

Manipulating the light/dark cycle: effects on dopamine levels in optic lobes of the honey bee (*Apis mellifera*) brain

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Abstract This study examines the relationship between cyclical variations in optic-lobe dopamine levels and the circadian behavioural rhythmicity exhibited by forager bees. Our results show that changing the light–dark regimen to which bees are exposed has a significant impact not only on forager behaviour, but also on the levels of dopamine that can be detected in the optic lobes of the brain. Consistent with earlier reports, we show that foraging behaviour exhibits properties characteristic of a circadian rhythm. Foraging activity is entrained by daily light cycles to periods close to 24 h, it changes predictably in response to phase shifts in light, and it is able to free-run under constant conditions. Dopamine levels in the optic lobes also undergo cyclical variations, and fluctuations in endogenous dopamine levels are influenced significantly by alterations to the light/dark cycle. However, the time course of these changes is markedly different from changes observed at a behavioural level. No direct correlation could be identified between levels of dopamine in the optic lobes and circadian rhythmic activity of the honey bee.

Keywords Circadian rhythms · Insect visual system · Biogenic amines

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Introduction

Honey bees have a remarkable sense of time that contributes significantly to their success as foragers. Their use of the sun as a navigational cue relies on the forager bee's ability to compensate for the sun's movements across the sky during the course of the day, and their sophisticated time sense also allows foragers to learn the times of day when nectar or pollen is most abundant at particular food sources (Renner 1960; von Frisch 1967; Moore and Rankin 1985, 1993; Moore et al. 1998; Moore 2001). This “time-memory” is based on an endogenous circadian timing system that regulates a wide variety of behavioural, physiological and metabolic processes in the honey bee.

Activity patterns of forager bees show strong rhythmicity characterised by intensive foraging activity during the day and relative inactivity in the hive at night (Moore and Rankin 1985; Frisch and Aschoff 1987; Moore et al. 1989). The endogenous nature of this rhythm is revealed when foragers are removed from their natural environment and placed into conditions of constant darkness or constant light. Rhythmic activity continues under these conditions, but peak activity appears earlier on each successive day (Moore and Rankin 1985). This ‘free-running’ pattern is normally prevented by *Zeitgebers* such as light and temperature, which drive the entrainment or synchronization of the bees' activities to the natural day (Moore and Rankin 1993; Crailsheim et al. 1996). Activity rhythms of individual bees are also synchronised by social interactions within the colony (Southwick and Moritz 1987; Frisch and Koeniger 1994).

Rhythmic behavioural patterns in honey bees develop with age. Young bees that provide around-the-clock care

of larvae, for example, show no overt behavioural rhythmicity (Kaiser and Steiner-Kaiser 1983; Moore and Rankin 1985; Kaiser 1988; Crailsheim et al. 1996; Moore et al. 1998), but prior to the onset of foraging, and independent of cues experienced outside the hive, the activity of young workers becomes increasingly rhythmic (Spangler 1972; Stussi 1972; Toma et al. 2000). Recent studies have shown that mRNA levels for the clock gene *period* are higher in foragers than in young bees performing ‘in-house’ tasks (Toma et al. 2000; Bloch et al. 2001) and the number of *period*-expressing neurons in the brain is reported to increase with age (Bloch et al. 2003). It is likely that these developmental changes in circadian clock machinery contribute to the transformation from arrhythmic to rhythmic behavioural patterns in young worker bees. Responsiveness to entraining signals, such as light, also change with age (Ben-Shahar et al. 2003).

Intense effort has been directed towards localising the oscillators that control the timing of behavioural rhythms in insects (reviewed by Page 1985; Helfrich-Förster et al. 1998). In some species, such as the blowfly, *Calliphora vicina*, there is evidence suggesting that the pacemaker is located in the central brain (Cymborowski et al. 1994; Cymborowski and King 1996), but in other species evidence points to the optic lobes as being the site of the circadian pacemaker. Removal of both optic lobes from the cockroach, *Leucophaea maderae*, for example, results in complete behavioural arrhythmicity (Sokolove 1975; Page 1982; Stengl and Homberg 1994), but circadian locomotor activity in the cockroach can be restored by transplantation of the optic lobes (Page 1982, 1983) or more specifically, ectopic transplantation of the accessory medulla (Reischig and Stengl 2003). Compelling evidence in *L. maderae* indicates that locomotor activity rhythm in this insect is controlled by two bilaterally paired and mutually coupled endogenous circadian pacemakers (Nishiitsutsuji-Uwo and Pittendrigh 1968; Sokolove 1975; reviewed by Helfrich-Förster et al. 1998). Neurons postulated to serve a pacemaker function in this species are immunoreactive to antibody raised against pigment-dispersing factors (PDF) (Homberg et al. 1991, 2003; Stengl and Homberg 1994; Helfrich-Förster et al. 1998). PDF-immunoreactive (PDF-ir) lateral neurons in the fruitfly, *Drosophila melanogaster*, have been shown to contain the clock proteins PERIOD and TIMELESS (Ewer et al. 1992; Helfrich-Förster and Homberg 1993; Frisch et al. 1994) and ablation of PDF neurons causes severe abnormalities in the circadian rhythms of the fly (Renn et al. 1999). PDH-ir neurons located in a region tentatively defined as the accessory medulla of the honey bee have

recently been identified (Bloch et al. 2003) but their role in regulating circadian rhythmicity in bees has yet to be determined.

Attention has also been drawn to correlations between the intensity of locomotor activity and levels of modulatory amines, such as dopamine (DA) and serotonin, in the insect brain. Early reports identified a correlation between brain serotonin and activity levels in the cricket, *Acheta domesticus* (Muszynska-Pytel and Cymborowski 1978a, b) and subsequent studies revealed that injection of serotonin into the optic lobe of the cockroach shifts the circadian locomotor activity rhythm of this insect in a phase dependent manner (Eskin et al. 1982; Page 1987). Cyclical variations in levels of the catecholamine, DA, have been identified also in the optic lobes of crickets (Germ and Kral 1995), moths (Linn et al. 1994) and honey bees (Purnell et al. 2000), and in the cockroach, *Periplaneta americana*, changes in brain DA levels have been correlated with behavioural activity rhythms (Prée and Rutschke 1983). DA levels in the optic lobes of forager honey bees peak around midday when bees are actively foraging (Purnell et al. 2000). An apparent correlation between high DA levels and intense foraging activity in these insects suggests that DA may play a role in modulating the circadian rhythmic activity of the honey bee.

In this study we explore the relationship between cyclical variations in DA levels and the circadian behavioural rhythmicity of forager bees. We examine the consequences, both on behaviour and on brain DA levels of altering the light/dark (LD) regimen to which bees are exposed. Our results show that not only activity patterns of forager bees, but also DA levels in optic lobes of the brain are influenced by changes to the LD cycle. Interestingly, shifts in the timing of peak DA levels do not mirror changes in the behavioural activity patterns of forager bees.

Materials and methods

Experiment 1: pilot study

A pilot study was undertaken to examine whether DA levels are affected by altering the LD cycle. The study was undertaken in late spring (October/November). Two hives that had been established the previous season were used: the first (the *outside hive*) was maintained outdoors under normal light conditions, and the second (the *inside hive*) was placed indoors within a large gauze flight cage (ca. 4 m × 3 m) under an altered LD cycle. For bees in the outside hive, time of sunrise

was around 0630 h and time of sunset was around 2030 hours giving a LD cycle of approximately LD14:10. For bees maintained indoors, a timer was used to switch lights on at 1610 hours and off at 0410 hours. In this preliminary experiment, lighting inside the flight room was provided by six 120 W spotlights focussed onto the flight-room walls, which were covered in aluminium foil. This provided a light intensity within the room of ca. 200 lux. The inside colony was maintained for 3 weeks in a LD cycle of LD12:12 before the levels of DA present in the brain of adult worker bees were assayed. The indoor colony was provided daily with fresh sugar solution, milled pollen and water at two feeding stations. The temperature in the flight room was kept constant at 20°C and relative humidity ranged from 31 to 50%. Bees from the outdoor colony foraged in the normal manner. Ambient temperatures outside ranged from 14 to 23°C and relative humidity ranges from 26 to 48%.

Measurement of activity patterns

The numbers of worker bees entering and leaving each hive were recorded over a 36 h period using a light-emitting diode (LED, RS 585–242) set at the hive entrance. The infrared beam of the LED was detected using an infrared phototransistor (RS 585–220). When a bee entered or exited the hive the infrared beam of the light diode was interrupted and registered as an activity event. This gives an indirect estimate of the number of bees foraging at any time. Although non-foraging bees engaged in tasks at the hive entrance such as guarding will also be counted, this was assumed to be a small proportion of the number of bees entering and leaving the hive. Younger bees carrying out orientation flights may also be included in these data, although such flights are reported to be relatively infrequent compared to the number of bees foraging (Lindauer 1961).

Collection of brain samples

Prior to the collection of brain samples, forager bees from the colony located outside were collected as they returned from foraging flights. These bees were marked on the thorax with a dot of white acrylic paint and then returned to the hive. This enabled the identification of forager bees at night. Bees that had been collecting pollen (identified by a full load of pollen in their corbiculae) were not used in this study. Forager bees from the indoor colony were captured at feeding stations during “lights on” and marked in the same way. Twenty marked bees were collected from each hive at 4 hourly intervals over a 24 h period. The bees were

cold-anaesthetized by chilling at -4°C prior to removing the head and embedding it in wax. A window was cut in the head capsule and the brain (supraoesophageal ganglion) cleared of glandular tissue and retinal tissue (Fig. 1a). The brain was then removed from the head capsule, placed in an eppendorf tube and immediately frozen in liquid nitrogen. The optic lobes (Fig. 1b) were separated from the rest of the supraoesophageal ganglion for separate analysis. The tissue was stored at -80°C prior to assaying for DA content.

High performance liquid chromatography

Reverse-phase high-performance liquid chromatography with electrochemical detection (HPLC) was used to determine the levels of DA in each sample. Details of the methodology used have been described in detail elsewhere (Kokay and Mercer 1997; Purnell et al. 2000). In brief, tissue was sonicated for 6–8 s in 50 μl ice-cold 0.4 mol/l HClO_4 containing 2.6 mmol/l sodium metabisulphite and 2.7 mmol/l EDTA then centrifuged

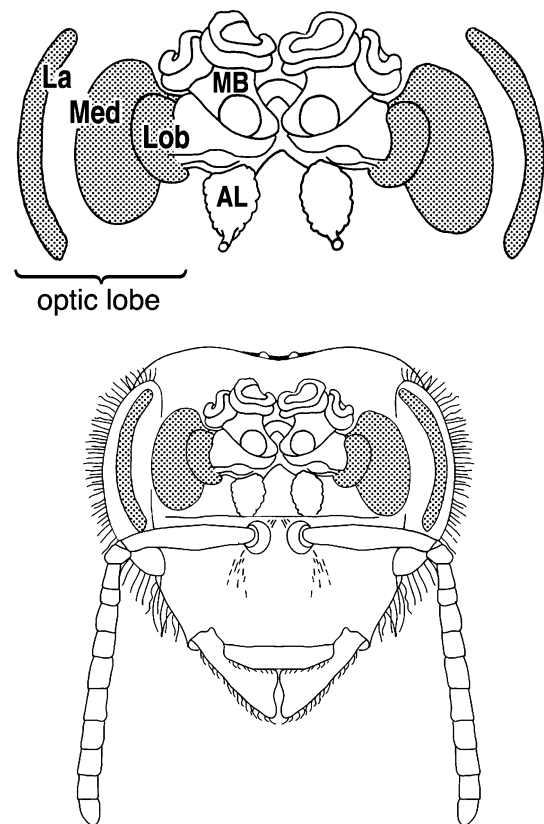


Fig. 1 Brain of the honey bee, *Apis mellifera*, showing the optic lobes. *Top* A schematic of the bee brain (modified from Mobbs 1982) shows the three optic ganglia that comprise the optic lobes, the lamina (*la*), medulla (*me*) and lobula (*lob*). The photoreceptor layer (retina) was removed in this study. *AL* antennal lobe, *MB* mushroom body, *OL* optic lobe. *Bottom* A window cut in the head capsule shows the position of the optic lobes of the brain

for 20 min at -4°C . Supernatant (20 μl) was injected directly onto the HPLC column. The HPLC equipment used in this study consisted of a Shimadzu LC-10AD pump, a Rheodyne injector, a C8 column (4.6 \times 100 mm with 5 μm packing) and an ESA model 5100A coulometric detector. The mobile phase consisted of (mmol/l): 20 sodium acetate, 100 sodium dihydrogen orthophosphate, 0.3 EDTA, 0.9 octanesulfonic acid (sodium salt) and 11% (v/v) acetonitrile adjusted to pH 2.5. The working potential was set at +0.3 V and a flow rate of 1.5 ml min^{-1} was used.

Calibration curves using dopamine HCl and *N*-acetyl-dopamine monohydrate standards were determined at the beginning of each assay run. Standards were also included at intervals during the assay run to confirm sample peak retention times. Stock solutions of standards were prepared in 0.4 mol/l HClO_4 and frozen at -80°C . These were further diluted into 0.4 mol/l HClO_4 prior to each assay run. For experiment 1, tissue samples were pooled (five brains minus optic lobes, or five pairs of optic lobes per sample) for analysis and DA levels are expressed in pmol per mg of protein (see below).

Protein analysis

In the cockroach, significant diel fluctuations in total brain protein levels correlate with levels of activity in this insect (Edwards 1980; Owen et al. 1987). For this reason, protein levels in the bee brain were examined over a 24 h period. The protein content of the pellets obtained after centrifugation of samples for HPLC was measured using the colorimetric method described by Peterson (1977). Before analysis, pellets were dissolved in 250 μl of 1 N NaOH containing 2.5% sodium dodecyl sulphate. Bovine serum albumin was used as the standard.

Experiment 2: manipulating the LD cycle: effects on activity patterns of foragers and DA levels in optic lobes of the brain

To monitor activity patterns more closely and their correlation with changes in DA levels in optic lobes of the brain, an observation hive (650 \times 57 \times 110 cm) was established together with facilities for continuous monitoring of activity levels. The observation hive held two brood frames. Double glass was fitted on both sides of the hive for observation purposes. For most of the time, the glass was covered using wooden panels that were used to insulate the hive and to keep it dark. Following the establishment of the new colony, the observation hive was placed outdoors for a period of time to enable the colony to stabilize. Throughout this time bees were able to enter and leave the hive at will. The

entrance of the hive was equipped with plastic tubes that the bees were required to walk through to enter or leave the hive. A feeding platform was constructed at the end of these tubes and a petri dish containing sugar water was placed on the platform.

In place of the flight room used in experiment 1, a 2 m \times 1.2 m \times 1.2 m perspex flight cage was constructed with holes for ventilation. A fan installed inside the chamber ensured continuous airflow within the flight room and the temperature was held at 22°C throughout the experiment. Illumination was initially provided by 6 UV-emitting fluorescent 8 Watt tubes (Philips TL 40/05) with a frequency of 33 kHz, a maximal spectral emission of 365 nm and a spectral energy distribution of 254 $\mu\text{Wm}^2/\text{s}$. As in experiment 1, foil placed on the walls of the flight chamber was used to create a diffusing reflector. Bees were initially exposed to LD12:12 for 1 week. On the seventh day, the LD cycle was reversed (DL12:12). It became apparent that the light intensity within the flight chamber was not strong enough to reset activity patterns as rapidly as was desired. For this reason, a further 40 Watt UV-emitting fluorescent lamp was added on day 13. The bees immediately adjusted their activity to the new reversed LD cycle. The bees were maintained under a reversed LD cycle for 13 days and then maintained for a further 8 days under constant darkness.

Activity measurements

Two tubes connecting the hive with the flight chamber contained light emitting diodes (LEDs) similar to those described in experiment 1. Rates of interruption of the infrared beams were recorded at 10 min intervals over the 28 days of the experiment and the data were collected on a BBC Archimedes 400 series computer. Raw activity counts were used to construct actograms, and the data were analysed using a periodogram analysis program based on Enright's periodogram (Enright 1965) and modified by Williams and Naylor (1978).

DA levels in optic lobes of forager bees

The brains of bees seen to be actively foraging from food sources inside the flight chamber were collected for analysis (10–15 per time point) during light hours. In order to collect bees during dark periods foragers were collected during the day and placed in a clear container provided with ventilation holes and ample food. The container was then left within the flight cage until the appropriate time for collection. Levels of DA were analysed as described above except that optic lobes were analysed per pair rather than pooled (five pairs) as in

experiment 1. As no significant variations in mean protein level could be identified in the optic lobes over a 24 h period (experiment 1), no further analysis of brain protein content was undertaken. DA levels in experiment 2 are expressed as pmol per pair of optic lobes.

Experiment 3: DA levels in the optic lobes of young worker bees

Optic lobes of young worker bees from an outdoor colony were examined in order to provide preliminary information as to whether differences in mean DA levels in the optic lobes apparent in foragers (Purnell et al. 2000, present investigation) are also apparent in young ‘house’ bees. Optic lobe sample were collected at two time points only, at midday (1200 hours) and at 1600 hours.

Data analysis

For statistical analysis the data were log transformed. To test the significance of changes in amine levels and protein levels data were subjected to ANOVA followed by post hoc pairwise comparisons to identify where differences lay. Tukey’s tests were used to compare groups in which sample sizes were equal. For sample sizes that were unequal, Dunn’s post hoc pairwise comparisons were used. Significance was defined at $P = 0.05$.

Results

Pilot study: activity measurements

Activity measurements under a normal LD cycle (experiment 1)

The number of bees entering and leaving the outside hive maintained under natural conditions was highest during daylight hours and peaked around midday (Fig. 2a). Activity levels remained high until 1700 hours after which they declined. By 1900 hours, 90 min before sunset (2030 hours), the number of bees entering and leaving the outside hive had reached low levels, and very little activity was observed at the hive entrance during scotophase.

Activity patterns under an altered LD cycle (experiment 1)

When subjected to an altered LD cycle, the activity levels of bees were synchronized to “lights on” and remained at a high level throughout the subjective daylight hours (Fig. 2b). Peak activity occurred around

2400 hours. Activity declined abruptly at 0400 hours coinciding with “lights off”.

Pilot study: DA levels in the brain and optic lobes

Normal LD cycle

Levels of DA measured in optic lobes of forager honey bees sampled from hives maintained outside in a normal LD cycle fluctuated significantly over the sampling period (ANOVA, $F = 2.81$, $P = 0.05$). The highest mean level of dopamine in the optic lobes (130 pmol/mg) was found in bees dissected at midday, significantly higher than the mean level detected at 0800 hours (37.3 pmol/mg; Fig. 3a). Small variations in DA levels in the optic lobes of bees at the other sampling times were not statistically significant. In contrast to the optic lobes, there were no significant fluctuations in DA levels in samples

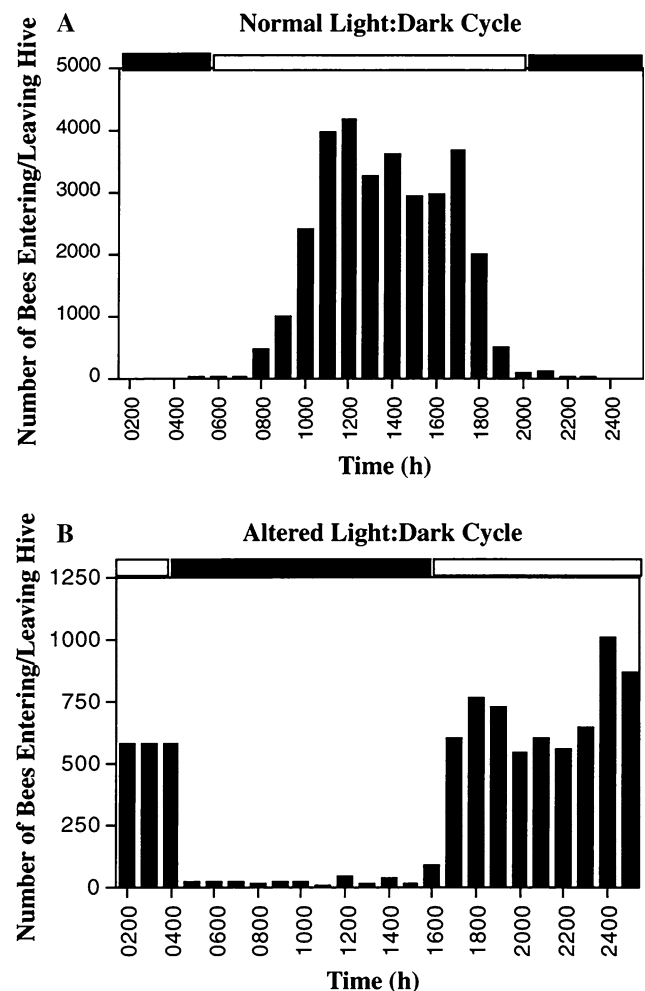


Fig. 2 Activity patterns measured in the pilot study over a 24 h period in bees maintained under **a** a normal LD cycle (LD14:10), and **b** an altered LD cycle (LD12:12). Photophase (white bars) and scotophase (black bars) are indicated above each graph

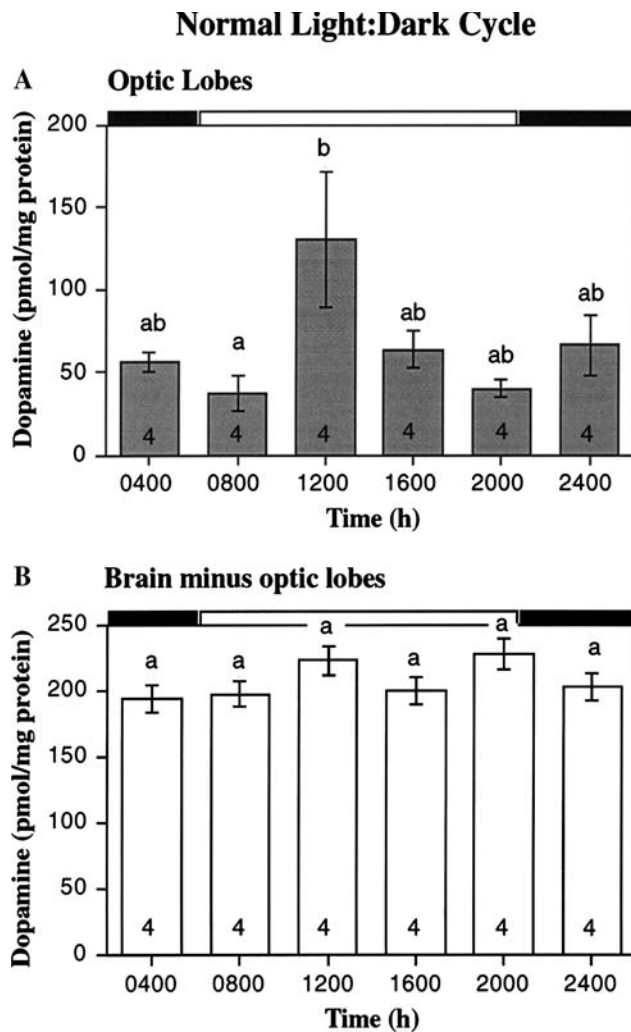


Fig. 3 Results of the pilot study examining DA levels observed under a normal LD cycle in **a** optic lobes and **b** brain minus optic lobes of forager bees over a 24 h period. Samples were taken at four hourly intervals over a natural LD cycle (LD 14:10). Each value represents the mean level (\pm SEM) of DA in pmol/mg protein. At each time interval, four samples of five pooled pairs of optic lobes, or five pooled brains minus optic lobes were analysed. Overall statistical significant was determined by ANOVA followed by Tukey's tests for post hoc comparisons. Letters that appear above each graph indicate whether or not differences between groups are significant. Groups that do not differ significantly share at least one letter. Groups with significantly different values have different letters. Photophase (white bars) and scotophase (black bars) are indicated above each graph

containing the brain minus the optic lobe (Fig. 3b). The highest mean DA level was detected at 2000 hours (227.6 pmol/mg) but this level was not significantly higher than samples dissected at other sampling times.

Altered LD cycle

In bees maintained under an altered LD cycle, the highest mean level of DA in the optic lobes was

recorded during the subjective darkness period, just prior to "lights on" (Fig. 4a). This level was significantly higher (ANOVA, $F = 4.61$ $P < 0.01$) than the mean DA level detected at 0400 hours (Fig. 4a). Although the peak DA level recorded in the hive maintained under an altered LD cycle was lower than the peak value recorded from bees held under a normal LD cycle, these values are not significantly different. In the brain minus optic lobe samples from the inside hive there were no significant diel fluctuations in

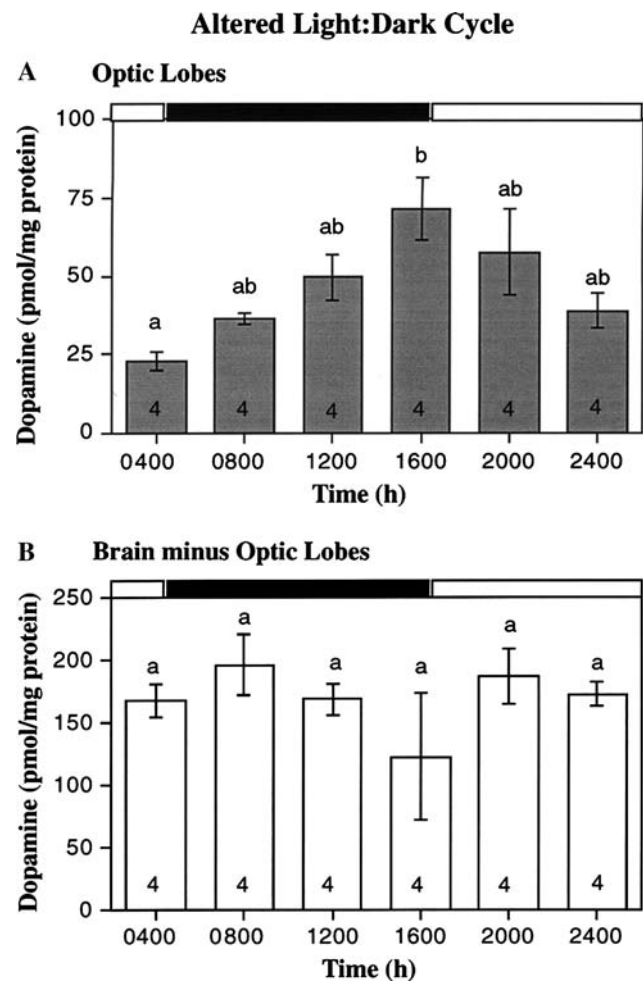


Fig. 4 Levels of DA measured during the pilot study in **a** the optic lobes and **b** the brain minus optic lobes of forager honey bees at four hourly intervals over an altered LD cycle (LD 12:12). Each value represents the mean level (\pm SEM) of DA in pmol/mg of protein. At each time interval, four samples of five pooled pairs of optic lobes, or five pooled brains minus optic lobes were analysed. Overall statistical significant was determined by ANOVA followed by Tukey's tests for post hoc comparisons. Letters that appear above each graph indicate whether or not differences between groups are significant. Groups that do not differ significantly share at least one letter. Groups with significantly different values have different letters. Photophase (white bars) and scotophase (black bars) are indicated above each graph

DA levels (Fig. 4b) although these levels were more variable than those found in the outside hive. The highest mean DA level was recorded at 0800 hours (196.3 pmol/mg) while the lowest mean level was detected in the 1600 hours sample (123 pmol/mg).

Protein levels in the brain

No significant variations in protein content were detected in the optic lobes of bees held under a normal LD cycle (Fig. 5a: top graph), or in bees maintained under altered LD conditions (Fig. 5b: top graph). In bees from the outside hive, the mean amount of protein in the brain minus the optic lobes measured at 0800 hours was higher (ANOVA, $F = 3.78$, $P < 0.05$) than at midday and 1600 hours (Fig. 5a: bottom graph), but these differences were not apparent in bees maintained indoors under an altered LD cycle (Fig. 5b: bottom graph). As no significant variation in protein content was detected in optic lobes of the brain over a 24 h period, dopamine levels in

optic lobes are expressed simply in pmol per pair of optic lobes in experiment 2.

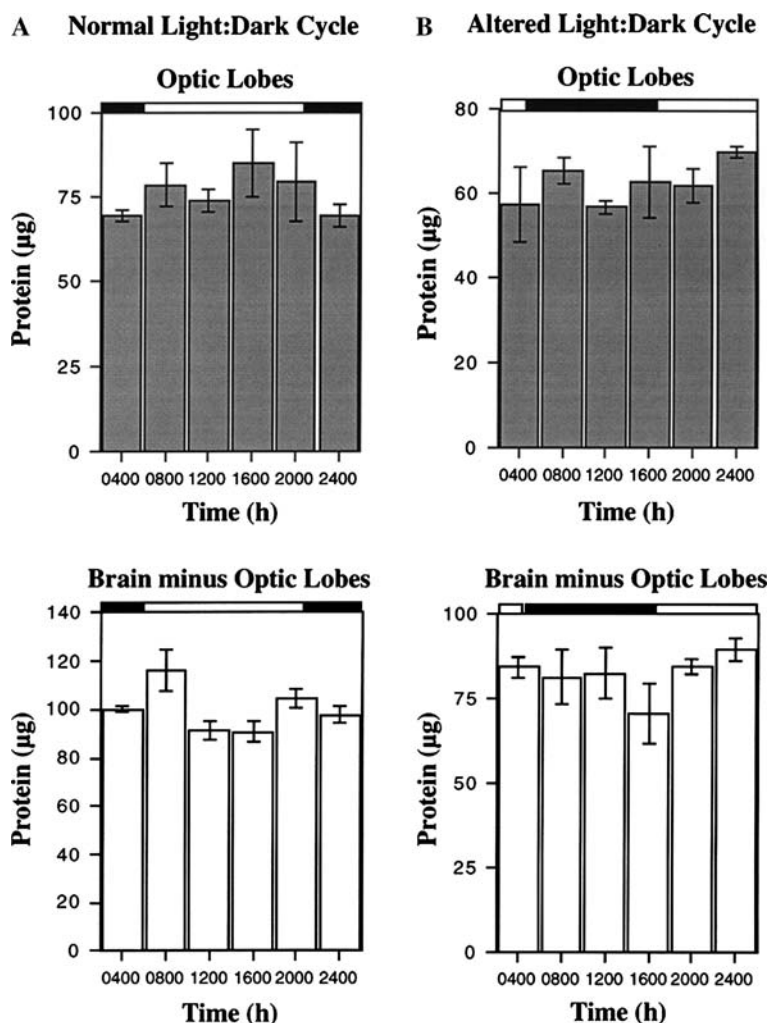
Experiment 2: Reversing the LD cycle and exposure to constant darkness

Manipulating the LD cycle: effects on activity patterns

In experiment 2, activity levels were recorded continuously for 28 days and the activity recordings plotted in an actogram (Fig. 6). Activity patterns recorded from days 1 to 6 are governed by a ‘normal’ LD cycle (LD 12:12). During this period, low levels of activity first become apparent around 1000 hours, activity peaked between 1400 and 1600 hours and then ceased abruptly around 1800 hours (Figs. 6, 7a).

From days 7 to 19, bees were exposed to a reversed LD cycle (DL12:12). Initially, the activity patterns lost their circadian rhythmicity (Fig. 6), but the introduction of additional lighting on day 13 saw the return of

Fig. 5 Levels of protein in the supraoesophageal ganglia of forager bees at four hourly intervals over 24 h LD cycle. Data are expressed as microgram protein per brain minus optic lobe or microgram protein per pair of optic lobes. *Left column a* Mean (\pm SEM) protein levels of bees in the hive maintained in a normal LD cycle (LD 14:10). *Right column b* Mean (\pm SEM) protein levels of bees in the hive maintained in an altered LD cycle (LD 12:12). Overall statistical significance was determined by ANOVA followed by Tukey’s tests for post hoc comparisons. There were no significant differences between mean protein levels in samples from bees from the hives maintained in the altered LD cycle or in protein levels in the optic lobes of bees from the hive maintained in the normal LD cycle. Mean protein levels from the brain minus the optic lobes of bees in the normal LD cycle were significantly higher at 0800 hours than at 1200 and 1600 hours



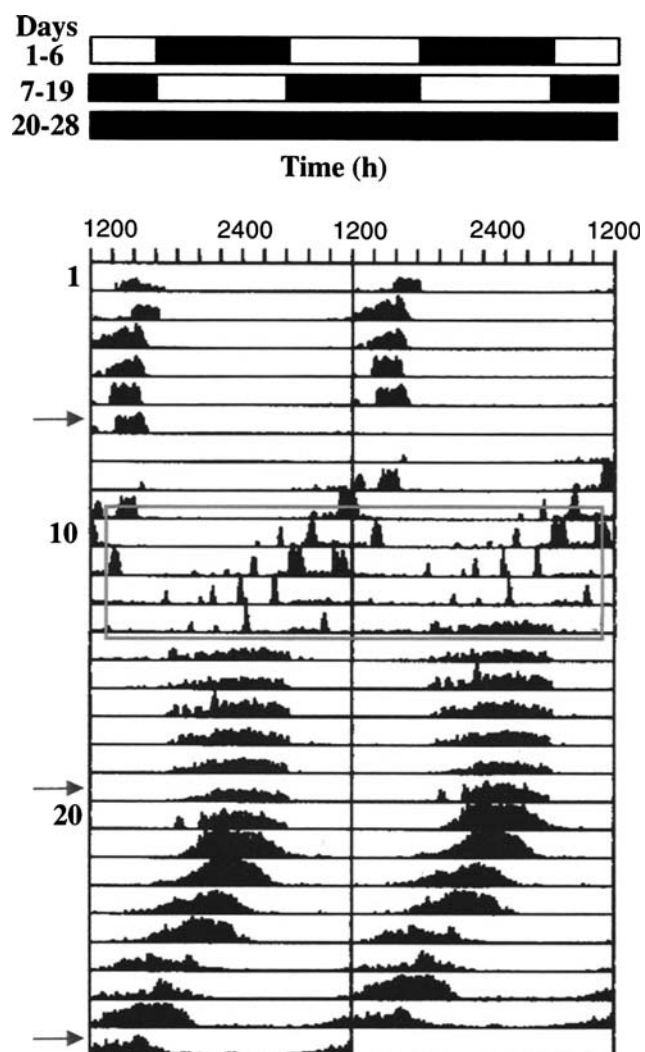


Fig. 6 Actogram depicting locomotor activity recorded over 28 days under three different light/dark regimes. Photophase (white bars) and scotophase (black bars) are indicated above the actogram. From days 1 to 6 bees were maintained under a normal LD cycle (LD12:12). From day 7 to day 19 bees the LD cycle was reversed (DL12:12). From day 20 onward bees were exposed to constant darkness. The grey box around days 10–13 indicates random activity following the implementation of the alternative LD cycle prior to the addition of extra illumination. On day 13 extra illumination was added to the flight cage and entrainment of activity occurred. Arrows on the left hand side indicate days on which optic lobe samples were collected for analysis of DA levels

rhythmic patterns of activity that coincided with the new subjective day. In contrast to the patterns of activity seen during the first 6 days of the experiment, relatively high levels of activity were detected throughout the period during which the lights were on.

For the final 8 days of the experiment (days 20–28), bees were exposed to constant darkness (DD12:12). Under these conditions, periods of peak activity shifted progressively towards earlier periods of the subjective day (Fig. 6).

Manipulating LD cycles: effects on DA levels in optic lobes of the brain

To examine the impact of manipulating the LD cycle on DA levels in the optic lobes, samples were collected from bees on day 6 at the end of the ‘normal’ LD cycle, on day 19 following the reversal of the LD cycle, and on day 28 after exposure to 8 days of constant darkness.

Bees exposed to a ‘normal’ LD cycle (LD12:12) sampled at day 6 were exhibiting circadian patterns of activity (Fig. 7a) and periods of highest activity were occurring on average every 23 h and 50 min (Fig. 7b). Under these conditions, high levels of DA were detected in the optic lobes at 1200 hours (midday), 2000 hours and at midnight (2400 hours) and low levels were detected at both 1600 and 0400 hours (Fig. 7c). Differences between levels of DA at these different time points are significant (ANOVA, $H = 67.450$, $P < 0.001$). Post hoc pairwise comparisons reveal that DA levels are significantly lower at 0400 hours than at all other time points. Levels of DA recorded at 1600 hours are also significantly lower than those detected at 1200, 2000, and 2400 hours.

Bees exposed to a reversed LD cycle (DL12:12) sampled at day 19 were exhibiting patterns of activity that were the reverse of those seen in the first 6 days of the experiment (Fig. 8a). A periodogram reveals that peak activity was recurring every 24 h (Fig. 8b). DA levels in the optic lobes under these conditions peaked during the dark phase (at 1600 hours), but were not significantly higher at this time than the levels of DA detected at any of the other time points examined that day (Fig. 8c). In marked contrast to the cyclical variations observed under normal environmental conditions (Fig. 3), peak levels of DA observed during photophase in the flight chamber (experiment 2) were generally no higher than peak levels of DA observed during scotophase.

Under conditions of constant darkness (DD12:12), bees began to ‘free-run’ (Fig. 9a) with time of peak activity occurring earlier each day. Periodogram analysis shows that on average, peak activity was occurring every 22 h and 40 min (Fig. 9b). Optic lobes were collected from bees on day 28. The activity pattern exhibited by bees at this time was similar to that seen on day 6, the day that samples were collected from the ‘normal’ LD phase of the experiment (see Fig. 6). Overall, the levels of DA in the optic lobes of bees held under conditions of constant darkness appeared relatively low (Fig. 9c). Nonetheless, significant differences were detected between levels of DA recorded at different time points (ANOVA, $F = 15.376$,

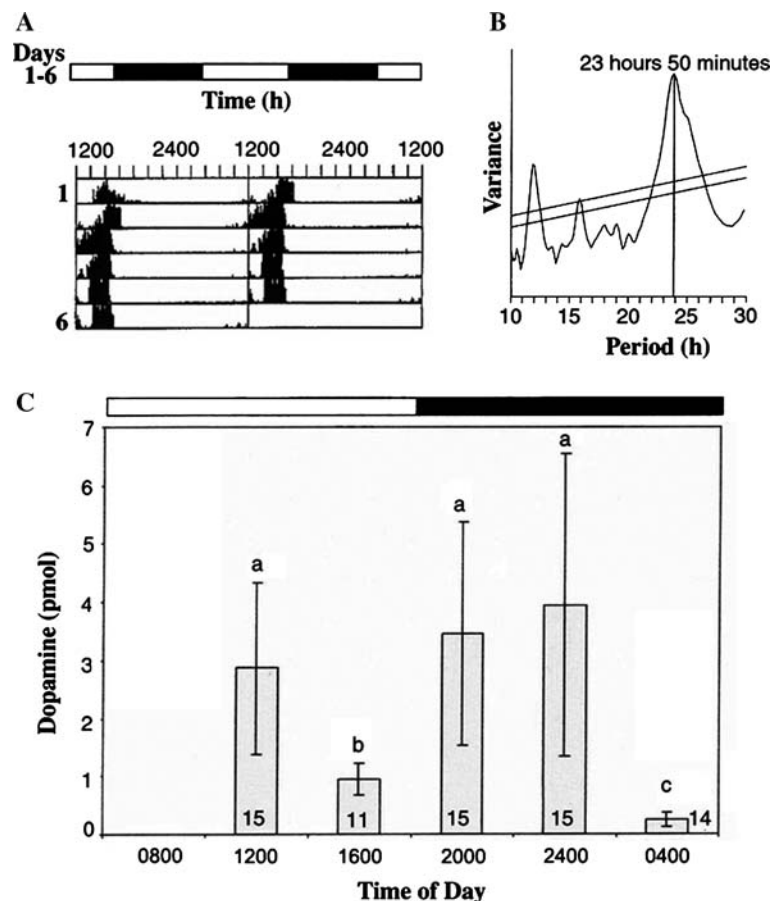


Fig. 7 Activity patterns and DA levels observed in bees maintained under a ‘normal’ LD cycle (LD12:12). **a** Actogram depicting locomotor activity recorded over days 1–6. **b** A significant ($P = 0.01$) circadian rhythm is indicated by a peak above the *diagonal lines* on the periodogram. Peak activity during days 1–6 is occurring every 23 h and 50 min. **c** Levels of DA in the optic lobes of forager bees examined at four hourly intervals. Each value represents the mean level (\pm SD) of DA in pmol per pair of optic lobes. The n values for each group are indicated in each bar.

Overall statistical significance was determined by ANOVA followed by Dunn’s test for post hoc comparisons. Letters that appear above each bar indicate whether or not differences between groups are significant. Groups that do not differ significantly share the same letter. Groups with significantly different values have different letters. Bars above the group indicate photophase (white bar) and scotophase (black bar). Data for 0800 hours were unusable because of HPLC contamination

$P < 0.001$). Post hoc comparisons show that the levels of DA detected at 0800 and 1600 hours are significantly lower than the levels recorded at 1200, 2000, 2400 and 0400 hours.

Experiment 3: DA in the optic lobes of young worker bees

Optic lobes of young worker bees from an outdoor colony were examined at two time points, midday (1200 hours) and at 1600 hours. Levels of DA in the optic lobes of 11 day-old adults from the colony were significantly lower than those detected in the lobes of 5-day old bees. In neither case, however, was there a difference between DA levels detected at 1200 and 1600 hours (Fig. 10).

Discussion

Our study supports the following conclusions: first, as shown elsewhere (Spangler 1972, 1973; Moore and Rankin 1985, 1993; Moore 2001), foraging activity displays all the properties of a circadian rhythm: it is entrained to daily light cycles to periods close to 24 h (Figs. 6, 7a,b), it changes predictably in response to phase shifts in light (Figs. 2, 6, 8a,b), and it is able to free-run in constant conditions (Figs. 6, 9a,b). Second, cyclical variations in DA levels in the optic lobes are affected by alterations to the LD cycle, but our results suggest that they are modulated also by factors other than light. Third, effects of altering the LD cycle on endogenous DA levels do not mirror those observed at a behavioural level (Figs. 7, 8, 9). No direct correlation

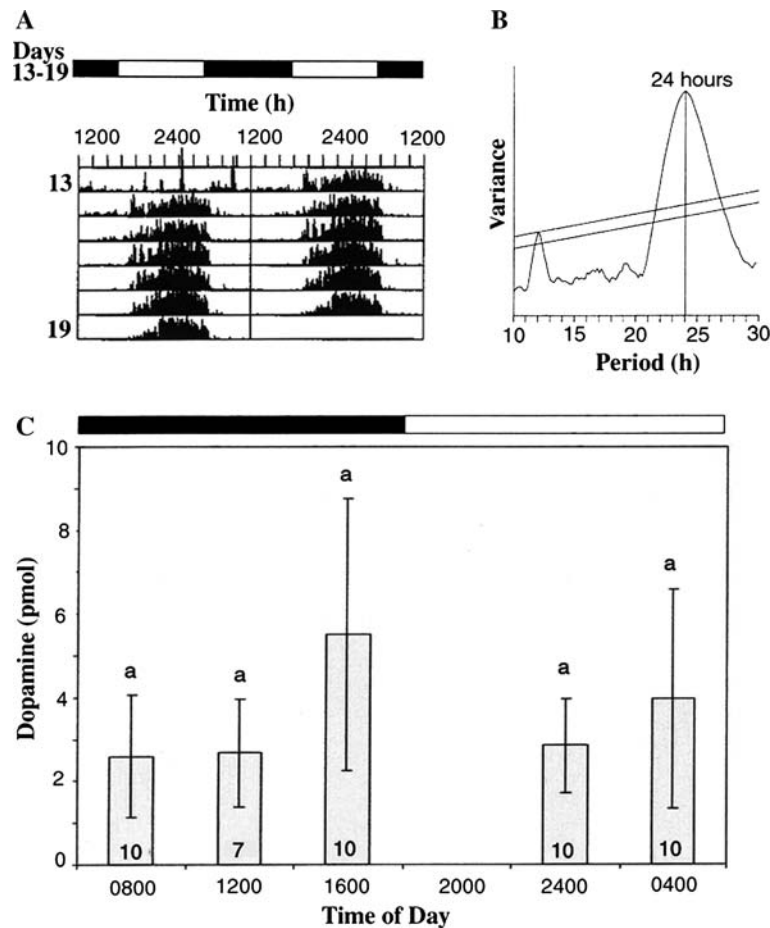


Fig. 8 Activity patterns and DA levels observed in bees maintained under an altered (reversed) LD cycle (DL12:12). **a** Actogram depicting locomotor activity recorded from days 13–19. **b** Periodogram shows peak activity is occurring every 23 h and 50 min during this period. **c** Levels of DA in the optic lobes of forager bees examined at four hourly intervals. Each value represents the mean level (\pm SD) of DA in pmol per pair of optic lobes. The *n* values for each group are indicated in each bar. Overall

statistical significance was determined by ANOVA followed by Dunn's test for post hoc comparisons. Letters that appear above each bar indicate whether or not differences between groups are significant. Groups that do not differ significantly share the same letter. Groups with significantly different values have different letters. Bars above the group indicate photophase (white bar) and scotophase (black bar). No measurements were obtained at 2000 hours

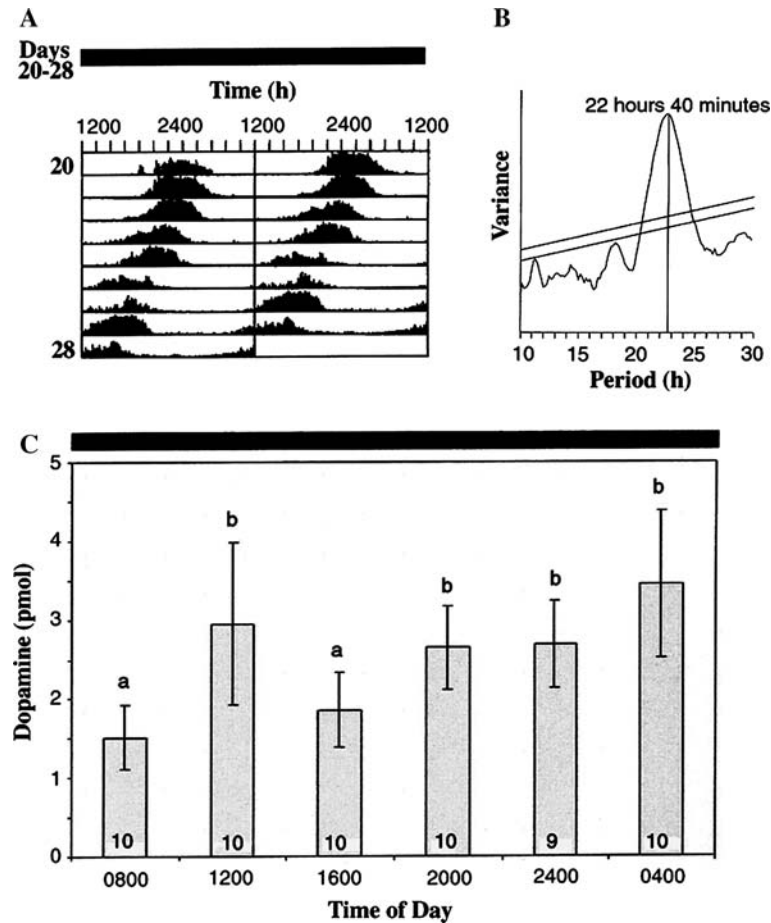
could be identified between DA levels in optic lobes of the brain and the circadian behavioural rhythmicity of bees.

During summer months, levels of DA in the optic lobes of forager bees are highest around midday (Purnell et al. 2000; Fig. 3) when bees are actively foraging (Fig. 2). Our results reveal, however, that if bees are exposed to an altered LD regimen the apparent correlation between endogenous DA levels and levels of foraging activity breaks down. Reversing the LD cycle reverses the rhythmical patterns of behavioural activity exhibited by foragers almost immediately, but the effects on endogenous DA levels are more complex. Rather than occurring midway through photophase, under a reversed LD cycle peak DA levels were observed earlier, immediately preceding photophase

(Figs. 4, 8). In real time, this peak was found 4 h after the midday peak in DA levels recorded in bees maintained under a normal LD regime (Fig. 3). Consequently, under altered LD conditions the peak in mean DA levels no longer coincided with times of peak activity. Interestingly, in the optic lobes of the cabbage moth *Trichoplusia ni*, DA levels are low during times of peak activity (Linn et al. 1994). While DA levels in the moth fall during scotophase, the moth unlike the honey bee, is nocturnally active.

It is possible that conditions required for robust manipulation of endogenous DA levels were not optimal in this study. The rate at which behavioural entrainment occurs, for example, depends on light intensity: the weaker the intensity of light, the longer is the time required for behavioural entrainment (Tomioka

Fig. 9 Activity patterns and DA levels observed in bees maintained under constant darkness (DD12:12). **a** Actogram depicting locomotor activity recorded from days 20 to 28. Peak activity shifts progressively each day. **b** Periodogram shows the time interval between peaks occurs every 22 h 40 min. **c** Levels of DA in the optic lobes of forager bees examined at four hourly intervals. Each value represents the mean level (\pm SD) of DA in pmol per pair of optic lobes. The *n* values for each group are indicated in each bar. Overall statistical significance was determined by ANOVA followed by Tukey’s test for post hoc comparisons. Letters that appear above each bar indicate whether or not differences between groups are significant. Groups that do not differ significantly share the same letter. Groups with significantly different values have different letters



and Chiba 1989). Under different conditions, or with additional time, peak DA levels in the optic lobes of bees exposed to an altered LD cycle may have shifted to a more ‘normal’ position, midway through the photophase. At a behavioural level, however, bees responded to changes in light cues immediately. The lack of correlation between cyclical variations in DA levels and shifts in locomotor activity suggests the two are independently regulated.

DA synthesis in the vertebrate retina is affected by light intensity (Proll and Morgan 1982; Proll et al. 1982) and our results suggest that this may be true also in the optic lobes of forager bees. DA levels in the optic lobes were lowest overall in foragers exposed to constant darkness (Fig. 9), and in marked contrast to the clear peak in DA levels occurring around midday under normal LD conditions (Fig. 3), DA levels in the optic lobes of bees held under constant darkness failed to peak around any particular time point (Fig. 9). Nonetheless, even under conditions of constant darkness cyclical fluctuations persist, suggesting that DA levels in the optic lobes of the bee are not modulated solely by exogenous light cues. Measurements of DA in the optic lobes of young ‘in house’ bees (Fig. 10,

Kokay and Mercer 1997) support this view. Age-related changes in DA levels have been identified in the optic lobes of very young ‘in house’ bees (e.g. 1- to 2-days post adult emergence; Kokay and Mercer 1997) and such variations in optic lobe DA levels are difficult to explain in terms of differential exposure to light cues. Whether diel fluctuations in DA levels occur in the optic lobes of young ‘in house’ bees remains unclear but if they do, our results indicate that the pattern of cyclical changes must be different from that seen in foragers (Fig. 3) as in young bees, unlike foragers, DA levels in optic-lobe samples collected at 1200 hours are very similar to those recorded in samples collected at 1600 hours (Fig. 10).

In the vertebrate eye, DA has been implicated in a vast array of modulatory events that ensure the information gathering properties of the eye remain optimal. The strength of lateral inhibition and centre-surround antagonism in the vertebrate retina, for example, is regulated by DA as a function of adaptive state (reviewed by Dowling 1991). While it is clear from this study that levels of DA in the optic lobes of forager bees are subject to daily modulation by external factors, the functional significance of cyclical variations in DA levels remains

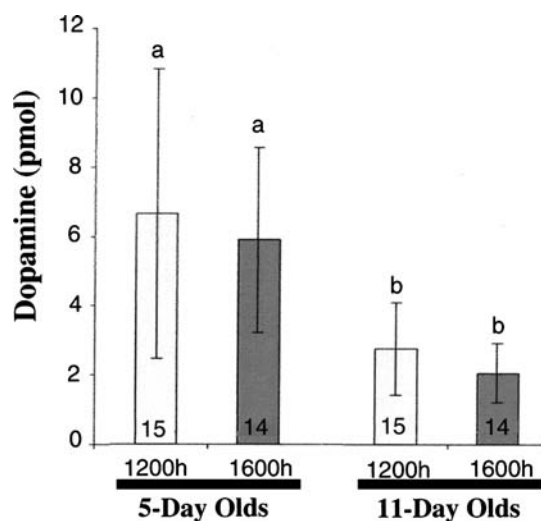


Fig. 10 Levels of DA in the optic lobes of non-foraging honey bees. Newly emerged adults were marked with paint and returned to the hive, then collected 5 or 11 days later. Bees of both age groups were sampled at 1200 hours (light grey bars) and 1600 hours (dark grey bars). Each value represents the mean level (\pm SD) of DA in pmol per pair of optic lobes. The *n* values for each group are presented within each bar. Overall statistical significance was determined by one-way ANOVA followed by Tukey's post hoc comparisons. Groups that do not differ significantly share the same letter. Groups with significantly different values have different letters

unclear. Circadian oscillations in neural activity, and changes in the sensitivity of optic lobe interneurons to visual stimuli have been reported in several insect species (Colwell and Page 1990; Tomioka et al. 1993), including the honey bee (Kaiser and Steiner-Kaiser 1983) and in the optic lamina neuropil of the housefly, circadian changes in neuronal structure have also been identified (reviewed by Meinertzhagen and Pyza 1996, 1999). The contribution that DA makes to such fundamentally important processes has yet to be revealed. There is tantalizing evidence, however, that levels of DA receptor expression in the brain of the honey bee change also over a 24 h period (Purnell et al. 2000), and in the central nervous system of the fruitfly, DA receptor responsiveness is under circadian control (Andretic and Hirsh 2000). The identification of DA receptors and their distribution in the brain of the honey bee (Blenau et al. 1998; Kokay and Mercer 1996; Kokay et al. 1998; Kurshan et al. 2003) and the cloning and characterization of honey bee DA-receptor genes (Blenau et al. 1998; Humphries et al. 2003; Beggs et al. 2005) has provided tools that will greatly enhance our ability to establish the roles played DA in optic lobes of the brain.

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