

General anesthesia alters time perception by phase shifting the circadian clock

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Following general anesthesia, people are often confused about the time of day and experience sleep disruption and fatigue. It has been hypothesized that these symptoms may be caused by general anesthesia affecting the circadian clock. The circadian clock is fundamental to our well-being because it regulates almost all aspects of our daily biochemistry, physiology, and behavior. Here, we investigated the effects of the most common general anesthetic, isoflurane, on time perception and the circadian clock using the honeybee (*Apis mellifera*) as a model. A 6-h daytime anesthetic systematically altered the time-compensated sun compass orientation of the bees, with a mean anticlockwise shift in vanishing bearing of 87° in the Southern Hemisphere and a clockwise shift in flight direction of 58° in the Northern Hemisphere. Using the same 6-h anesthetic treatment, time-trained bees showed a delay in the start of foraging of 3.3 h, and whole-hive locomotor-activity rhythms were delayed by an average of 4.3 h. We show that these effects are all attributable to a phase delay in the core molecular clockwork. mRNA oscillations of the central clock genes *cryptochrome-m* and *period* were delayed by 4.9 and 4.3 h, respectively. However, this effect is dependent on the time of day of administration, as is common for clock effects, and nighttime anesthesia did not shift the clock. Taken together, our results suggest that general anesthesia during the day causes a persistent and marked shift of the clock effectively inducing “jet lag” and causing impaired time perception. Managing this effect in humans is likely to help expedite postoperative recovery.

chronobiology | anesthesiology | post-operative sleep disruption

Ever since “Ether Day” in 1846, when William Morton administered the first general anesthetic, anesthesia has been used to alleviate pain and enable a wide range of surgical procedures that were not previously possible. Today, an estimated 234 million operations requiring anesthesia occur around the world each year (1). Despite the ubiquity and importance of general anesthesia, the mechanisms by which anesthetics work to “put you to sleep” remain unclear. Recent evidence suggests that most general anesthetics act, at least in part, on the same brain centers as those involved in the control of sleep (2) and that they may “hijack” endogenous GABA-ergic (γ -aminobutyric acid) sleep-controlling pathways to exert their effects on consciousness (3). The effect of anesthesia on brain activity parallels some of the features of sleep (3). However, there are obvious differences. For example, a common patient response on emerging from anesthesia is disorientation and the feeling that time has not passed. This is in stark contrast to sleep, where one often wakes up just before the alarm sounds aware that time has passed during the night.

Daily sleep timing relies on the endogenous circadian clock, which drives daily rhythms in biochemistry, physiology, and behavior. In animals, this clock is controlled by a set of conserved and well-characterized clock genes, the mRNA and protein levels of which oscillate to generate a rhythm of ~24 h. Environmental

time cues such as daylight, temperature, and social interactions entrain the clock to exactly 24 h on a daily basis (4). Even transient desynchronization between the circadian clock and the environment, as experienced for example during travel across time zones or shift work, can have major impacts on our well-being (5), with long-term health consequences (6–8).

The honeybee (*Apis mellifera*) provides an unparalleled model with which to test the effects of anesthesia on time perception and the circadian clock. The potency of most anesthetic drugs varies very little between different species, and even different phyla. This observation is reliable enough that insects have been successfully used as models of anesthetic drug potency in mammals (9). Furthermore, bees show a “time sense” based on their ability to continuously consult their endogenous circadian clock, as we might consult a wrist watch to determine the time of day (10, 11). Using this time sense, bees exhibit an extraordinary array of complex and accurately timed behaviors in their normal daily life, navigating using the sun compass and visiting flowers at times of maximum nectar production (10, 12). Furthermore, the bee molecular circadian clockwork is remarkably similar to that of mammals both in genetic structure and function (13–15).

Here, we have taken advantage of the time sense of bees and their well-characterized molecular clockwork to investigate the effects of general anesthesia on time perception and the circadian clock. Specifically, we use three complimentary approaches, analyzing the effects of the common inhalational anesthetic isoflurane on orientation behavior in the field and timed foraging behavior in addition to the effects on circadian activity rhythms and the expression patterns of three clock genes.

Results

Isoflurane Systematically Alters Sun Compass Navigation. Bees orient using the sun compass, determining their flight direction relative to the position of the sun. As the Earth rotates, the position of the sun appears to move on average 15° per hour. Bees account for this using their circadian clock-based time sense (12). On foraging excursions, this information is used to calculate the directional component of the flight between the nectar source and the hive. This typically fast and straight flight is defined as the vector-flight (16). Bees that are trained to visit a feeder at a particular distance and orientation from the hive, when released at

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a new location, show the same vector-flight that would normally return them to the hive. Bees translocated to a novel release site rely on the sun compass to orient (10). Thus, if an extended period of anesthesia suspends the time sense of a bee, the direction of the vector-flights should show a systematic change (17).

To investigate this hypothesis, we first recorded vanishing bearings (the compass bearing at which a bee disappears from sight) to measure the direction of vector-flights of bees. Bees trained to a feeder 100 m from the hive at a location in the Southern Hemisphere (Auckland, New Zealand) were caught at the feeder and anesthetized for either 30 min (control; see *Materials and Methods*) or 6 h (treatment) with 2% isoflurane in air. During anesthesia, these bees were translocated 6 km away to a novel location. The vanishing bearings of both control and treatment bees were nonrandomly distributed (Raleigh uniformity test; control: $P < 0.0001$; treatment: $P < 0.0001$). On release, control bees ($n = 80$) showed an average orientation angle of 346° [95% confidence interval (CI): 335–358°; Raleigh mean vector length: 0.650], which would have returned them to the hive. By contrast, bees anesthetized for 6 h ($n = 108$) showed an average orientation angle of 260° (95% CI: 234–285°; Raleigh mean vector length: 0.291) (Fig. 1). This represents a statistically significant mean anticlockwise shift in the vanishing bearing in the treatment group of 87° (95% CI: 59–116°; nonparametric bootstrap: $P < 0.001$). This shift in angle is of a magnitude and direction consistent with time sense of bees being suspended during anesthesia (Fig. 1).

To track entire flight paths, we then used a unique harmonic radar system (18) in the Northern Hemisphere (Klein-Lüben, Brandenburg, Germany). This technique allowed for the high-resolution analysis of the bee flights to determine the effect of anesthesia on the immediate homeward vector-flight. In contrast to the Southern Hemisphere, where the sun travels from east to west through the northern half of the sky, after the same 6-h anesthesia in the Northern Hemisphere, we would predict bees to make the opposite (clockwise) shift in orientation behavior. Individuals trained to a feeder 300 m from the hive and then displaced 500 m in a different compass direction to the training direction (Fig. 24) were separated into three groups (control: 30-min anesthesia; treatment: 6-h anesthesia; catch and release: no anesthesia), fitted with a radar transponder and released (Fig. 24).

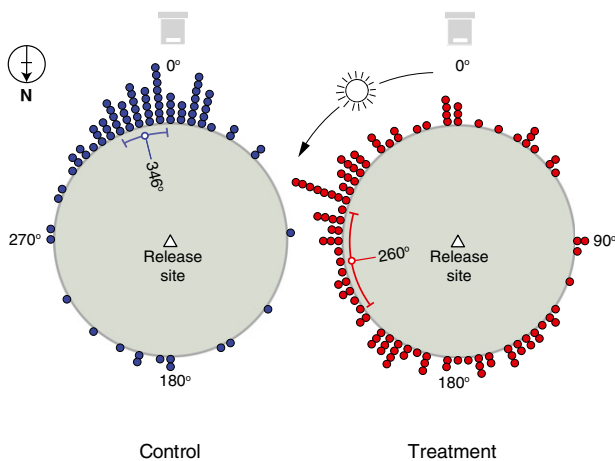


Fig. 1. Vanishing bearings of individual bees in the Southern Hemisphere. Data are shown with respect to the expected homing direction to the hive (defined as 0°, gray hive symbol). Bees caught departing from a feeder to which they had been trained were translocated to a release site outside of their home range. Vanishing bearings were recorded after either 30-min anesthesia (blue circles, control; $n = 80$) or 6-h anesthesia (red circles, treatment; $n = 108$). The mean bearing of each group is represented by the white circle with 95% CI scribed by the arc.

The vector-flight angles of all three groups were nonrandomly distributed (Raleigh uniformity test; treatment: $P < 0.0001$; catch and release: $P < 0.0001$; control: $P < 0.0001$). The mean angle of vector-flights of treatment bees ($n = 24$) was 80° (95% CI: 63–97°; Raleigh mean vector length: 0.750) compared with a mean angle in catch and release bees ($n = 13$) of 18° (95% CI: 15–21°; Raleigh mean vector length: 0.996) and control bees ($n = 25$) of 22° (95% CI: 18–27°; Raleigh mean vector length: 0.978) (Fig. 2B and *SI Appendix*, Figs. S1 and S2 and Table S2). This represents a mean clockwise shift in the vector-flight angle of treatment bees of 62° (95% CI: 44–77°) with respect to catch and release and 58° (95% CI: 38–74°) with respect to the control group. The mean vector-flight angle of the treatment bees was significantly different from both the catch and release and control bees (nonparametric bootstrap: $P < 0.001$).

The greater variance in the treatment groups in both sun-compass navigation experiments compared with controls may result from: (i) some bees using secondary external cues [that were not excluded despite the featureless environment (19)] to re-compensate their direction during the vector-flight; (ii) differences in drug efficacy possibly reflecting genetic differences between individuals; or (iii) general effects on the performance of the bees attributable to long-term (6-h) anesthetic exposure.

Isoflurane Delays Foraging Time. Bees can be trained to forage to a food source at the same time on consecutive days, and this foraging continues for several days after the food source is removed (food anticipatory behavior) (20). To investigate whether the anesthetic effect on time perception, as seen in the sun-compass experiments, persists beyond the day of treatment, we examined the effect on food anticipatory behavior. Forager bees fitted with radio frequency identification (RFID) tags were trained to a feeder between 0900 and 1000 hours each day for 4 d in natural light/dark cycles. The timing of individual bee visits over 3 d to an unrewarded feeder following whole-hive anesthesia [control: 30-min anesthesia ($n = 9$); treatment: 6-h anesthesia ($n = 11$)] were compared with preanesthesia visits using RFID.

On the day following a 6-h anesthetic, the mean delay in foraging onset time was 3.3 h ($P < 0.001$) compared with 1.0 h in the controls ($P = 0.13$) (Fig. 3). This difference between control and treatment visits is significant ($P = 0.019$). After 3 d in light/dark cycles, bees re-compensated and visited the unrewarded feeder at the original training time, with a mean time of onsets of 0930 hours in the treatment group ($n = 8$) compared with 0830 hours in the controls ($n = 8$) (Fig. 3). Significantly, this result demonstrates that the effect of general anesthesia on time perception persists for several days despite the presence of strong time cues (i.e., light/dark cycles), and it takes at least 3 d to retrain.

Isoflurane Phase Shifts Daily Activity Rhythms and the Circadian Clock. To test whether the anesthetic effect on time perception results from an effect on the circadian clock underpinning the time-compensated behaviors, we conducted a series of experiments to determine the effect of isoflurane on an output rhythm from the clock (locomotor activity) and the clock itself (clock gene expression).

During the day, honeybee foragers actively fly between the hive and food sources. The strong diurnal activity of the hive can be measured by recording locomotor activity at the hive entrance. Because this activity rhythm is endogenously controlled by the circadian clock, it continues under constant conditions (21).

In the following experiments, beehives were maintained in the laboratory in constant conditions (dim light, ≤ 2 lux) to assess the effect of anesthesia on the clock in the absence of external time cues. The activity rhythm of the hive was measured at the hive entrance using an infrared motion detector. A 6-h anesthetic during the subjective day caused a mean phase delay in activity rhythms of 4.3 h (95% CI: –2.2 to –6.4; $n = 6$ hives), whereas a 30-min control

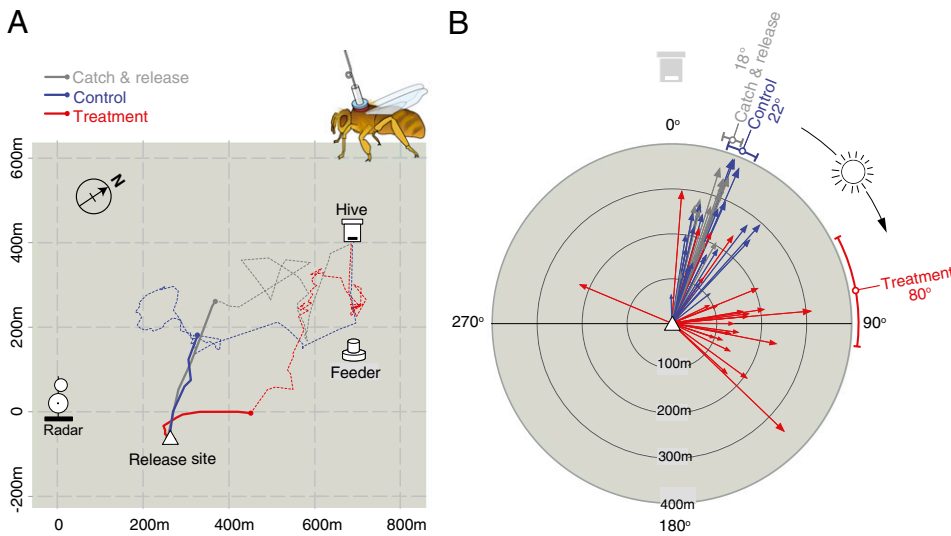


Fig. 2. Flight paths of individual bees tracked by harmonic radar in the Northern Hemisphere. Data are shown with respect to the expected homing direction to the hive (defined as 0°, gray hive symbol). (A) Typical flight paths of three bees from release site to hive. Each bee represents one of three experimental groups: catch and release; 30-min anesthesia (control); and 6-h anesthesia (treatment). The vector-flight is indicated by the solid line terminating at the filled circle. The remainder of the flight is shown as a broken line. (B) The distribution of vector-flights of all individual bees. The vector-flight of each bee is represented by a line indicating both the direction (angle) and duration (length) of the flight. Mean angle of orientation of each of the three groups (catch and release, $n = 13$; control, $n = 25$; treatment, $n = 24$) is indicated by the white circle with 95% CI scribed by the arc.

anesthetic resulted in no discernable shift (0.3 h; 95% CI: -0.3 to 0.9 ; $n = 3$ hives) (Fig. 4A and *SI Appendix, Table S3*).

We serially sampled forager bees from one of these hives (every 3 h, 36 h before and after anesthesia) and determined the effect of general anesthesia on the mRNA levels of three clock genes: mammalian-type *cryptochrome* (*Cry-m*), *period* (*Per*), and *clock* (*Clk*). *Cry-m* and *Per* mRNA levels are known to cycle in forager bee brains over the course of 24 h, whereas *Clk* mRNA levels are constant (13). Quantitative real-time PCR (RT-qPCR) analysis of individual bee brains ($n = 6$ per time point; 29 time points) revealed a large phase delay of mRNA oscillations of *Cry-m* and *Per*; *Cry-m* expression was delayed by 4.9 h (95% CI: -3.8 to -6.1 ; $P < 0.001$), and *Per* expression was delayed by 4.3 h (95% CI: -2.2 to -6.4 ; $P < 0.001$). There was no overall change in the constitutive expression of *Clk* following anesthesia (Fig. 4B). The corresponding phase delay in activity of this hive was 5.3 h (Fig. 4A). These gene expression data indicate that the activity phase shifts observed result from isoflurane affecting the molecular clockwork in the brain.

Both the magnitude and direction of clock phase shifts are well known to depend on the time of the administration. Therefore,

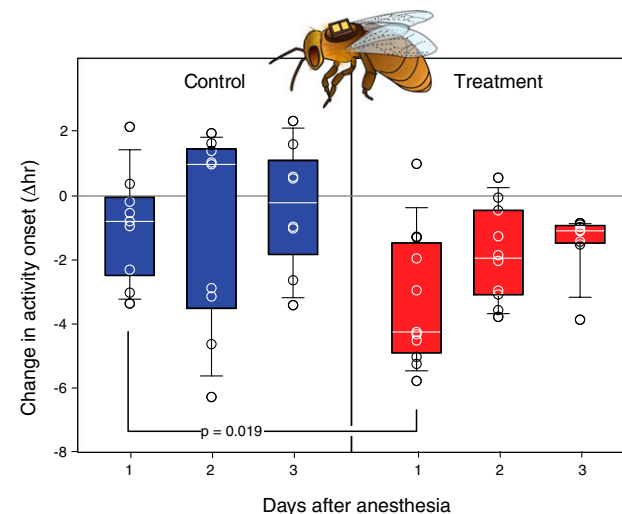


Fig. 3. Difference in onset of foraging time between the day before anesthesia and each of 3 d after anesthesia. Blue: 30-min anesthesia (control, $n = 9$); red: 6-h anesthesia (treatment, $n = 11$).

we performed the same experiments as above but changed the time of treatment from the subjective day to the subjective night. Importantly, the 6-h nighttime anesthetic did not induce a significant phase delay in hive-activity rhythms and, if anything, produced a small phase advance (1.4 h; 95% CI: 0.2 – 2.6 ; $n = 3$ hives). The controls also showed no phase shift (-0.1 h; range: -0.7 – 0.6 ; $n = 2$) (Fig. 4C and *SI Appendix, Table S3*). Furthermore, neither *Cry-m* (-0.4 h; 95% CI: -2.0 – 1.2 ; $P = 0.34$) nor *Per* (-1.2 h; 95% CI: -3.9 – 1.5 ; $P = 0.25$) mRNA oscillations were shifted by anesthesia at night (Fig. 4D). The marked difference between a daytime and nighttime anesthetic strongly supports the conclusion that isoflurane influences key molecular components of the circadian clock and acts by phase shifting the clock.

Discussion

The agreement between the effects of general anesthesia on orientation behavior, timed foraging, circadian activity rhythms, and clock gene expression is striking. The magnitude and direction of the shifts in time-compensated behavior in the field [vanishing bearings: 87° (corresponding to approximately -5.8 h); radar-determined vector-flights: 58° (corresponding to approximately -4 h); food anticipatory behavior: -3.3 h] correspond closely to the phase delays seen in activity rhythms (-4.3 h) and in clock gene expression (-4.3 and -4.9 h). Thus, we have demonstrated that general anesthesia phase shifts the circadian clock and, as a consequence, influences both daily behavior and time perception.

However, like other factors that shift the clock (*zeitgeber*), the effect of isoflurane is time-dependent. We have shown that a nighttime isoflurane anesthetic had little or no effect on hive activity rhythms and clock gene expression. The reason for this differential effect can be understood by realizing that the magnitude and direction of a clock shift depends on the state of the system at the time of treatment. For example, if isoflurane causes an increase in mRNA expression, because levels are already in the rising phase of their oscillation, then it will cause a phase advance. Conversely, if administered during the declining phase, then it will result in a delay. In contrast, there will be no effect if isoflurane is administered during the high point of the oscillation, because it is already at its highest possible level. The latter might be the case for our nighttime isoflurane anesthesia (Fig. 4B and D). Hence, the absence of isoflurane-induced phase shifts during the night supports our conclusion that general anesthesia influences the circadian clock. Interestingly, the phase-shifting effects of isoflurane contrast with those of light. Whereas isoflurane administered during the subjective day elicits a strong phase delay (Fig. 4), a light pulse at the same circadian time

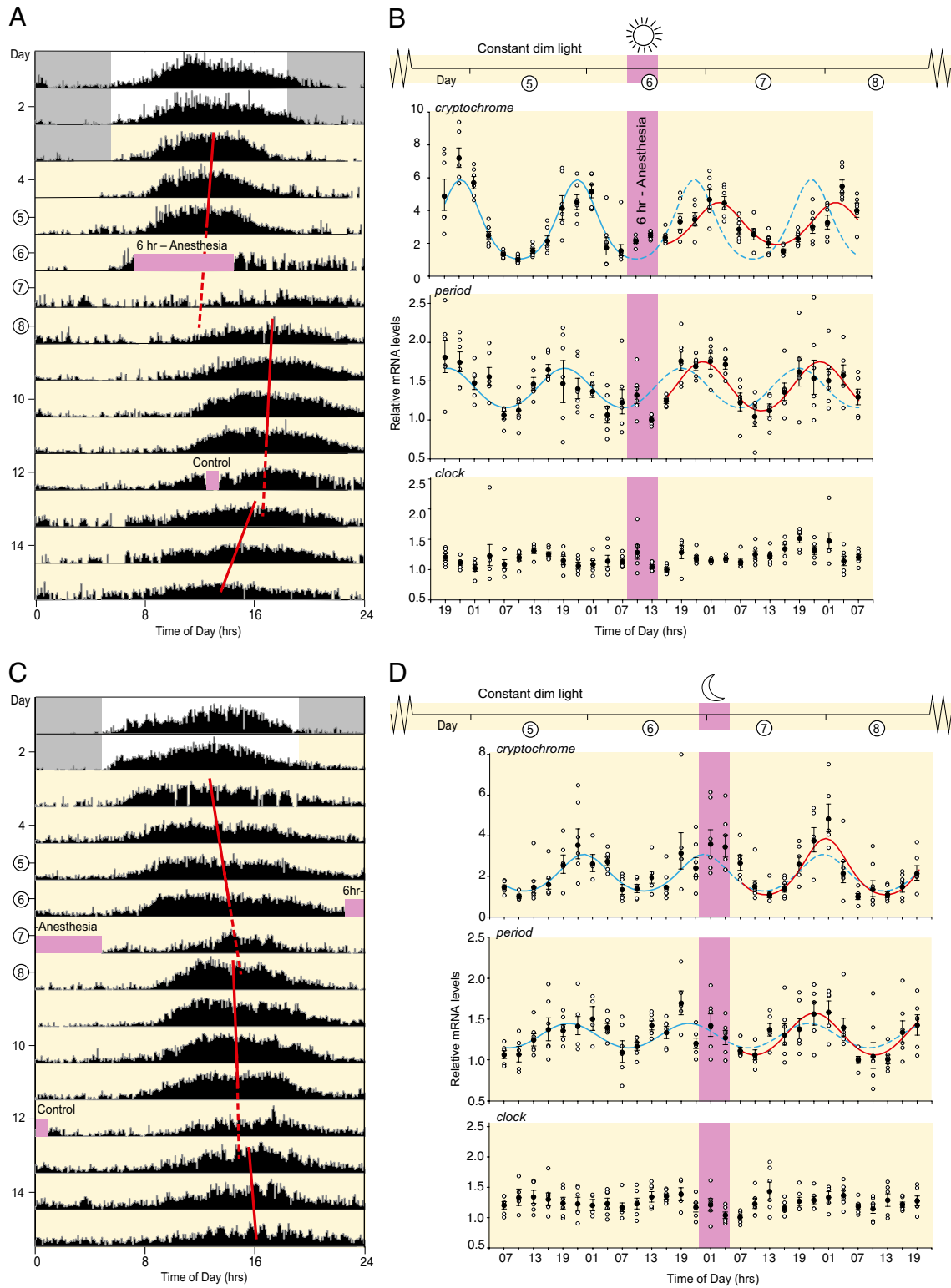


Fig. 4. Hive-activity rhythms and clock gene expression. (A) Hive locomotor activity before and after a 6-h daytime anesthetic and a 30-min control anesthetic. Black bars represent the activity of bees entering and leaving the hive every 5 min. Regression line through the peaks (acrophases) is shown in red. Background colors indicate level of illumination (gray: dark; white: bright light; yellow: dim light). Samples represented in B were collected during the period indicated by circled days. (B) Brain mRNA levels of clock genes *cryptochrome*, *period*, and *clock* from bees sampled before and after daytime anesthesia during the recording of the actogram in A. Open circles represent individual data points ($n = 6$ per time point); filled circles represent the geometric means \pm SEM. Solid lines display a cosinor model fitted to the log-transformed data (blue: preanesthesia; red: postanesthesia); broken lines are an extrapolation of the model. (C) Hive locomotor activity before and after night anesthesia. Samples represented in D were collected during the period indicated by circled days. (D) Brain mRNA levels of clock genes *cryptochrome*, *period*, and *clock* from bees sampled before and after nighttime anesthesia during recording of the actogram in C.

produces a clear phase advance (22). Isoflurane administration during the subjective night does not cause a shift of the clock, but light causes a phase delay (22).

An obvious question arising from our findings is why the magnitude of the shift does not correspond to the duration of the anesthesia. Specifically, why does a 6-h anesthetic elicit only a 4–5-h shift in time perception, activity rhythms, and clock gene expression? A plausible explanation is that the clock is not stopped but shifted or slowed down during the period of anesthesia. Like other factors that shift the clock (chronobiotics), which typically have a “ceiling effect,” isoflurane might also have a maximum phase-shifting limit. This observation further supports our contention that isoflurane acts on the circadian clock to exert the observed effects.

Previous work has shown that some hypoxic agents such as carbon dioxide and nitrogen can influence timed foraging in honeybees (23) or timed emergence in fruit flies (24). Furthermore, some anesthetics can affect behavioral and physiological rhythms in rodents. Whereas the benzodiazepine midazolam and the opiate fentanyl can cause phase advances and delays in locomotor activity depending on the time of administration (25), the anesthetic propofol induces only phase advances (26). Moreover, the inhalational anesthetic sevoflurane has been shown to cause phase delays in activity of ~1 h when administered during the subjective day (27). Until the work described here, however, it had not been shown that any of these effects result from a phase shift in the molecular clockwork.

A possible neurobiological mechanism by which isoflurane could shift the circadian clock is through its action on GABA receptors. General anesthetics including isoflurane are well known to potentiate the activation of GABA receptors of vertebrates and invertebrates (28, 29), and GABA is known to be an important neurotransmitter in both the entrainment and generation of circadian rhythms of mammals and insects. Pharmacological manipulations of GABA receptors phase shift the circadian clock and modify clock responses to light (30). In mammals, GABA and GABA receptor agonists such as diazepam have been shown to cause small phase delays of circadian rhythms in locomotor activity and, specifically, to block light-induced phase advances of behavioral rhythms (31). By contrast, GABA antagonists such as dehydroepiandrosterone sulfate (DHEAS) have been shown to cause phase advances (32), whereas the antagonist bicuculline specifically blocks light-induced phase delaying (33). In insects, injection of GABA into the brain can cause both phase advances and delays. During the active phase, GABA injection into the accessory medulla of cockroaches produces phase delays of similar magnitude to those reported here (34). Thus, the isoflurane-induced clock effect could plausibly result from a state of hyperactivation of the GABA receptor.

The importance of our work lies in the demonstration that isoflurane, the most commonly used clinical inhalational anesthetic in the world, causes a substantial shift of the circadian clock by acting on the expression of clock genes and that this effect persists for days. The unique nature of the honeybee model has also enabled us to understand how this phase shift affects time perception.

In conclusion, our results demonstrate a substantial, clear, and persistent effect of isoflurane anesthesia on the perception of time and the clock. The universal effectiveness of anesthesia, the similarity of the bee and mammalian circadian clock, and parallel findings in rodents suggest that our results in honeybees can be extrapolated to the mammalian and human system. In the human context, our results provide an explanation why, on waking from an anesthetic, many patients feel that they have just been “put to sleep.” Furthermore, our results suggest that postoperative patient management may need to account for the fact that patients have an altered circadian clock that is out of phase with their environment. This means that in addition to the physical insult of surgery, patients may also be suffering from anesthesia-induced “jet lag.” This anesthesia-induced jet lag is likely to have adverse conse-

quences for the physiology, metabolism, and general well-being of patients (5). Addressing the effects of general anesthesia on the circadian clock may significantly expedite postoperative recovery. Given the involvement of GABA in light entrainment of circadian rhythms, and the ability of GABA-ergic drugs to block circadian light resetting an obvious and important extension of the current work would be the concurrent administration of light and isoflurane to examine whether general anesthesia-induced jet lag can be treated during the anesthetic.

Materials and Methods

A more detailed experimental description can be found in the *SI Appendix*.

General Anesthesia. Honeybees (*Apis mellifera*) were anesthetized for 6 h with 2% isoflurane (Aerrane; Baxter) in air. The behavioral marker of anesthesia was immobility. In orientation experiments, forager bees were anesthetized in a light- and gas-proof container (3.5 L; flow rate: 0.5 L/min). For general anesthesia of entire colonies, hives were filled with the anesthetic gas mixture at 8 L/min for 5 min before anesthetized bees were transferred to an anesthetic chamber (22.3 L). Here, anesthesia was maintained at a flow rate of 2–3 L/min until bees were returned to their hive to recover.

In all experiments, our control consisted of bees anesthetized for 30 min. This procedure controlled for the following: (i) the physiological insult of anesthetic induction (e.g., disorientation, amnesia); (ii) exposure to the pungent odor of isoflurane; (iii) handling of bees (including transfer of bees between their hive and anesthetic chamber); and (iv) any other nonspecific effects that might be induced by anesthesia.

Time-Compensated Sun Compass Navigation. Vanishing bearings (Southern Hemisphere). Bees trained to a feeder (devoid of artificial scent; 0.5–2 M sucrose) 100 m geographically due north of the hive were caught at the feeder and anesthetized for 6 h (from 0900 hours, treatment) or 30 min (controls). Following recovery and displacement (6 km), individuals were released and the vanishing bearing of each bee recorded independently by two observers. **Harmonic radar tracking of vector-flights (Northern Hemisphere).** Bees trained to a feeder devoid of scent 300 m geographically east-south-east of the hive were caught at the feeder, displaced 500 m south, and released following the attachment of a radar transponder. Treatment bees (6-h anesthesia, from 0900 hours) were compared with two different groups: catch and release (caught at the feeder, displaced, and released immediately) and controls (30-min anesthesia). The vector-flight for each bee was determined by two observers blinded to the treatment group using consistency of angle, together with relative speed (first high-speed component) and distance traveled (18). The straightness ratios were calculated as described previously (18). **Data analysis.** Distributions of vanishing bearings and vector-flight paths were tested against random distributions using standard circular statistics methods including Raleigh uniformity test (35) (Oriana version 3.11; Kovach Computing Services). Inference about the mean difference between control and treatment vector-flight angles (i.e., *P* value and 95% CIs) was calculated using a nonparametric bootstrap distribution of the average difference (based on 9,999 random resamples). To facilitate interpretation, all flight directions were normalized to the position of the hive (defined as 0° in Figs. 1 and 2).

Food Anticipatory Behavior. Bees time-trained to a feeder box between 0900 and 1000 hours for 4–7 d were tagged with RFID chips (Mic3-TAG 64-D; Microsensus), and the timing of their foraging visits was measured by an RFID reader at the entrance to the feeder box for three pretreatment days. The entire hive was then anesthetized (from 0800 hours), and RFID recording of visits to the feeder box (containing no sucrose) continued a further 3–4 d. Paired *t* tests were used to analyze the difference in timing of foraging visits.

Hive Activity Rhythms. Experimental colonies (6,000–10,000 workers, 1 queen) were housed in 2-frame observation hives in a temperature- and light-controlled cabinet each. Bees foraged ad libitum for sugar water (50% vol/vol sucrose) in a flight cage in the same cabinet. After initial days of light/dark cycles upon transfer indoors, hives were maintained in constant dim light (LL_{dim}) with 2 lux at the cage top and 0.5 lux at the cage bottom. Population locomotor activity was detected at the hive entrance via an infrared light gate and recorded with ClockLab (Actimetrics).

Daytime anesthesia (*n* = 6 hives, of which three also underwent control treatment; *SI Appendix, Table S3*) was performed in the subjective morning after onset of activity, commencing at circadian time (Ct) 1–2 (Ct 6, predicted acrophase in activity). Night anesthesia (*n* = 3 hives, of which two received

control treatment) took place during the inactive period starting at Ct 14. Colonies free-ran under LL_{dim} for 3–8 d before and after the anesthetic, except for two daytime anesthesia experiments (designed for a different purpose; hive 4, 5), where LL_{dim} began only 24 h before the anesthetic.

Data were analyzed in 5-min time bins using ClockLab. Phase was attained by linear regression (least squares) through daily phase markers [activity onset, offset, and acrophase (only acrophase reported in *Results*; peak of the sine function with a period of 24 h fitted to the activity of each day)]. Phase shifts were calculated on the day after the anesthetic intervention by comparing the regression lines from before and after treatment.

Clock Gene Expression Analysis. Sample collection. Three hourly samples were taken from indoor colonies hive 3 and hive 9 (*SI Appendix, Table S3*) over a period of ~39 h before and after anesthesia, frozen in liquid nitrogen, and stored at –80 °C. Collections took place under LL_{dim} and a dim red-LED light source. Because only forager bees display robust oscillations in clock gene expression (36), we aimed to collect bees older than 22 d. These could be identified by a paint dot on their thorax that we had applied after their eclosion in an incubator (age, ≤24 h). All bees within an experimental hive came from the same source hive and queen.

Quantitative real-time PCR protocol. Clock gene mRNA levels were quantified in individual bee brains via RT-qPCR. A detailed experimental description satisfying the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (37) can be found in the *SI Appendix*.

Cerebrum and subesophageal ganglion were dissected on dry ice from frozen bee samples with atrophied hypopharyngeal glands [indicative of foraging activity (38)]. Following extraction of total RNA using silica-matrix

spin columns (RNeasy Mini Kit; Qiagen) and on-column DNase treatment (DNase I, RNase-free; Qiagen), cDNA synthesis was performed from 200 ng of RNA and primed with random hexamers using the SuperScript III first-strand synthesis system (Invitrogen). RT-qPCR was carried out with SYBR chemistry (Power SYBR Green PCR Master Mix; Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) including a melting curve. Reactions contained an equivalent of 6.3 ng of transcribed RNA as template and a final primer concentration of 200 nM. (Primer details are listed in *SI Appendix, Table S1*.) *Cry-m*, *Per*, and *Clk* mRNA levels were normalized against the stably expressed reference genes *EF1 α* , *GAPDH*, and *Tub* (day anesthesia) or *EF1 α* and *Tub* (night anesthesia) and converted to relative quantities with qBasePlus (version 1.5 and 2.0; Biogazelle) using the efficiencies listed in *SI Appendix, Table S1*; interrun calibration was performed with three calibrator samples.

Data analysis. Cosinor models were fitted to log-transformed data for *Per* (least squares) and *Cry-m* (generalized least squares with an AR1 error structure because of autocorrelated residuals) using the period of the pre-anesthesia activity of the hive (day anesthesia: 23.8 h; night anesthesia: 24.4 h). Changes in phase were tested and quantified according to the description of Bingham et al. (39).

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