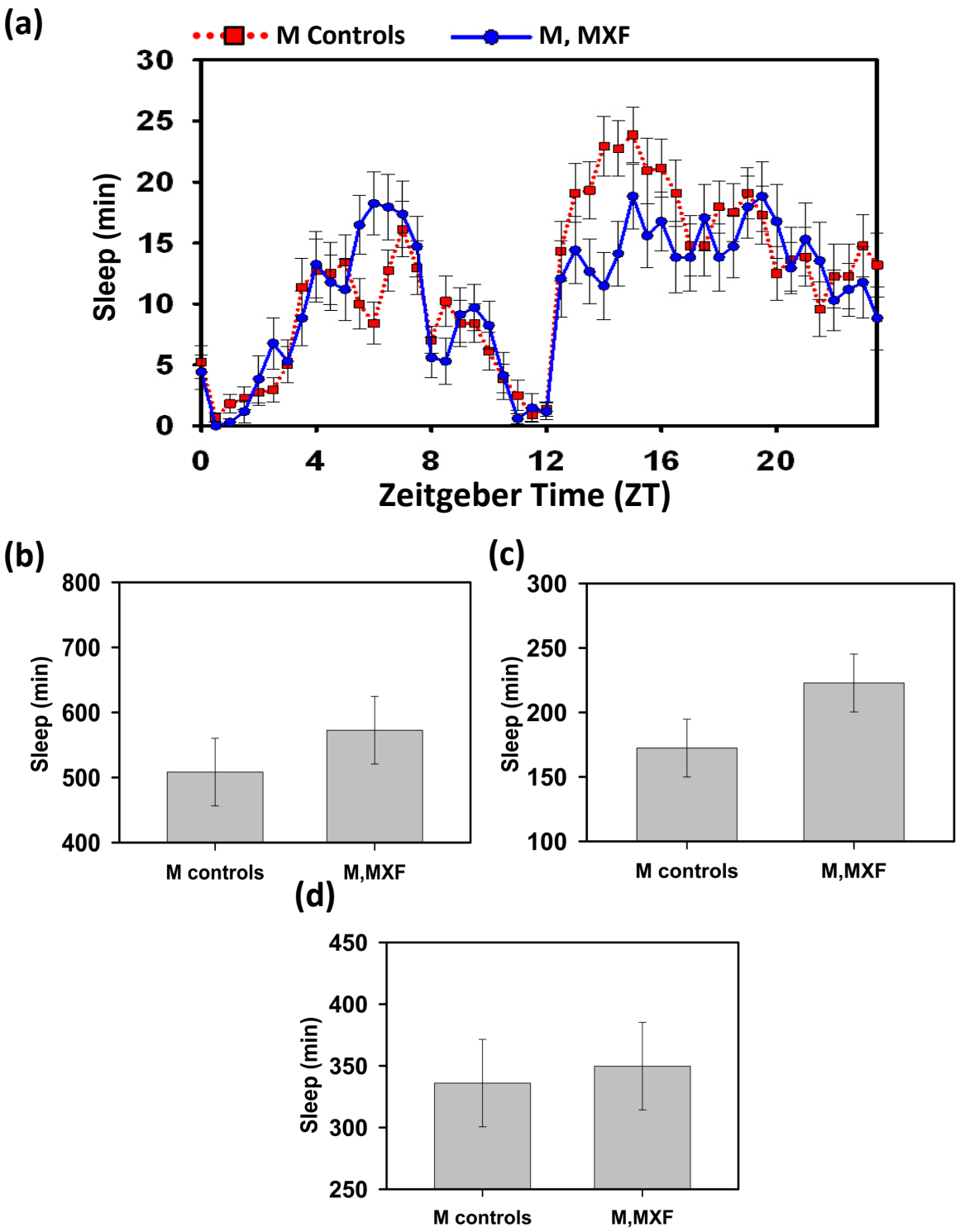

Figure 7. Figure shows a comparison of (a) Sleep profile (b) Total sleep (c) Daytime sleep (d) Nighttime sleep of males that were made to interact with females 4 days post eclosion and their isolated controls (Stage 2 flies). Error bars in (a) represent SEM, in case of (b),(c),(d) represent 95%CI.

controls. These results are also similar to what was observed by Lone et al. (2016) who speculated that the females require a larger group size for sleep levels to increase as a consequence of same sex interactions. This may be true for the experiments that we have conducted as well and remains to be verified. These results highlight the sex-specific effects of social interactions on sleep levels. Such differences between males and females are not surprising considering the fact that both sleep as well as effects of other social and sexual behaviours are sexually dimorphic (Huber et al., 2004; Lone et al., 2012; Vartak et al., 2015).

In case of interactions between opposite sexes in either Stage 1 or Stage 2 flies, we do not find a difference in either total, daytime, or night time sleep levels of males or females involved in the interactions, when compared to their respective controls. There is however a trend among both the males as well as the females involved in opposite sex interactions where the males and females undergoing interaction in both Stage 1 as well as Stage 2, shows a trend of increase in daytime sleep compared to the controls though the differences were marginally not significant. Such an increase in daytime sleep is consistent with the effects of group-wise interactions (Ganguly-Fitzgerald et al., 2006) but not with sex peptide-dependent increase in midday activity in mated females (Isaac et al., 2009). The reason for the lack of statistical significance may be the short duration of interaction (4-days), which may not be sufficient. Alternatively, the group size may not be large enough to evoke significant changes in sleep levels. We could verify these possibilities by carrying out a similar experiment for a longer duration while keeping the group size the same or we could carry out an experiment where the duration of interaction is the same but the group size is increased.

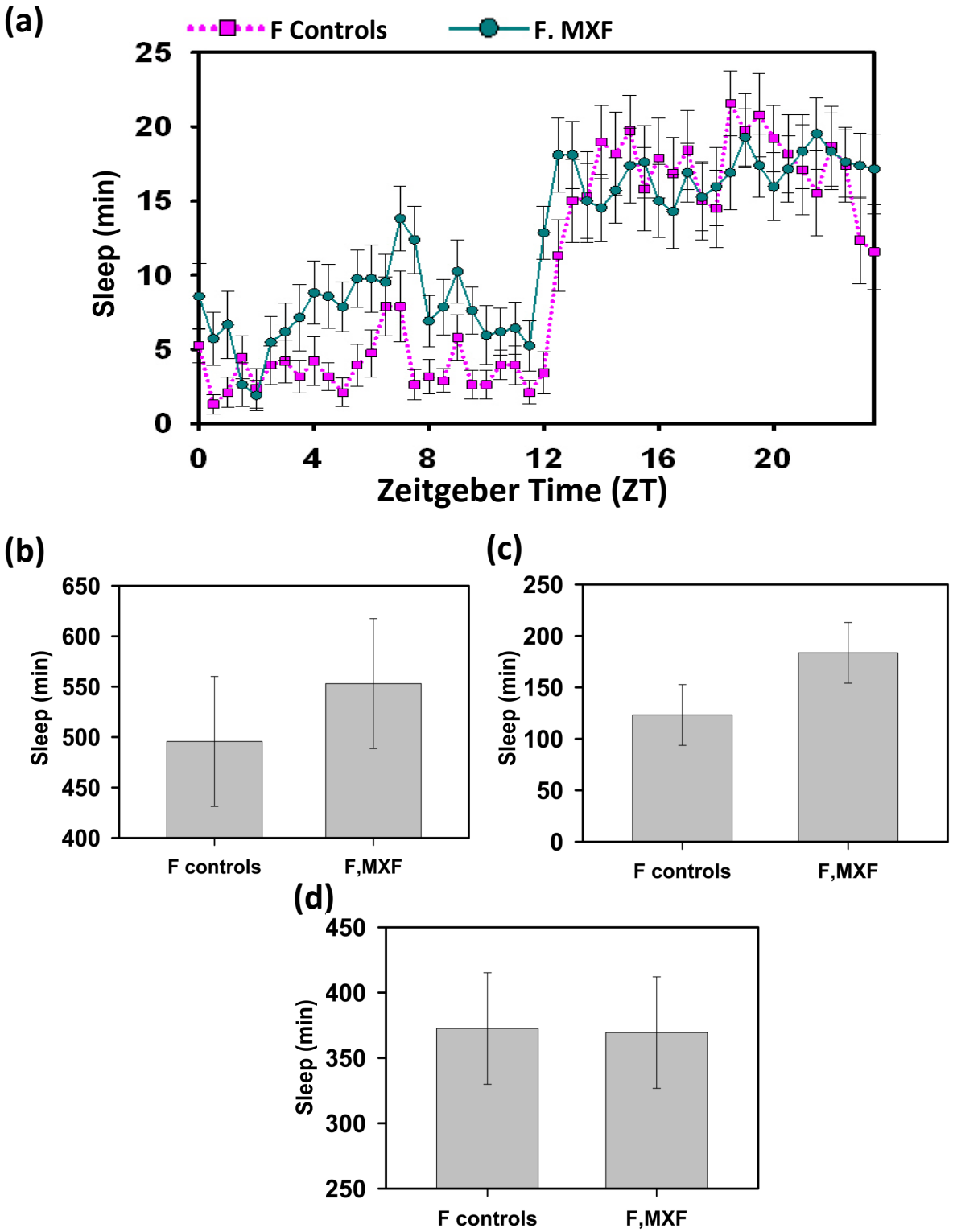

Figure 8. Figure shows a comparison of (a) Sleep profile (b) Total sleep (c) Daytime sleep (d) Nighttime sleep of females that were made to interact with males 4 days post eclosion and their isolated controls (Stage 2 flies). Error bars in (a) represent SEM, in case of (b),(c),(d) represent 95%CI.

In summary, male-male interactions appear to cause an increase in sleep, especially daytime sleep, post interactions immediately after eclosion or 4 days later. However, such differences in sleep compared to solitary controls are only significant in the stage 2 pair-wise interactions (may be because of high levels of daytime sleep in solitary controls of the stage 1 flies (Shaw et al., 2000)). Females do not appear to show significant differences in sleep levels post interactions with the same sex. However, both males and females show trends of increased daytime sleep post interactions with the opposite sex, though social interactions with greater number of flies or interaction for longer durations may be necessary to cause significant increases in sleep levels in such individuals.

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