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ORIGINAL PAPER

The Effect of Mating and the Male Sex Peptide on Group Behaviour of Post-mated Female *Drosophila melanogaster*

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Abstract

Sleep is a highly conserved state in animals, but its regulation and physiological function is poorly understood. *Drosophila melanogaster* is an excellent model for studying sleep regulation and has been used to investigate how sex and social interactions can influence wake-sleep profiles. Previously we have shown that copulation has a profound effect on day time activity and quiescence (siesta sleep) of individual post-mated females. Here we have the studied the effect of mating and the transfer of the 36 amino acid sex peptide in the seminal fluid on the behavior of mated female *Drosophila* populations, where there will be on-going social interactions. The locomotor activity and sleep patterns of virgin and post-mated female *D. melanogaster* from three laboratory strains (Oregon-R, Canton-S and Dahomey) were recorded in social groups of 20 individuals in a 12–12 h light–dark cycle. Virgin female populations from all three fly strains displayed consolidated periods of low activity in between two sharp peaks of activity, corresponding to lights-on and lights-off. Similar light-correlated peaks were recorded for the mated female populations, however, the low afternoon activity and siesta seen in virgin populations was abolished after mating in all three strains. In contrast, night activity appeared unaffected. This post-mating effect was sustained for several days and was dependent on the male SP acting as a pheromone. Evidence from mixed populations of virgin and mated females suggests that the siesta of non-mated females is not easily disturbed by the presence of highly active post-mated females.

Keywords *Drosophila* · Sleep · Social behaviour · Sex peptide · Seminal fluid

Introduction

Sleep, a rapidly reversible resting state accompanied by reduced response to sensory stimuli, is an enigmatic behaviour that is highly conserved in animals from nematodes to humans [1–4]. Sleep is critical for animal well-being, however its function and neuronal regulation is poorly understood [3, 5, 6]. Two important characteristics of sleep is the regulation by an internal circadian clock as well as a homeostatic mechanism that can compensate for sleep disturbance [7]. *Drosophila melanogaster* is proving to be a model *par excellence* in resolving the cellular mechanism of the circadian clock and, more recently, for identifying genes involved in sleep regulation and in unravelling the neuronal networks involved in promoting wake and sleep states [4, 8–10]. The importance of neuropeptide signalling in the clock cell network and in promoting and maintaining sleep in *D. melanogaster* is becoming increasingly apparent and has highlighted mechanisms by which wake-sleep profiles can be coordinated with other physiological events, such as feeding and reproduction [11–16].

In a previous study we showed that a male 36 amino acid peptide, known as the sex peptide (SP), can change the wake-sleep pattern of post-mated female *D. melanogaster*. SP is a well characterised male modulator of female *Drosophila* behaviour serving as a multi-functional signalling molecule that is passed to the female in the ejaculate [17, 18]. The change in female behaviour by SP is triggered by the silencing of sensory neurons of the female reproductive tract that communicate with the peptidergic cells of the *pars intercerebralis*, a brain neuroendocrine centre homologous to the vertebrate hypothalamus and known to be involved in the regulation of sleep as well as feeding and reproduction

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[19, 20]. SP is synthesised in the male accessory gland of adult flies as a 55 amino acid preprohormone [39]. After processing, the mature peptide is secreted into the seminal fluid and on mating is transferred in the ejaculate to the female where it elicits numerous post-mating behavioural and physiological responses (PMRs) [21, 22], including increased rate of egg-laying, reduced attractiveness to and rejection of courting males, stimulation of juvenile hormone synthesis [23–25], yolk accumulation in oocytes [26], increased appetite [27] and altered food preferences [28–31], elevated rate of excretion [32], loss of day-time sleep [33], enhanced female aggression [34], release of stored sperm [35] and modulation of the female innate immune system [36].

A notable structural feature of SP is a Trp-rich N-terminal region, which is not required for biological activity, but is responsible for SP binding to the surface of sperm tails. This attachment means that SP is transferred with sperm to the sperm storage organs and can be released over time by proteolytic cleavage at a trypsin-like cleavage site [37, 38]. This provides a mechanism by which the male can extend its influence on female behaviour to several days by the gradual release of the active SP from the sperm surface. In addition to the sperm-binding domain, there are two other distinct functional domains; a central region comprising the five 4-hydroxyproline residues and a modified isoleucine, and a C-terminal section ($SP^{21–36}$) responsible for receptor binding and initiation of many of the female PMRs [21]. The 4-hydroxyproline-rich central domain appears to have a role in eliciting an early (24 h after mating) female immune response to mating [36, 38]. The C-terminal signalling domain ($SP^{21–36}$) is critical for activating the G protein-coupled receptor expressed in sensory neurons of the female uterus that result in silencing of their neuronal activity. This signalling domain includes a peptide ring structure with a disulphide bridge between cysteine^{24} and cysteine^{36} [39, 40], which is not necessary for receptor activation, but probably protects the peptide from degradation by seminal fluid peptidases [41].

Our observation that the male SP can change the sleep behavior of post-mated females, added another response to the long list of PMRs triggered by the transfer of this male pheromone to the female during copulation [33]. When adult *D. melanogaster* are placed in a light/dark cycle, they display two periods of intense wakeful locomotor activity, one at lights-on and the other around the time of the light–dark transition. In between these peaks of activity there are periods of quiescence, or sleep, a behavior that is sexually dimorphic with males sleeping more than females during the afternoon or siesta period [7, 42]. The flies' siesta is a possible adaptation for survival during hot afternoons which might place individuals at risk from desiccation [43, 44]. Females as well as males should benefit from inactivity during the siesta, but females differ from males in that they need to balance risks with the demands of reproduction, which include foraging, to satisfy an increase in appetite and the need for a high-protein diet to sustain egg production, as well as the seeking of egg laying sites [45]. However, virgin females are not under the same pressures and therefore appear to reduce exposure to environmental risks by reducing locomotor activity and increasing levels of day-time quiescence to levels similar to that seen in males. SP appears to be the molecular switch that changes the behavior of post-mated females by increasing locomotor activity and reducing sleep [33]. A similar response to mating by female *D. melanogaster* was also observed in other studies and in the related fruit fly *D. suzukii*, which also receives SP in the male seminal fluid [20, 46, 47].

The circadian timing of locomotor rhythmic activity and the wake-sleep architecture of *D. melanogaster* are influenced by social interactions experienced by couples or flies housed in larger groups [48, 49]. For example, Ganguly-Fitzgerald et al. showed that a 5-days enriched social experience amongst same and mixed sex adults can substantially increase the amount and quality of sleep of individuals compared to flies that have been deprived of any social interactions from eclosion [48]. This effect of social interaction on wake-sleep balance resulted mainly from an increase in day-time sleep and could be reproduced when flies were kept in same-sex pairs for 3 or more days, resulting in enhanced day-time sleep for male, but not female, *D. melanogaster*. Recently, population activity monitors have been employed to show that sleep in male and female populations of 50 flies are regulated by both circadian and homeostatic mechanisms, as reported for individual flies [50]. This study, however, revealed some sleep differences between populations and individuals, possibly from olfactory communication between flies within a population. Sexually dimorphic sleep behavior was also reported with males sleeping more during the day than females, however, the study did not investigate any impact of mating on sleep on the female population [50].

In the present study, we have extended our earlier investigation of the effect of mating on female sleep from individuals to populations. We now show that mating has a profound effect on the activity of socially enriched female flies resulting in increased day light locomotor activity and loss of siesta sleep. Males lacking SP in their ejaculate do not elicit a strong sleep PMR in the female population. Locomotor data collected from mixed populations of virgin and mated females suggests that the siesta sleep of virgin flies is robust and is not disturbed by the afternoon excitable activity of the post-mated population.

Materials and Methods

Fly Strains

Oregon-R were from an established stock maintained in our laboratory for over 20 years. Canton-S and Dahomey wildtype strain were provided by S. T. Sweeney, University of York, U.K. and T. Chapman, University of East Anglia, U.K., respectively. SP null mutants (SP^0) and control wildtype flies (SP^+) were generated as described previously using mutant stocks, provided by S. Wigby, University of Oxford, U.K. and originating from the laboratory of E. Kubli [34, 37].

Fly Culture

Flies were cultured on oatmeal/molasses/yeast/agar medium at 25 °C in 12:12 h light–dark cycle and were sexed at the pupal stage on the basis of presence/absence of male sex combs.

Recording Locomotor Activity and Sleep of Fly Populations

All experiments were conducted at 25 °C in a 12h:12 h light–dark cycle. Unless stated otherwise, female flies (1 day-old) were mated with males by placing 10 virgin females with 10 virgin males in vials (95 × 25 mm) containing oatmeal/molasses/agar diet for 3 days. Virgin females were kept in groups of 20 for the same length of time under identical conditions. After 3 days, these flies were lightly anaesthetised using CO_2, separated by sex and placed in glass vials (95 × 25 mm) containing 6 ml of 2% (w/v) agar and 5% (w/v) sucrose. Vials (95 × 25 mm) were placed in *Drosophila* population activity monitors (DPM, Trikinetics Inc. Waltham, U.S.A.) that use three arrays of infrared (IR) beams, each set comprising 15 beams and placed in three positions along the length of the glass vial to detect movement as the fly walks along the glass tube (Fig. 1a). The apparatus was kept in a vertical position with the bottom array one positioned just above the agar/sucrose, the middle array two recorded movement half-way along the vial and the top array three was located close to the cotton plug at the open end of the vial. The total number of beam breaks was obtained by summing the data for all three sets of IR beams in 5 min or 30 min time-bins for each sex and strain, and the data analysed using microsoft excel. Flies were allowed to acclimatise for 12 h before data were utilised for analysis. For the purpose of this study, group sleep was defined as a period of 5 min with no locomotor activity detected by any of the three arrays of IR beams. Statistical analysis was carried out using GraphPad Prism 7.01.

Results

The Trikinetics DPM monitors allow the recording of locomotor activity of adult insects as they break three sets of IR beams positioned (i) just above the food, (ii) half way along the length of the population vial and (iii) just below the cotton plug (Fig. 1a). DPMs were used to compare the activity of populations of virgin and post-mated female *D. melanogaster* from three common laboratory strains (Oregon-R, Canton-S and Dahomey) in a 12:12 h light–dark cycle and constant temperature and humidity. Flies were placed in monitoring vials (20 females per vial) and were allowed to acclimatise for 12 h before activity data were collected for analysis. Virgin females of all three strains displayed two prominent peaks of population activity around lights-on (morning) and lights-off (late afternoon/evening) (Fig. 1b-d), separated by periods of very low activity that lasted for up to 6 h during the middle (afternoon) of the light period and for up to 9 h during lights-off (night). Mated female populations behaved similarly to their virgin counterparts during night time, but during day-light hours the populations remained very active during the afternoon, which contrasted with the quiescence of the virgin flies at the same time of day (Fig. 1b–d). All three arrays of detector beams were repeatedly broken by moving flies showing that the increased daytime activity of the post-mated population was not restricted to any one position in the vial, although relatively greater activity was usually detected by array three which was furthest away from the food (Fig. 2e, f).

To assess the influence of the male SP on the behaviour of mated female populations, Oregon-R females were mated with SP^o males that do not make SP, but otherwise have normal seminal fluid The resulting PMR was compared with that of populations of females mated with genetically matched control flies (SP^+) and virgin females [34, 37]. The high afternoon activity of the mated female population recorded previously was reproduced when SP^+ males producing normal levels of SP were used for insemination, but not when mated with SP null (SP^o) males (Fig. 2a). The high level of SP-induced afternoon activity of the female population progressively declined with time until at around 7 days it reached the same level of day light activity recorded for the virgin female population (Fig. 2b). Night activity also declined steadily over this period, but there was no apparent difference in the population activity between virgin females and females mated with either SP^+ or SP^o males (Fig. 2c), emphasising that this SP-induced PMR only occurred during the afternoon period.

The locomotor activity data was transformed to provide a measure of sleep, defined as 5 min time bins in which no movement in the entire population was detected by any of the three sets of IR arrays (Fig. 3). For the first 6 days

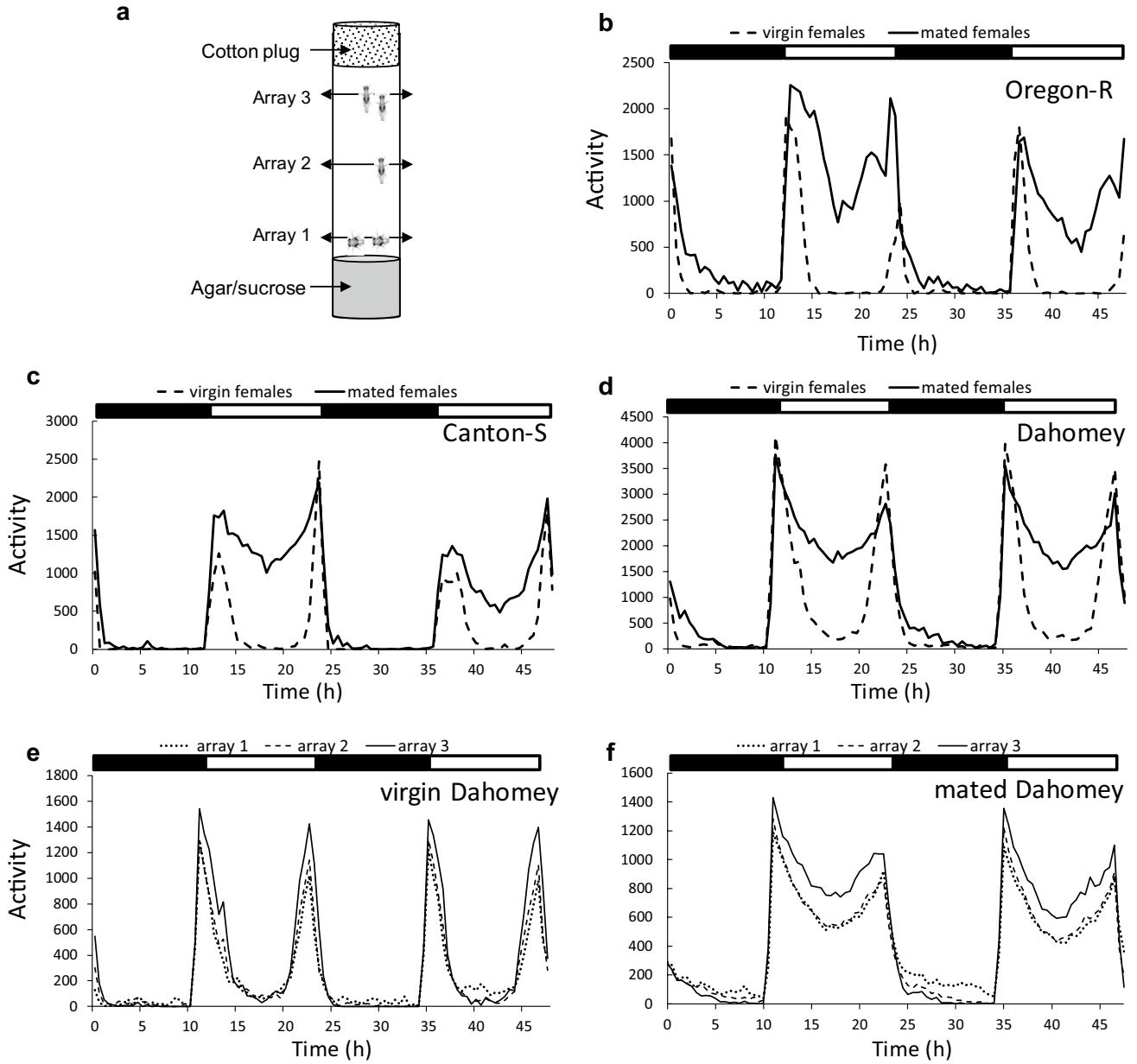

Fig. 1 Mating elevates afternoon locomotor activity in populations of female *D. melanogaster*. The activity of virgin (- -) and mated (--) populations was monitored over 48 h using DAM population monitors that record the movement of flies breaking three arrays of IR beams positioned as indicated in **a**. **b**–**d** Activity of populations (20 females) of Oregon-R, Canton-S and Dahomey strains, respectively. Populations were maintained in a 12:12 h light–dark cycle indicated by the open (lights-on, day light) and solid black (lights-off, night) bars. The activity is the sum of the beam breaks for all three arrays of IR beams recorded in 30 min time bins. **e, f** Beam breaks/30 min recorded by each of the three arrays for **e** virgin Dahomey population and **f** mated Dahomey population. These activities were summed to generate the data plotted in **d**

of the experiment, the afternoon siesta, a characteristic of the virgin female population, was essentially abolished for females mated with control SP^+ males. In contrast, the female population mated to males lacking SP (SP^o) did sleep during the afternoon, although the total sleep was not as great as that experienced by the virgin female population. The relatively low sleep value for day 1 probably reflects poor acclimatisation after transfer to the DAM vials. Mating with males expressing SP, but not with SP null males, also appeared to trigger a reduction in night-time sleep, however, only by around 25%.

To investigate possible day light social interactions between virgin and mated females, the locomotor activity

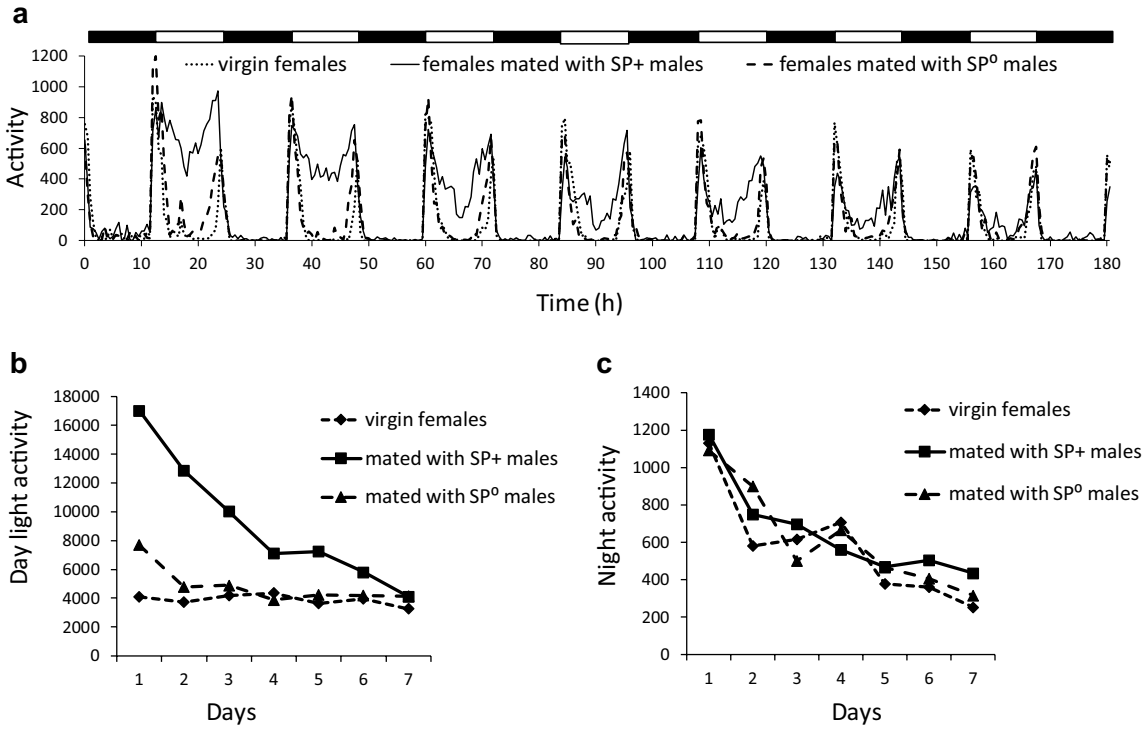

Fig. 2 SP of the male seminal fluid is necessary for elevating the afternoon activity in populations of post-mated *D. melanogaster* females. **a** Population activity of virgin Oregon-R females (●●●) and females mated to either SP null (SP^0, - - -) or control (SP^+, ---) males kept in a 12:12 h light–dark cycle indicated by the open (day light) and solid black bars (night). **b** Data from **a** plotted as total population activity in the 12 h of day light. **c** Data from **a** plotted as total population activity in the 12 h of night

of a mixed population comprising ten mated and ten virgin females was compared with a population of 20 virgins (20) and a population of ten mated females for 2 days in the standard 12:12 h light–dark cycle. The expected peaks of morning and evening activity were observed for all three populations as well as the mating-induced rise in afternoon activity in the mated fly population compared to the virgin population (Fig. 4a). Increasing the population of flies from 10 to 20 by mixing mated and virgin females increased the activity levels at lights-on and lights-off, but not during the afternoon siesta period. To emphasise this point and provide statistical support, the day light activity for the three female populations was split into four 3 h periods. Period 1 covers the morning, period 2 and 3 is the midday afternoon and period 4 is the late afternoon/evening. As expected, mixing ten mated and ten virgin females raised activity levels during peak periods 1 and 4 when compared to the levels recorded for ten mated females. In contrast, mixing populations of mated females (ten) with virgin females (ten) made no significant difference to the activity levels during the siesta periods 2 and 3 compared with ten mated females, suggesting that the virgin flies remain quiescent despite the elevated activity of the co-housed ten mated females (Fig. 4b).

Discussion

Previous studies have shown that socially enriched individuals sleep more compared to flies that are socially deprived [48, 51]. These studies focused on the social experience prior to the monitoring of sleep and wakeful activity in individual flies and therefore differed from the present study and that of Liu et al. [50], who studied social behaviour in populations where flies experience ongoing interactions with other members of the community. Liu et al. used the LAM25H Trikinetics activity equipment which allowed monitoring of the activity/sleep behaviour of populations of 50 adult *D. melanogaster* [50]. This system although using vials of the same dimension as those used in the present study, differed significantly from our population monitors (DPM) in that the LAM25H has just one set of IR beams and detectors positioned to detect moving flies in the central axial region of a horizontal population vial. DPMs have three sets of IR beams/detectors positioned not only to detect flies crossing the middle of the vials, but also to detect flies moving close to the food surface and at the cotton plug interface. The study of Liu et al. showed that sleep/wake behaviour of same-sex populations was under both circadian and homeostatic control and like individual flies was sexually dimorphic [50]. Some differences between the behaviour

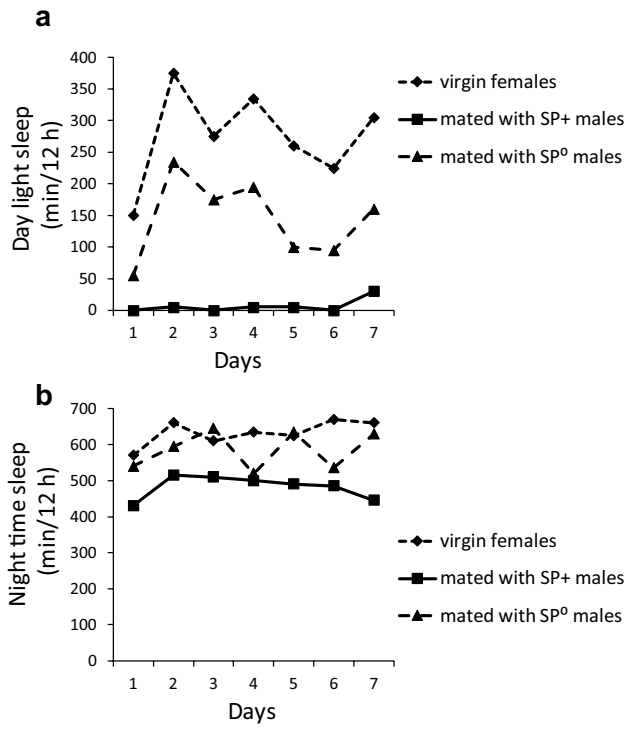

Fig. 3 The siesta sleep of a virgin female population is abolished after mating with males expressing SP, but not when mated with males lacking seminal fluid SP. Data from Fig. 2 was transformed to minutes of sleep/12 h, calculated from the number of 5 min periods of zero beam breaks recorded by any of the three sets of arrays of the DAM population monitors. **a** amount of day light (siesta) sleep and **b** night sleep experienced by virgin females and females mated to either SP null (SP^o) males or control (SP^+) males over 7 days

of individuals and populations were however reported (e.g. more rapid synchronisation of sleep onset in populations) and these were likely the result of social interactions mediated by multiple sensory stimuli including visual, tactile and olfactory [50]. Interestingly, when females were placed with males in a mixed-sex population (female to male ratio of 2:1) total day time sleep was much lower than that recorded for both single sex populations, suggesting that sexual encounters were stimulating activity of both sexes.

The present study focused on the behaviour of female-only populations of *D. melanogaster* and the impact of mating status on the sleep/activity states of different laboratory strains (Oregon-R, Canton-S and Dahomey). This study allowed comparison with our previous published work describing the role of SP in abolishing the siesta sleep of individual virgin females [33]. The stimulating social environment of a population might be expected to increase the level of activity and reduce quiescence in the population, especially during the afternoon when sleep is less intense. Our data shows that for all three strains of *D. melanogaster*, virgin females display synchronised activity and sustained quiescence during both night and the afternoon siesta period despite the obvious potential for disruptive social interactions with other individuals in the population. Higher activity levels were noted for the Dahomey strain compared to the other two. The reason for this difference is not clear, however it has been previously reported that strains and even sub-strains can have markedly different locomotor behaviour [20, 52].

We have previously shown that mating results in increased locomotor activity and concomitant loss of sleep during the afternoon for individual females and that the male SP is the principal molecule responsible for switching female behaviour [33]. The same mating-induced loss of a siesta has now been reproduced in female populations for three strains of *D. melanogaster*. This change in population behaviour, at least for Oregon-R, is SP-dependent and persists for up to 1 week, presumably because of the previously reported slow-release of the peptide from stored sperm in the female [38]. A similar persistence of the post-mating response was observed in the earlier study of individually housed post-mated females [33]. Mixing of equal numbers of mated flies with high afternoon activity with virgin females experiencing a siesta period resulted in significant increase in the morning and evening peaks of activity because of the larger population size. This increase was not 100%, probably because the relationship between number of beam breaks and population size is not linear due to the greater chance of multiple flies breaking beams in the same time bin as the number of individuals in the vial increases. In contrast, doubling the population size did not increase the afternoon activity of the mixed population at all, suggesting that the siesta of virgin females is robust and not easily disrupted by the presence of the more excited post-mated females in the population.

In summary, this study has established that the SP-induced loss of the afternoon siesta by female *D. melanogaster* that was first observed in individual flies, can be replicated in populations experiencing ongoing social interactions and that the change of behaviour mated females is independent of fly strain. Furthermore, the siesta of virgin females appears to be maintained in mixed populations comprising equal number of the resting virgins and the very active post-mated females.

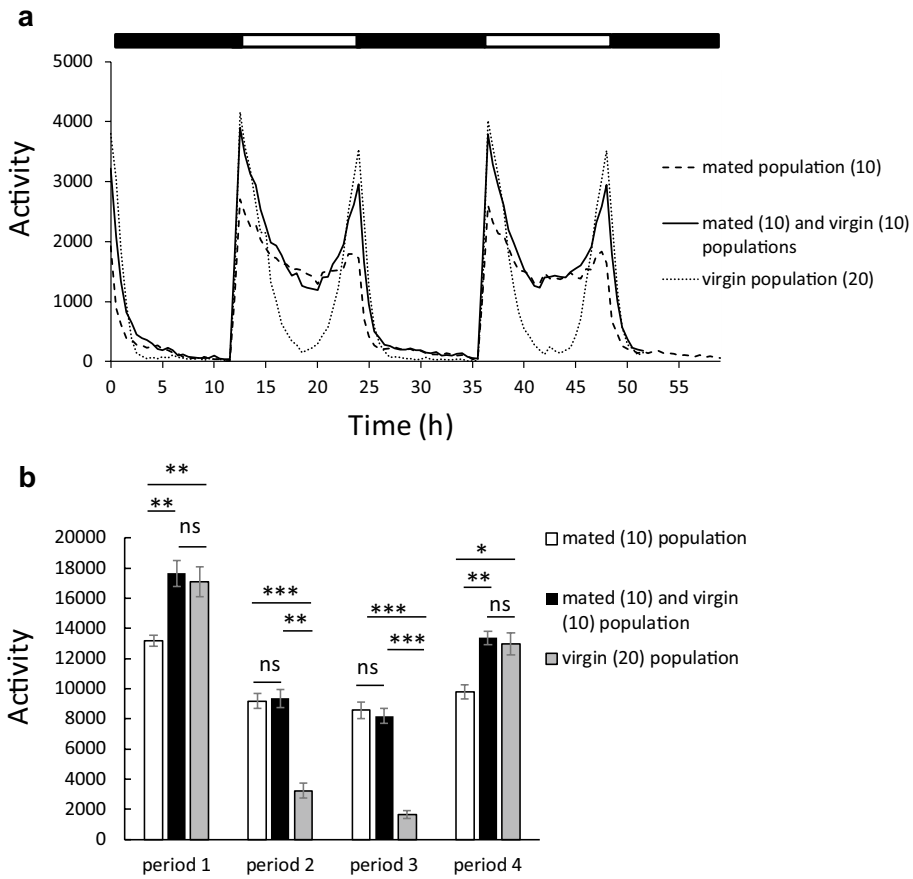

Fig. 4 The effect of mixing mated and virgin female populations on locomotor activity. **a** The population activity expressed as total beam breaks/h for ten mated females (- -), 20 virgin females (●●●) and a mixed population of ten virgin and ten mated females (--). The plotted activities are the means of data collected over 48 h from four separate experiments using flies of the Dahomey strain. The data points and error bars have been omitted for clarity. Open and solid black bars indicate the light and dark periods, respectively. **b** Activity data from the first day in **a** were summed into four 3 h day light periods (period 1, 13–15 h; period 2, 16–18 h; period 3, 19–21 h; period 4, 22–24 h) and expressed as the mean ± s.e.m. (n = 4). Statistical analysis was conducted using student's t test and one-way ANOVA (GraphPad Prism 7.01). ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Similar results were obtained when the second 24 h of data was analysed in the same way (plots not shown)

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