Report

Current Biology

Bumble Bee Workers Give Up Sleep to Care for Offspring that Are Not Their Own

Highlights

- Bumble bee workers show the essential characteristics of sleep
- Sleep is reduced in the presence of pupae that do not need to be fed
- Empty cocoons induce transient sleep loss, suggesting involvement of brood pheromones
- Worker bees give up sleep to care for offspring that are not their own

Authors

Moshe Nagari, Ariel Gera, Sara Jonsson, Guy Bloch

Correspondence

guy.bloch@mail.huji.ac.il

In Brief

Nagari et al. report that bumble bee workers reduce sleep in the presence of larvae that need to be fed or pupae that do not. Emptied cocoons induce a similar but transient effect, suggesting that pupal substances mediate the brood effect on sleep. This is the first evidence that animals give up sleep to care for offspring that are not their own.

Nagari et al., 2019, Current Biology 29, 1–6
October 21, 2019
https://doi.org/10.1016/j.cub.2019.07.091

CellPress

Please cite this article in press as: Nagari et al., Bumble Bee Workers Give Up Sleep to Care for Offspring that Are Not Their Own, Current Biology (2019), https://doi.org/10.1016/j.cub.2019.07.091

Current Biology
Report

Bumble Bee Workers Give Up Sleep to Care for Offspring that Are Not Their Own

Moshe Nagari,[1] Ariel Gera,[1] Sara Jonsson,[1] and Guy Bloch[1,2,*]
[1]Department of Ecology, Evolution, and Behavior, The A. Silberman Institute of Life Sciences, Hebrew University, Jerusalem 91904, Israel
[2]Lead Contact
*Correspondence: guy.bloch@mail.huji.ac.il
https://doi.org/10.1016/j.cub.2019.07.091

SUMMARY

Sleep is ubiquitous in vertebrates and invertebrates, and its loss is typically associated with reduced performance, health, or survival, for reasons that are yet unclear [1–3]. Nevertheless, some animals can reduce sleep for increasing foraging time [4], under predation risk [5–8], during seasonal migration [9–11], or for having greater mating opportunities [12, 13]. Here, we tested the hypothesis that social bumble bee (*Bombus terrestris*) workers give up sleep for improving brood care. We combined video recordings, detailed behavioral analyses, sleep-deprivation experiments, and response-threshold assessments to characterize the sleep behavior of worker bees and showed that immobility bouts of ≥5 min provide a reliable proxy for sleep. We next used this index to study sleep with an automated video-based activity monitoring system. We found that isolated workers severely reduce sleep time in the presence of both larvae that need to be fed and pupae that do not. Reduced sleep was also correlated with around-the-clock activity and wax-pot building, which are typical for nest-founding mother queens. Cocoons, from which we removed the pupae, elicited a similar but transient sleep loss in tending workers, suggesting that the pupa effect on sleep is mediated by pheromonal signals. Sleep time increased following brood removal but remained lower compared to control bees, suggesting that the brood modulated sleep need. This first evidence for brood modulation of sleep in an insect suggests that plasticity in sleep can evolve as a mechanism to improve care for dependent juveniles, even in social insect workers that do not care for their own offspring.

RESULTS

Bumble Bees Show Sleep-like Behavior Similar to that of Other Vertebrates and Invertebrates

Using video records of workers isolated with or without a pupa (experiment 1; Figure S1A), we found that stationary bumble bees might show one of three distinct behaviors that we termed "immobile-active," "incubation-like," and "sleep-like" (see Table S1; Figure S2; Videos S1 and S2; STAR Methods for detailed descriptions). Incubation-like behavior was defined based on previous descriptions [14–16], our own detailed observations, and thermal imaging (Figure S2). Sleep-like behavior was defined based on previous detailed studies with honeybees and other insects [17–19]. Bees in sleep-like state showed few antennal movements, and sleep-bout duration was longer compared with the other two stationary behaviors (Figures 1A, 1B, and S3A; Videos S1 and S2). Sleep-like behavior constituted 91.1% of the time spent in immobile bouts lasting 5 min or more (Figure S3B) or 86.6% of the number of bouts ≥5 min (Figure 1C). We did not record any incubation-like bouts lasting 5 min or more. Although one-third of total sleep-like time was in bouts lasting less than 5 min (Figure S3A), these constituted only 1.9% of the total short immobile bouts (Figure 1C). Thus, using immobile bouts shorter than 5 min as a proxy for sleep would significantly increase false detection, and we selected immobility bouts ≥5 min as a conservative index for sleep-like behavior in the bumble bee. We next recorded the response (movement) of stationary isolated bees to a series of light pulses with increasing intensity directed to their eyes (experiment 2). We found that the response threshold of sleeping bees was ~20- to 150-fold higher compared to incubating and immobile-active bees (Figure 1D). We also tested whether the bumble bee sleep-like state is homeostatically regulated (experiment 3) following disturbance with a series of three substrate vibration sessions (Figure S4). There was substantial variation between individuals, with some disturbed bees not reducing their amount of sleep (≥5 min of immobility) during the vibration sessions (Figure S4A). However, when we closely observed the bees during a vibration session (orange line in Figures S4A–S4C), we noticed that some stationary bees were nevertheless awake and moved their antennae, suggesting that their sleep was disturbed. Consistent with this notion, disturbed bees slept significantly more during both the light and the dark phases on the 3 days following the last disturbance compared to before the disturbances (Figure S4E). Taken together, these findings suggest that bumble bee workers show homeostatic increase in sleep amount following sleep disturbance.

Brood-Tending Workers Reduce Sleep Amount in the Presence of Brood

We used an automatic data-acquisition system to test the influence of male larvae (offspring of orphan "queenless" workers) and female larvae (offspring of mated queens; "queenright") on the sleep shown by tending workers (experiment 4; Figure S1B). Given that egg-laying workers tended to have reduced daily

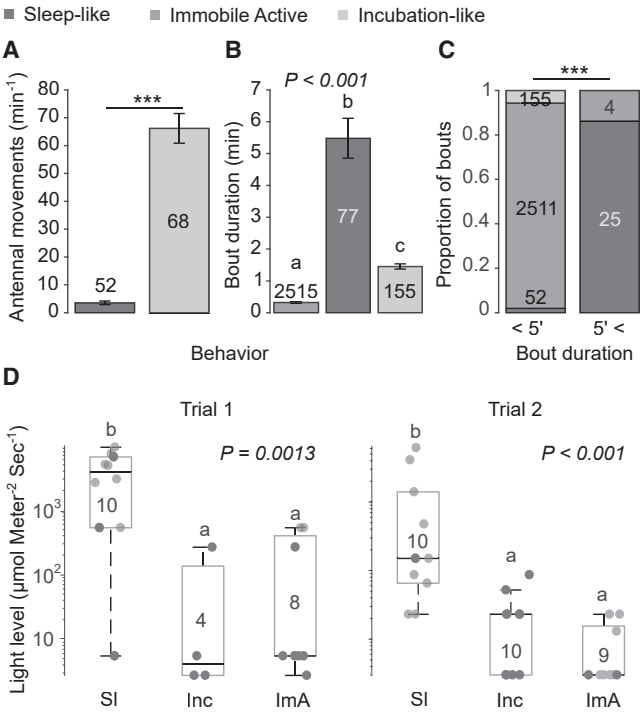

Figure 1. Bumble Bees Show Typical Sleep-like Behavior

We used video analyses (A–C) and response threshold experiments (D) to compare three behaviors shown by stationary worker bees.

(A) Antennal movements (means ± SEM; two-sample t test).

(B) Bout duration (means ± SEM; Kruskal-Wallis test followed by Games-Howell post hoc tests).

(C) The proportion of behavioral bouts lasting ≥5 min or <5 min (χ^2 test for independence).

(D) The response threshold to a light pulse. Each circle depicts the mean minimal light intensity at which an individual bee responded to a light pulse directed to her eye. Dark and light filled circles indicate bees housed with or without a pupa, respectively. The central black line shows the median, the bottom and top box outlines depict the 25th–75th percentiles, and the whiskers extend to the most extreme data points not considered outliers. Boxes marked with different letters are significantly different in Kruskal-Wallis followed by Games-Howell post hoc tests. ImA, immobile-active; Inc, incubation-like; SI, sleep-like. ***p < 0.001.

See also Videos S1 and S2 and Figures S2–S4.

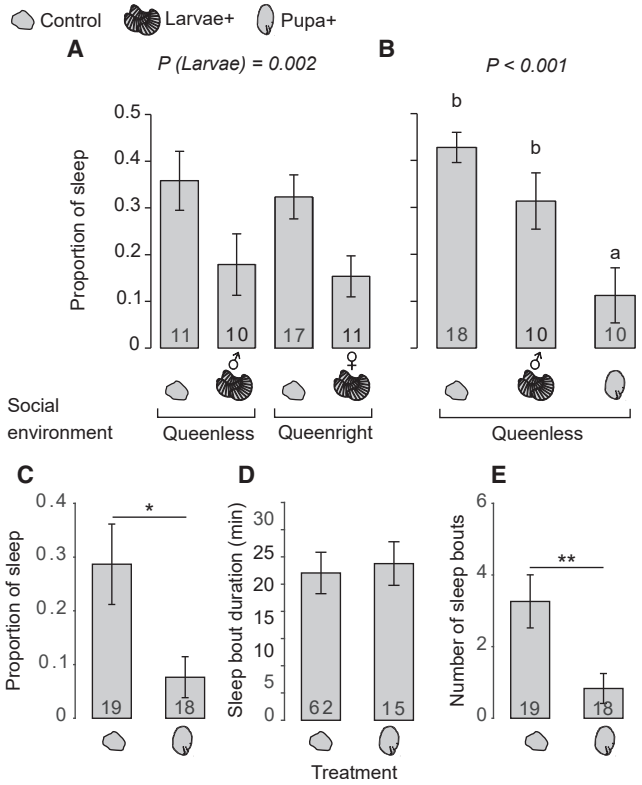

Figure 2. Bumble Bee Workers Reduce Sleep Amount in the Presence of Larvae or Pupae

(A) The proportion of daily sleep in bees placed with male or female larvae or with a control piece of wax (experiment 4). Two-way ANOVA with the presence of larvae and previous social experience (i.e., queenless or queenright) as factors is shown. p (social experience) = 0.58; p (larvae X social experience) = 0.93. The preceding social experience was confounded with larvae gender.

(B) The proportion of daily sleep in bees placed with larvae that need to be fed or pupae that do not (experiment 5). One-way ANOVA followed by LSD post hoc test is shown.

(C–E) Analyses of 1-h video-recording sessions (experiment 1).

(C) Proportion of time asleep per recording session.

(D) Sleep bout duration.

(E) Number of sleep bouts per recording session.

(C and E) Two-sample t test; *p < 0.05; **p < 0.01. (D) Mann-Whitney test. Control, a bee isolated with a piece of wax and no brood; Larvae+, a bee isolated with ~8 larvae; Pupa+, a bee isolated with a single live pupa. All the plots show mean ± SEM; n inside bars.

sleep and weaker circadian rhythms in locomotor activity, we excluded them from following analyses (STAR Methods). Worker bees slept significantly less in the presence of both male and female larvae ("larvae+" treatment) compared to a control piece of wax with no effect of larval gender (Figure 2A). Therefore, in experiment 5, we used only male larvae and compared their effect to that of pupae (Figure S1C). We found that workers with a pupa, which they do not need to feed, nevertheless slept less compared to control broodless bees (Figure 2B). To confirm these findings, we analyzed the video records of experiment 1 and found that the proportion of time asleep and the number of sleep bouts, but not bout duration, were significantly reduced in bees housed with a pupa (Figures 2C–2E). These detailed analyses confirm that sleep amount is severely reduced in bees tending even a single pupa. The presence of a pupa also affected the temporal organization of behavior (Figure 3). Control bees with no brood were overall more active during the subjective day ("total activity" and "immobile-active"; see Table S1 for details; Figure 3A). Workers isolated with a pupa tended the brood ("on pupa"; Figure 3) and were overall similarly active during the day and night ("total activity"), which is consistent with the activity pattern shown by brood-caring bees in free-foraging colonies [20, 21]. However, in these bees, "mobile-active" and "immobile-active" (p = 0.068) behaviors were higher during the subjective day and incubation-like behavior was higher during the subjective night (Figure 3B).

Bees Placed with Emptied Cocoons Show a Reduction in Sleep Duration that Fades over Time

We recorded sleep duration for individually isolated workers housed with an empty cocoon, from which we removed the

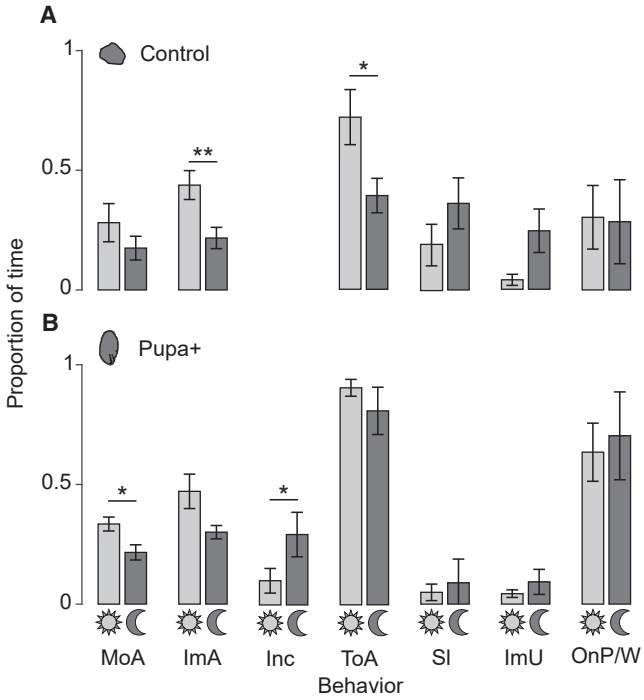

Figure 3. The Presence of a Pupa Influences the Temporal Organization of Worker Behavior

(A) Bees without a pupa (Control).

(B) Bees with a pupa (Pupa+). ImU, immobile-unknown; MoA, mobile-active; OnP/W, on-pupa/wax; ToA, total-activity. Light and dark bars, subjective day or night, respectively. The data summarize detailed analyses of 1 h video-recording sessions (experiment 1); mean ± SEM; n = 5 bees/treatment; paired t test: *p < 0.05; **p < 0.01.

pupa ("empty"); a cocoon in which we made a similar incision but did not remove the pupa ("sham"); a live intact pupa ("pupa+"); or only a piece of wax ("control"; experiment 6; Figure S1D). We repeated this experiment three times, each with bees and brood from different source colonies. Bees that laid eggs during the monitoring sessions were excluded from analyses (for more details, see experiment 6 in the STAR Methods). As in experiments 1 and 5, bees slept less in the presence of a live pupa (sham and pupa+) compared to control bees, which had only a piece of wax in their cage (Figure 4A; not statistically significant for the sham treatment in trial 3). The day ($p < 0.001$) and the "day X treatment" interaction ($p = 0.001$, $p = 0.01$, and $p = 0.08$ in trials 1, 2, and 3, respectively) had a significant influence, suggesting that the effect of time differed between treatments. Indeed, bees with an empty cocoon showed a distinct pattern: They slept similarly to bees that were housed with a live pupa (sham and pupa+) but significantly less than the control bees during the first day (Figure 4A). Thereafter, their sleep duration increased up to a level comparable to the control bees on day 4. Thus, the effect of empty cocoons faded over time. Removal of the empty, sham, and pupa+, but not control, treatments on day 5 resulted in a significant increase in the amount of sleep on the following day (Figure 4A; in trial 2, the effect was statistically significant only for the pupa+ treatment), lending additional credence to the hypothesis that signals from the pupae modulate the sleep of tending workers. The bees that had live pupae at the first part of the experiment slept less than control bees even after treatment removal (in trials 1 and 2), and sleep did not increase further on the following days. These observations are not consistent with sleep rebound, which would be expected if the bees were sleep deprived by the presence of live pupae in the first part of the experiment. Rather, they suggest that the brood modulates sleep need in tending bees. Bees with a live pupa (sham and pupa+) were more likely to construct wax pots compared to control bees (Figure 4B; not recorded in trial 1). Wax-pot builders slept less compared to non-builders in a pooled sample of bees from all treatment groups (insets in Figure 4A). To avoid possible confounding effects of the pupae, we further analyzed only bees housed without a live pupa (i.e., control and empty treatments) and found similar significant differences in sleep proportion between bees that built or did not build wax pots in trial 3 (mean ± SEM = 0.08 ± 0.016 and 0.15 ± 0.025, respectively; two-sample t test, $p = 0.036$) and a similar trend in trial 2 (0.11 ± 0.02 and 0.16 ± 0.03, respectively; $p = 0.25$).

DISCUSSION

Using video recordings, response threshold evaluation, sleep disturbance experiments, and detailed behavioral analyses, we established that the sleep-like state of bumble bee (*Bombus terrestris*) workers shows the essential behavioral and physiological characteristics of sleep and is particularly similar to that of the honeybee [17–19, 22, 23] (Video S2). Sleeping bumble bees have a significantly higher response threshold and show a homeostatic increase in sleep duration following sleep disturbance. Our analyses show that bouts of inactivity lasting 5 min or more provide a reliable proxy for sleep, an index similar to that developed for automated sleep monitoring of honey bees and fruit flies [17, 24].

Bumble bee sleep is incredibly sensitive to the presence of brood. The brood effect is specific and dynamic, as was best exemplified by the increase in sleep duration on the day following brood removal in experiment 6 (Figure 4A). This effect of brood on sleep is reminiscent of studies with several species of mammals [25, 26]. For example, human mothers sleep less and more intermittently in close proximity to their babies [25, 27, 28]. Rat pups search for their mother's nipples and by that apparently interrupt her sleep [29], which later returns to normal after pup weaning [30]. The findings in mammals and bees make functional sense because their newborns are helpless and reliant on adult care. Whereas in humans and rats the newborn influence is associated with their activity, food begging, or acoustic signals [25, 29], the bumble bee pupa does not move, is not known to emit acoustic signals, and does not need to be fed. How do the apparently passive pupae reduce the sleep of tending adults? Visual signals are not likely because the nest cavity is typically dark, and we used dim red light that bumble bees do not see well [31]. Sound, vibrations, and temperature may also be excluded because the effect of empty cocoons in experiment 6 faded gradually, although they were empty already on the first day. Although we cannot exclude gradual changes in the texture of emptied cocoons, we suggest that the most plausible explanation for the sleep reducing effect is that pupal substances were left on emptied cocoons and gradually lost their effect due to degradation or evaporation. This explanation is

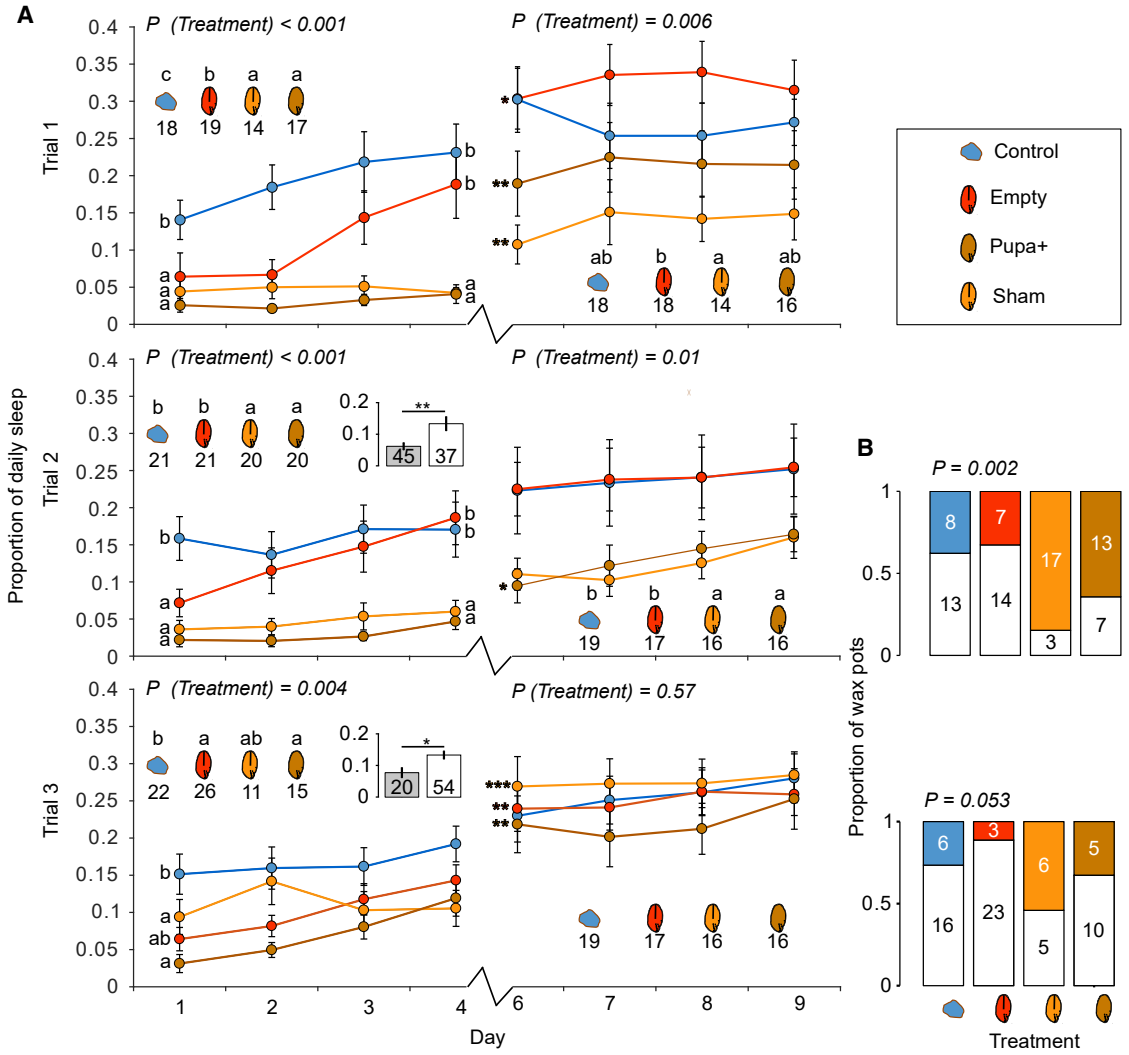

Figure 4. Workers Tending Pupae or Emptied Cocoons Showed Reduced Sleep Duration and Pupa Presence Was Associated with Increased Wax-Pot Building

Each row depicts a trial with bees from different source colonies.

(A) The influence of brood stimuli on sleep time (mean ± SEM; sample sizes are shown below the legend icons). Legend icons with different letters are statistically different in two-way repeated-measures ANOVAs with treatment and day as factors, done separately for days 1–4 and days 6–9, and followed by least significant difference (LSD) post hoc tests for the treatment factor. Different small letters inside the plots on the 1st or 4th day indicate a statistically significant difference in one-way ANOVA followed by LSD post hoc tests. The colored asterisks indicate significant differences in sleep amount between the day before and the day after the removal of the treatment stimuli from the monitoring cages (paired t tests). The insets in trials 2 and 3 show the amount of sleep for bees that did (gray bars) or did not (open bars) build wax pots during the monitoring session (two-sample t test).

(B) The influence of brood stimuli on wax-pot building (not determined for trial 1). Empty, a bee isolated with a cocoon from which the pupa was removed; Sham, a bee housed with a similarly treated cocoon from which the pupa was not removed. Other details are as in Figure 2 or provided in the text. *p < 0.05; **p < 0.01; ***p < 0.001 in all plots.

consistent with the hypothesis that brood pheromones emitted from the pupae modulate the sleep of bumble bee workers. Interestingly, the brood has somewhat different effects in honey bees: the presence of brood attenuates circadian rhythms of nurse bees but does not affect their amount of sleep [32, 33]. To the best of our knowledge, the only other evidence for pheromonal modulation of sleep is the female sex pheromones of *Drosophila* that reduce sleep in males [13]. The sex-pheromones-aroused males are thought to increase the number of females they sire and overcome the assumed fitness cost of reduced sleep [12, 13]. To our knowledge, the present study provides the first evidence for pheromonal modulation of sleep in animals caring for dependent juveniles.

Why does the brood need to be tended around the clock, even at a cost of a significant sleep loss for tending adults? The most intuitive explanation is frequent feeding, which enables accelerated growth rate, but this cannot account for the strong influence of pupae that do not require feeding. The tending bees may need to tightly regulate brood temperature and perhaps additional constituents of the microenvironment. In honey bees, in which

the pupae temperature is tightly regulated [34], a slight deviation from optimal temperature impairs pupal development [35, 36]. The significance of brood incubation in bumble bees has long been appreciated [14–16], and our video analyses indeed show that the isolated workers frequently incubated the pupae (Video S1), particularly during subjective night, a time when broodless bees typically sleep (Figure 3). Brood-tending workers show additional behaviors, such as wax-pot construction and pupal grooming, that may also contribute to the need to be active around the clock and reduce sleep time.

A remarkable aspect of our findings is that the workers gave up sleep to care for brood that was not their own offspring. Brood tending bees showed several additional behavioral and physiological characteristics. For example, they were more likely to build wax pots (Figure 4B) and had weaker circadian rhythms (data not shown). The few workers that laid eggs were active around the clock, and tended to sleep less. This suite of characteristics is remarkably similar to the association between comb construction, egg laying, and around-the-clock brood care in nest-founding queens [16, 37]. Egg-laying bumble bee queens switch to around-the-clock activity already before building an eggcup, suggesting that it can stem from altered physiology, in addition to direct response to brood signals. These similarities between brood-tending workers and mother queens are consistent with the reproductive and ovarian ground plan hypotheses. These hypotheses state that the evolution of insect sociality was associated with modifications in pathways regulating reproduction and maternal care in the solitary ancestors, such that they were co-opted to regulate sibling care in workers [38–42]. The strong association between reduced sleep duration and other maternal-like traits in brood-tending bumble bee workers suggests that sleep modulation is part of the set of maternal traits that were co-opted along the evolution of sociality in bees.

The importance of sleep for normal function and health is well established (e.g., [43]), yet brood-tending bumble bees dramatically reduce sleep duration. We found no evidence for sleep rebound after brood removal, in contrast to the increase in sleep after substrate vibrations (Figures 4 and S4). These observations suggest that brood-tending bees are not sleep deprived but rather are able to reduce their sleep need. Recent studies with *Drosophila* provide plausible neuronal bases for this observation by showing that activation of certain arousal neural circuits does not lead to subsequent homeostatic sleep rebound [44, 45]. Additional studies are needed to determine whether reduced sleep in brood-tending bumble bees is homeostatically compensated by a subsequent increase in sleep intensity or that it comes with a cost in the form of compromised cognitive performance or health. Nevertheless, our findings support and extend a small number of studies in ecologically relevant contexts, suggesting that some animals can perform well with little sleep over relatively long periods [4, 9–12, 26]. Taken together, these studies lend credence to the hypothesis that some animals had evolved means to substantially reduce sleep need in order to free time that they then devote to fitness-enhancing activities. Remarkably, in the bumble bee, the selection pressure to reduce sleep is linked to the evolution of sociality: sleep reduction enables "nurse" workers effective and almost continuous care for their siblings rather than for their own offspring.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Locomotor activity monitoring and sleep analyses
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Sleep and circadian rhythms analyses
  - Other statistical analyses
- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.07.091.

ACKNOWLEDGMENTS

We thank Barrett Klein for producing the bumble bee thermal images, Guy Aronov and Zvi Sagiv for setting the electronics for the substrate vibration apparatus, Hagai Shpigler for valuable discussions on experimental design, Mira Cohen for technical support in the laboratory, Nir Keren for kindly allowing us to use his photometer, and Noga Gross for assistance with video analyses. "Larva" icon (Figures 2 and S1) is by Yu Luck, "moon" icon (Figure 3) is by Marek Polakovic, "sun" icon (Figure 3) is by Marco Livolsi, "bumble bee" icon (Figure S1) is by Matt Hawdon, "crown" icon (Figure S1) is by Abdul Karim, and "camera" icon (Figure S1) is by Anas Ramadan. All these icons are from the Noun Project. The study was supported by the Israel Science Foundation (ISF) (number 1274/15 to G.B.).

AUTHOR CONTRIBUTIONS

Conceptualization, M.N. and G.B.; Methodology, M.N. and G.B.; Formal Analysis, M.N.; Investigation, M.N., A.G., and S.J.; Writing – Original Draft, M.N.; Writing – Review & Editing, M.N., A.G., and G.B.; Visualization, M.N.; Supervision, G.B.; Project Administration, G.B.; Funding Acquisition, G.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: February 14, 2019
Revised: July 9, 2019
Accepted: July 31, 2019
Published: October 3, 2019

REFERENCES

1. Cirelli, C., and Tononi, G. (2008). Is sleep essential? PLoS Biol. *6*, e216.
2. Eban-Rothschild, A., Giardino, W.J., and de Lecea, L. (2017). To sleep or not to sleep: neuronal and ecological insights. Curr. Opin. Neurobiol. *44*, 132–138.
3. Dubowy, C., and Sehgal, A. (2017). Circadian rhythms and sleep in *Drosophila melanogaster*. Genetics *205*, 1373–1397.
4. Rattenborg, N.C., Voirin, B., Cruz, S.M., Tisdale, R., Dell'Omo, G., Lipp, H.P., Wikelski, M., and Vyssotski, A.L. (2016). Evidence that birds sleep in mid-flight. Nat. Commun. *7*, 12468.
5. Rattenborg, N.C., Lima, S.L., and Amlaner, C.J. (1999). Facultative control of avian unihemispheric sleep under the risk of predation. Behav. Brain Res. *105*, 163–172.
6. Lesku, J.A., Bark, R.J., Martinez-Gonzalez, D., Rattenborg, N.C., Amlaner, C.J., and Lima, S.L. (2008). Predator-induced plasticity in sleep

architecture in wild-caught Norway rats (*Rattus norvegicus*). Behav. Brain Res. *189*, 298–305.

7. Dominguez, J. (2003). Sleeping and vigilance in black-tailed Godwit. J. Ethol *21*, 57–60.
8. Lendrem, D.W. (1984). Sleeping and vigilance in birds, II. An experimental study of the Barbary dove (*Streptopelia risoria*). Anim. Behav. *32*, 243–248.
9. Rattenborg, N.C., Mandt, B.H., Obermeyer, W.H., Winsauer, P.J., Huber, R., Wikelski, M., and Benca, R.M. (2004). Migratory sleeplessness in the white-crowned sparrow (*Zonotrichia leucophrys gambelii*). PLoS Biol. *2*, E212.
10. Jones, S.G., Paletz, E.M., Obermeyer, W.H., Hannan, C.T., and Benca, R.M. (2010). Seasonal influences on sleep and executive function in the migratory white-crowned Sparrow (*Zonotrichia leucophrys gambelii*). BMC Neurosci. *11*, 87.
11. Fuchs, T., Maury, D., Moore, F.R., and Bingman, V.P. (2009). Daytime micro-naps in a nocturnal migrant: an EEG analysis. Biol. Lett *5*, 77–80.
12. Lesku, J.A., Rattenborg, N.C., Valcu, M., Vyssotski, A.L., Kuhn, S., Kuemmeth, F., Heidrich, W., and Kempenaers, B. (2012). Adaptive sleep loss in polygynous pectoral sandpipers. Science *337*, 1654–1658.
13. Beckwith, E.J., Geissmann, Q., French, A.S., and Gilestro, G.F. (2017). Regulation of sleep homeostasis by sexual arousal. eLife *6*, e27445.
14. Heinrich, B. (1972). Physiology of brood incubation in the bumblebee queen, bombus vosnesenskii. Nature *239*, 223–225.
15. Heinrich, B. (1974). Thermoregulation in bumblebees I. Brood incubation by *Bombus vosnesenskii* queens. J. Comp. Physiol. *88*, 129–140.
16. Heinrich, B. (1972). Patterns of endothermy in bumblebee queens, drones and workers. J. Comp. Physiol. *77*, 65–79.
17. Eban-Rothschild, A.D., and Bloch, G. (2008). Differences in the sleep architecture of forager and young honeybees (*Apis mellifera*). J. Exp. Biol. *211*, 2408–2416.
18. Klein, B.A., Olzsowy, K.M., Klein, A., Saunders, K.M., and Seeley, T.D. (2008). Caste-dependent sleep of worker honey bees. J. Exp. Biol. *211*, 3028–3040.
19. Kaiser, W. (1988). Busy bees need rest, too: behavioural and electromyographical sleep signs in honeybees. J. Comp. Physiol. A *163*, 565–584.
20. Yerushalmi, S., Bodenhaimer, S., and Bloch, G. (2006). Developmentally determined attenuation in circadian rhythms links chronobiology to social organization in bees. J. Exp. Biol. *209*, 1044–1051.
21. Crall, J.D., Switzer, C.M., Oppenheimer, R.L., Ford Versypt, A.N., Dey, B., Brown, A., Eyster, M., Guérin, C., Pierce, N.E., Combes, S.A., and de Bivort, B.L. (2018). Neonicotinoid exposure disrupts bumblebee nest behavior, social networks, and thermoregulation. Science *362*, 683–686.
22. Kaiser, W., and Steiner-Kaiser, J. (1983). Neuronal correlates of sleep, wakefulness and arousal in a diurnal insect. Nature *301*, 707–709.
23. Helfrich-Förster, C. (2018). Sleep in insects. Annu. Rev. Entomol. *63*, 69–86.
24. Shaw, P.J., Cirelli, C., Greenspan, R.J., and Tononi, G. (2000). Correlates of sleep and waking in *Drosophila melanogaster*. Science *287*, 1834–1837.
25. Nishihara, K., and Horiuchi, S. (1998). Changes in sleep patterns of young women from late pregnancy to postpartum: relationships to their infants' movements. Percept. Mot. Skills *87*, 1043–1056.
26. Lyamin, O., Pryaslova, J., Lance, V., and Siegel, J. (2005). Animal behaviour: continuous activity in cetaceans after birth. Nature *435*, 1177.
27. McKenna, J.J., Thoman, E.B., Anders, T.F., Sadeh, A., Schechtman, V.L., and Glotzbach, S.F. (1993). Infant-parent co-sleeping in an evolutionary perspective: implications for understanding infant sleep development and the sudden infant death syndrome. Sleep *16*, 263–282.
28. Volkovich, E., Bar-Kalifa, E., Meiri, G., and Tikotzky, L. (2018). Mother-infant sleep patterns and parental functioning of room-sharing and solitary-sleeping families: a longitudinal study from 3 to 18 months. Sleep *41*, zsx207.
29. da Rocha, L.R.M., and Hoshino, K. (2009). Some aspects of the sleep of lactating rat dams. Sleep Sci. *2*, 88–91.
30. Sivadas, N., Radhakrishnan, A., Aswathy, B.S., Kumar, V.M., and Gulia, K.K. (2017). Dynamic changes in sleep pattern during post-partum in normal pregnancy in rat model. Behav. Brain Res. *320*, 264–274.
31. Briscoe, A.D., and Chittka, L. (2001). The evolution of color vision in insects. Annu. Rev. Entomol. *46*, 471–510.
32. Nagari, M., Brenner, Y., and Bloch, G. (2017). Nurse honeybee workers tend capped brood, which does not require feeding, around the clock. J. Exp. Biol. *220*, 4130–4140.
33. Shemesh, Y., Eban-Rothschild, A., Cohen, M., and Bloch, G. (2010). Molecular dynamics and social regulation of context-dependent plasticity in the circadian clockwork of the honey bee. J. Neurosci. *30*, 12517–12525.
34. Kronenberg, F., and Heller, H.C. (1982). Colonial thermoregulation in honey bees (*Apis mellifera*). J. Comp. Physiol. *148*, 65–76.
35. Tautz, J., Maier, S., Groh, C., Rossler, W., and Brockmann, A. (2003). Behavioral performance in adult honey bees is influenced by the temperature experienced during their pupal development. Proc. Natl. Acad. Sci. USA *100*, 7343–7347.
36. Groh, C., Tautz, J., and Rössler, W. (2004). Synaptic organization in the adult honey bee brain is influenced by brood-temperature control during pupal development. Proc. Natl. Acad. Sci. USA *101*, 4268–4273.
37. Eban-Rothschild, A., Belluci, S., and Bloch, G. (2011). Maternity-related plasticity in circadian rhythms of bumble-bee queens. Proc. Biol. Sci *278*, 3510–3516.
38. Linksvayer, T.A., and Wade, M.J. (2005). The evolutionary origin and elaboration of sociality in the aculeate Hymenoptera: maternal effects, sib-social effects, and heterochrony. Q. Rev. Biol. *80*, 317–336.
39. West-Eberhard, M.J. (1987). Flexible strategy and social evolution. In Animal Societies: Theories and Facts, Y. Ito, J.L. Brown, and J. Kikkawa, eds. (Japan Scientific Societies Press), pp. 35–51.
40. Amdam, G.V., and Page, R.E., Jr. (2010). The developmental genetics and physiology of honeybee societies. Anim. Behav. *79*, 973–980.
41. Johnson, B.R., and Linksvayer, T.A. (2010). Deconstructing the superorganism: social physiology, groundplans, and sociogenomics. Q. Rev. Biol. *85*, 57–79.
42. Bloch, G., and Grozinger, C.M. (2011). Social molecular pathways and the evolution of bee societies. Philos. Trans. R. Soc. Lond. B Biol. Sci. *366*, 2155–2170.
43. Rechtschaffen, A., Gilliland, M.A., Bergmann, B.M., and Winter, J.B. (1983). Physiological correlates of prolonged sleep deprivation in rats. Science *221*, 182–184.
44. Seidner, G., Robinson, J.E., Wu, M., Worden, K., Masek, P., Roberts, S.W., Keene, A.C., and Joiner, W.J. (2015). Identification of neurons with a privileged role in sleep homeostasis in *Drosophila melanogaster*. Curr. Biol. *25*, 2928–2938.
45. Thimgan, M.S., Suzuki, Y., Seugnet, L., Gottschalk, L., and Shaw, P.J. (2010). The perilipin homologue, lipid storage droplet 2, regulates sleep homeostasis and prevents learning impairments following sleep loss. PLoS Biol. *8*, e1000466.
46. Eban-Rothschild, A., and Bloch, G. (2015). The colony environment modulates sleep in honey bee workers. J. Exp. Biol. *218*, 404–411.
47. Friard, O., and Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol. Evol. *7*, 1325–1330.
48. Megevand, P. (2017). Games-Howell Post-Hoc Test for One-Way ANOVA (MATLAB Central File Exchange).
49. Quinn, G.P., and Keough, M.J. (2002). Experimental Design and Data Analysis for Biologists (Cambridge University Press).

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Bombus terrestris colonies	Yad-Mordechai Pollination Services, Yad-Mordechai, Israel	http://www.polyam.net
Experimental Models: Organisms/Strains		
Bumble bee (*Bombus terrestris*)	Yad-Mordechai Pollination Services, Yad-Mordechai, Israel	http://www.polyam.net
Software and Algorithms		
MATLAB R2017a	MATLAB (2017). version 9.2.0 (R2017a), The MathWorks, Natick, Massachusetts	http://www.mathworks.com
SPSS 21.0	IBM SPSS Statistics for Windows. Armonk, NY: IBM	http://www.ibm.com/analytics/spss-statistics-software
Big Brother	Big Brother, Actimetrics 1621 Elmwood, Avenue, Wilmette, IL 60091	http://www.actimetrics.com
ClockLab	ClockLab, Actimetrics 1621 Elmwood, Avenue, Wilmette, IL 60091	http://www.actimetrics.com
BeeSleep v.2.0	[46]	N/A
SleepPlotV2	This paper	http://guybloch.huji.ac.il

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Guy Bloch (guy.bloch@mail.huji.ac.il).

This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bombus terrestris colonies were obtained from Yad-Mordechai Pollination Services, Yad-Mordechai, Israel. Colonies were housed in wooden nest-boxes (30 X 23 X 20 cm) with transparent plastic covers. The bees were fed *ad libitum* with pollen (collected by honeybees) mixed with commercial sucrose syrup (Yad-Mordechai Pollination Services, Israel). The colonies were kept in an environmental chamber (27—29°C; relative humidity = 40—60%), under constant dim red light (illuminated with Edison Federal EFEE 1AE1 Deep Red LEDs, maximum and minimum wavelengths = 670 and 650, respectively; except for Experiment 1, see below) that bumble bees do not see well [31]. In Experiments 2, 3 and in Trial 3 of Experiment 6, the colonies were kept in 12 hours light: 12 hours dark (LD) illumination regime (see below). During the light phase, colonies were illuminated with 'white' LED lights (Optima Lighting IP65; 30W, 2700 lumens). Lighting intensity during the light phase was 600—700 lux. Each colony contained ~50 worker bees and a queen at the beginning of the experiments. In order to obtain bees of known age we collected callow (newly emerged, 0—24 hours of age) worker bees, identified by their light coloration, and marked each with a dot of acrylic paint-die (DecoArt, Stanford, KY, U.S.A, in Experiments 4, 5) or a number-tag (Experiments 1—3, 6) on the dorsal part of their thorax. To obtain enough callow bees, collection, marking and reintroduction were done over two or three (Experiment 1) consecutive days. We used 'white' LED flashlights (Energizer, St. Louis, Missouri, U.S.A) to facilitate callow-bee collection during daytime (between 8:00 and 17:00). We collected the marked focal bees from their colonies (Experiments 1—3, 6) or experimental cages (Experiments 4, 5) when they were 5—7 days of age, and placed each individually in a monitoring cage. The monitoring cages were made of modified Petri dishes (diameter: 90 mm, height: 30mm) with the exception of Experiments 1 and 2 (see below). We provisioned each cage with *ad libitum* pollen and sucrose syrup.

METHOD DETAILS

Locomotor activity monitoring and sleep analyses

We monitored locomotor activity of individually isolated bees using an automatic data acquisition system as previously described [20]. Briefly, the monitoring cages with the focal bees and the experimental stimuli were placed in an environmental chamber (26—28°C, relative humidity 50%—70%) kept under constant dim red light (Edison Federal EFEF 1AE1 Far (Cherry) Red LED;

maximum and minimum wavelengths were 750 and 730, respectively). In Experiment 3, the bees were monitored under 12:12 LD regime. Activity was recorded continuously over the entire duration of the monitoring session at a frequency of 1 Hz with four CCD cameras (Panasonic WV-BP334) and an image acquisition board (IMAQ 1409, National Instruments, U.S.A).

Experiment 1: Video analyses of the behavior of workers isolated with or without pupae

On days 1—3 of the experiment, we marked focal callow bees collected from a single source colony and without delay returned them to the colony (Figure S1A). On Day-4, we changed the illumination regime to 12 hours light: 12 hours dark (LD; lights on at 07:00). During the light phase, the room was illuminated with standard florescent light (500 lux) and during the dark phase, with dim red light (as above). On days 7, 8, and 9, we transferred focal bees (4 bees in each day, that were marked on days 1, 2, and 3, respectively), into individual cages, such that all 12 bees were 6—7 days of age when isolated. The cages (7.5 x 5 x 3 cm) had a glass wall that enabled video recording. Each set of four individual cages was placed in an environmental chamber illuminated with constant dim red light (DD). Into two of the four cages we introduced a small piece of bumble bee wax from nectar pots collected in the source colony and no brood (‘*Control*’). Each of the two other cages received a single live intact pupa that was collected from the same source colony (‘*Pupa+*’). The developmental stage of the pupae ranged from pre-pupa to purple-eyed pupa, as estimated by inspecting at least three neighboring pupae. On the day after focal bee introduction, we video-recorded each set of four cages using an infrared video camera (Sony DCR-TRV75E). We video recorded four 1-hour sessions; two during the subjective day (11:00 and 17:00; Group 1 was not recorded at 11:00) and two during the subjective night (23:00 and 5:00). .

We analyzed the videos using the BORIS behavior analysis software [47]. Table S1 summarizes the behaviors that we recorded in these analyses. We specifically focused on stationary bees because we were interested in developing a sleep index that can reliably distinguish sleep bouts from other stationary states in automatically collected locomotor activity data. Our own experience with honeybees [32] and previous studies in which the sleep-like behavior of honeybees and other insects was described [17–19, 23, 24] guided our identification of sleep-like behavior in bumble bees. Bees in ‘*Sleep-like*’ state had a relaxed body posture with reduced muscle tonus, their abdominal ventilation pumping was discontinuous, their antennae had an angle of $< 90^{\circ}$ between the flagellum and scape, or the antennae moved with a frequency of $\leq 20\ s^{-1}$ (recorded only when both antennae were clearly seen, see below). Bouts, in which the bee did not continuously change her position, but was clearly active and awake (but not incubating, see below) were defined as *‘Immobile-active’* bouts. ‘*Incubation-like’* behavior was identified based on the detailed description of Heinrich [14, 16] (see Video S2 and Table S1) and thermal imaging showing a clear increase in the abdominal temperature in a sample of bees performing this behavior (Figure S2). Incubating bees spread their abdomen tight on the pupa surface and wrapped their legs around it, while continuously vibrating their abdomen in high-frequency pumping movements. They occasionally moved and changed their position. Their antennae were typically extended with an angle of $> 90^{\circ}$ between the scape and flagellum, and they moved with a higher frequency compared to bees performing *‘Sleep-like’* behavior (see Results; Videos S1 and S2). Bouts in which the bee was clearly immobile but part of her body was hidden such that we could not unambiguously assign her behavior were classified as *‘Immobile-unknown’*. We used the BORIS software to record antennal movements for a subset of *‘Sleep-like’* and ‘*Incubation-like’* bouts, in which we could clearly see both antennae. ‘*Sleep-like’* bouts in which the frequency of antennal movement was $> 20\ s^{-1}$ were excluded from the analyses (10 out of 87 bouts).

One of the cages with a pupa (‘*Pupa+*’, from the 1st set of cages) was excluded from the analysis because the introduced pupa was not properly attached to the substrate, and could not be groomed or incubated by the focal bee. One broodless (‘*Control*‘) bee from the same set was excluded because she displayed frequent atypical twitching and shivering movements, which may suggest that she was not healthy. We additionally excluded the 5:00 observation of one ‘*Pupa+*’ bee in Group 3, because she was out of clear sight during most of the recording session. To confirm that the pupae were alive during the monitoring session, we kept them in an incubator after the end of video recordings, until adult emergence. We used two-sample t-test to compare the frequency of antennal movement for the two behavioral states and two-sample Kolmogorov-Smirnov test to compare bout length distribution of ‘*Immobile-active’*, *‘Sleep-like’* and ‘*Incubation-like*’ behaviors. Given that the distributions of bout length differed between these behaviors (see Results; Figure S1), we further used the non-parametric Kruskal-Wallis followed by Games-Howell post hoc tests [48] to compare bout lengths [49]. We compared the frequency of each immobile behavior between short (< 5 minutes) and long ($\geq$5 minutes) immobility bouts using χ^2 test of independence. We next compared the following indices between bees placed with or without a pupa: the proportion of sleep time, the number of sleep bouts per recording session (two-sample t tests) and sleep bout duration (Mann-Whitney test, given that sleep bout duration was not normally distributed). Finally, we compared the proportion of time spent performing each behavior between the subjective day and subjective night using paired t tests in bees with or without a pupa.

Experiment 2: The response threshold to a light pulse

We performed two trials. In both trials, we kept colonies under 12:12 LD illumination regime throughout the entire experiment (lights on at 20:00). In Trial 1, we collected 40 worker bees of unknown age from four source colonies. In Trial 2, we number-tagged and reintroduced newly-emerged workers from six colonies. We collected these bees for analyses when they were 6—7 days of age. In each trial, we placed each focal bee in an individual cage made of a modified 50 mm Petri dish that was provisioned with ad-libitum sugar syrup. We introduced a single pupa into 20 cages (‘*Pupa+*’ treatment), and in the remaining cages, placed a piece of wax (‘*Control*’ treatment). In the first trial, 8 and 13 bees from ‘*Pupa+’* and *‘Control’* treatments, respectively, died and were not included in the measurements or analyses. Each cage with a focal bee was mounted vertically in one of 40 Styrofoam cells (10 X 10 X 9 cm each) that together formed a grid. These cells were designed to minimize the leaking of light between chambers (see below). After placing all focal bees in their cells, we left them under a similar LD illumination regime to acclimate until the following morning. On the

next day, we turned off the lights (constant darkness, DD) and assessed the response threshold to light pulses during the subjective night, in which the bees are more likely to sleep. We performed three sessions in Trial 1 (ZT6:30, ZT10:30 and ZT1) and four sessions in Trial 2 (ZT1, ZT4, ZT7 and ZT9:30), each lasting 1.5—2 hours. During each session, we first used a night vision infrared camera (Panasonic HC-W850, 24 Mega-Pixels Full HD) to identify stationary bees (‘*Immobile-active’*, ‘*Incubation-like’* or ‘*Sleep-like’*). We selected bees standing with their lateral side facing toward the observer, and observed each bee for at least 1 minute before exposing her to the first light pulse. To generate the light-pulses, we used an optic glass fiber (Schott-Fostec, LLC, Elmsford, NY, USA) that produced a narrow light beam, which we directed to the bee eye. The fiber tip was placed at a distance of about 4 cm from the bee eye, and the light pulse lasted 5 s. If the bee did not respond, we increased the light intensity and delivered another pulse after approximately 15 s. We repeated this procedure until the bee responded, using 10 (Trial 1) or 12 (Trial 2) discrete light intensity levels. We recorded the lowest light intensity to which the bee responded. We defined a response as a bee starting to walk or turn her body during the interval starting when the light pulse was delivered until the next light pulse. We made sure that at least one hour spanned between repeated-measurements of the same individual bee. Records for each individual bee and behavioral state were pooled and averaged. We measured the light intensity at each level at a distance of 4 cm using LI-185A (Li-Cor, Lincoln, NE, USA) photometer. We used Kruskal-Wallis test followed by Games-Howell Post hoc tests to compare the response threshold of bees showing the three stationary states (‘*Immobile-active’*, *‘Sleep-like’* and ‘*Incubation-like*’).

Experiment 3: The influence of sleep disturbance on subsequent sleep duration

We kept three colonies under 12:12 LD illumination regime (lights on at 20:00). We collected 52 focal bees (5—7 days of age) and placed each one of them in an individual monitoring cage on one of two canvas sheets that were placed in our locomotor activity monitoring system (as described above). Under one of the canvas sheets, we attached a loudspeaker that generated pulses of 50Hz vibrations with adjustable intensity. Focal bees were randomly assigned to the ‘*Vibration*’ or ‘*Control’* sheets (n = 28 and n = 24, respectively). The cages with the bees were attached to the canvas using a piece of magnetic strip and faced up toward the cameras. The monitoring chamber was kept under 12:12 LD regime (lights on at 20:00) throughout the monitoring session. During the 4th (ZT20-24) and 5th ZT18-24) dark phases, we exposed the ‘*Vibration*’ treatment group to series of 1 min 50Hz vibrations at 5 min intervals and low intensity. On the 6th night (ZT3:30—3:50), we exposed the ‘*Vibration*’ treatment bees to 5 min intervals that included 3 min during which we applied vibrations with a higher intensity. These intervals were applied for 20 min. During this session, we entered the monitoring chamber and carefully inspected the behavior of the bees while subjected to strong vibrations. On the next dark phase (Day 7), we delivered similar 5 min intervals over a continuous 6-hour vibration session (ZT15—ZT18). After the end of this disturbance session, we continued monitoring the bees for additional three days.

For the data analysis, we first scanned the actograms and sleepograms of each individual bee and excluded from further analyses bees that did not show clear dial rhythms (n = 3 and n = 6 for ‘*Vibration*’ and ‘*Control’*, respectively) or that died (n = 3, n = 5) during the monitoring session. In order to avoid possible effects of age and time in isolation on worker sleep, we normalized the sleep data of bees subjected to the ‘*Vibration*’ treatment relative to the mean sleep of the ‘*Control*’ bees during the same period. To assess the influence of vibrations on sleep we used paired t-tests to compare normalized sleep of the ‘*Vibration*’ treated bees during the three hours before and after the beginning of the vibration disturbance sessions. We next used repeated-measures ANOVA and LSD *Post hoc* tests to compare the normalized sleep of ‘*Vibration*’ treated bees during the three days before and after the application of the vibration protocols. We performed separate analyses for the dark (night) and light (day) phases.

Experiment 4: The effect of larvae on sleep of tending workers

We paint marked all worker bees in six incipient colonies, and eight days later established 20 orphan (‘‘queenless’’) and 22 queenright cages (see Figure S1B). Into each queenless cage, we introduced two marked workers (> 8 days of age); into each queenright cage, we introduced a marked worker and a mated queen (obtained from Yad-Mordechai Pollination Services, Yad-Mordechai, Israel). We placed the bees in wooden cages with two removable glass walls (15 X 10 X 5 cm) and provisioned them with *ad libitum* pollen and sugar syrup. The cages were inspected daily for the presence of new eggcups and for monitoring brood development. When we began the experiment six days later, all the cages contained larvae. On the next two days (Day-1 and 2; Figure S1B), we introduced two additional paint marked sister callow-bees (the focal bees) into each one of the queenright and queenless cages. On Day-6, we collected the focal sister bees and placed each one individually in a monitoring cage with either a piece of wax (‘*Control*‘) or ~8 live larvae from her original cage (‘*Larvae+’*). Larvae from the queenright or queenless cages were assumed to be females or males, respectively. This experimental design produced four treatment groups differing in the type of stimulus (i.e., larvae or a piece of wax) and the social environment (i.e., queenright or queenless) they experienced before isolation. Thus, the social environment was confounded with the larvae sex (male in queenless cages and female in queenright cages; Figure S1B). The cages with the bees were then transferred to an environmental chamber, in which we monitored their locomotor activity over five consecutive days. At the end of the experiment, we inspected the larvae to confirm that they are alive. Seven of the 35 broodless control *(‘Control’)* workers laid eggs during the monitoring session. Egg-laying did not significantly affect the proportion of daily sleep (0.24 ± 0.066 and 0.34 ± 0.036, mean ± s.e.m in layers and non-layers, respectively; independent t-test, p = *0.23).* However, egg-layers had weaker circadian rhythms in locomotor activity compared to non egg-laying broodless workers (power of circadian rhythms = 52.8 ± 26 and 144 ± 25.9, p = *0.022*). Given the possible interactions between circadian rhythmicity and sleep, we excluded the egg-laying workers from following analyses. We used two-way ANOVA with larvae presence (Larvae) and previous social environment (Social environment; i.e., queenright or queenless conditions) as factors to assess the affect of treatment on the amount of sleep. We used LSD tests for *Post hoc* analyses.

Experiment 5: The effects of larvae and pupae on sleep of tending workers

We established 22 queenless cages, following the experimental procedure described for Experiment 4. We began the experiment, when all the queenless cages contained larvae (12 days after the establishment of cages), as in Experiment 2. On Day-1, we introduced into each cage three marked sister callow bees collected from seven source colonies (Figure S1C). On Day-5, we collected pupae from the same source colonies and introduced one pupa into each queenless cage. On the next day (Day-6), each focal bee was collected from its queenless cage and placed in an individual monitoring cage together with either a piece of wax ('*Control*'), ~8 larvae ('*Larvae+*') or a pupa ('*Pupa+*'), that were all collected from their original queenless cage (Figure S1C). We then monitored the locomotor activity of the isolated focal bees, as described above. At the end of the monitoring session, we confirmed that the pupae and larvae were alive, as described for Experiments 1 and 4, respectively. Cages in which the pupa did not eclose were excluded from the analyses. We used one-way ANOVA followed by LSD *Post hoc* tests to assess the influence of brood type on the proportion of daily sleep.

Experiment 6: The effects of live pupae and empty cocoons on the sleep of tending workers

Colonies were kept under DD (Trials 1 and 2) or 12:12 LD (Trial 3; lights on at 7:00) illumination regime. Focal callow bees were marked and reintroduced into their source colonies on Day-1 and −2 of the experiment (see outline in Figure S1D). On Day-7, we re-collected the focal bees and isolated each one of them in a separate monitoring cage with one of four treatments. The '*Control* 'and '*Pupa+*' treatments were similar to those described for Experiments 1 and 5. An additional treatment was an empty cocoon from which we gently removed the pupa through a longitudinal incision in the cocoon casing (termed '*Empty*'). '*Sham'* treated pupae, were subjected to a similar incision in the pupal cocoon, but the pupa was left intact. We used melted bumble bee wax to seal the incision in the cocoons of the 'Empty' and 'Sham' treatments. The focal bees in each of the three trials were collected from 5—7 different source colonies and were assigned randomly to treatment. We monitored locomotor activity over five consecutive days, and then entered the monitoring chamber and gently removed the treatment stimulus (i.e., *'Control', 'Empty', 'Sham*', or '*Pupa+*') from each one of the cages using a red flashlight. We continued monitoring locomotor activity for five more days with no treatment stimuli (Figure S1D, bottom). At the end of monitoring, we confirmed that the pupae are alive as described above. Cages with pupae from which bees did not emerge or in which the focal bee laid eggs were excluded from analyses. We inspected the cages and recorded the bees that started to build a wax pot in their cage, and compared the proportion of wax-pot building workers between the treatments using X-square test of independence. A few bees in Trials 2 and 3 (1, 2, 3, 3 and 4, 1, 0, 2 from the '*Control*', '*Empty*', '*Sham*' and *'Pupa+*' treatments in Trials 2 and 3, respectively) laid eggs during the monitoring sessions. We removed these bees from our analyses given that they showed a non-significant trend for reduced sleep (Trial 2: mean ± s.e.m = 0.078 ± 0.029 and 0.16 ± 0.031, respectively; two-samples t test p = *0.063;* Trial 3: 0.084 ± 0.032 and 0.16 ± 0.028; p = *0.11*) and significantly weaker circadian rhythms in locomotor activity in one of two trials (Trial 2: 61.6 ± 13.3 and 140.1 ± 30.4, p = *0.026;* Trial 3: 98.28 ± 30.29 and 196.89 ± 38.6, p = *0.058*). We compared the proportion of daily sleep between treatments and between the days in the presence of treatments, using two-way repeated-measures ANOVA followed by LSD *Post hoc* tests for the Treatment factor. We also compared sleep proportion on the 1st and 4th days of the monitoring session between treatments using one-way ANOVA and LSD *Post hoc* tests. Finally, we used paired t-tests to compare within each treatment, the proportion of sleep on the days before and after treatment stimuli removal.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sleep and circadian rhythms analyses

For circadian rhythms analyses we used the ClockLab circadian analyses software package (Actimetrics, IL, USA). We generated χ^2 periodograms using 10-minute bins and free running periods ranging between 20—28 hours. We used the 'Power' as a proxy for the strength of circadian rhythmicity. The power was calculated as the height of the periodogram peak above the p = 0.01 significance threshold. Bees with periodograms below the threshold line were assigned with a zero power value [20]. We estimated the sleep amount for individual bees using our custom-made algorithm SleePlotV2 (see Data and Code Availability section below), as well as the BeeSleep v.2.0 software described by Eban-Rothschild and Bloch [46]. Sleep was defined as a bout of $\geq$ 5′ of immobility based on the detailed video analyses in Experiments 1 and 2 (see Results).

Other statistical analyses

The statistical analyses used for each experiment, including the reasoning for sample exclusions, are described with detail in the different chapters of the Method Details section to facilitate reading. All statistical analyses were performed using MATLAB R2017a or SPSS (IBM SPSS Statistics V21.0). Significance threshold for all analyses (except for determining the Power of circadian rhythms, see above) was set to α = 0.05. We detailed the statistical tests used, *P values* and the results of *Post hoc* comparisons in each figure either in the Main text or in the Supplementary Information. For analyses that were not included in the figures, we presented the means, sample sizes (number of individuals- n), standard errors (s.e.m), statistical tests used, and *P values* in the main text.

DATA AND CODE AVAILABILITY

The MATLAB code used for sleep analysis termed SleepPlotV2 is available at the Guy Bloch Group website: https://guybloch.huji.ac.il/.

Current Biology, Volume *29*

Supplemental Information

Bumble Bee Workers Give Up Sleep to Care for Offspring that Are Not Their Own

Moshe Nagari, Ariel Gera, Sara Jonsson, and Guy Bloch

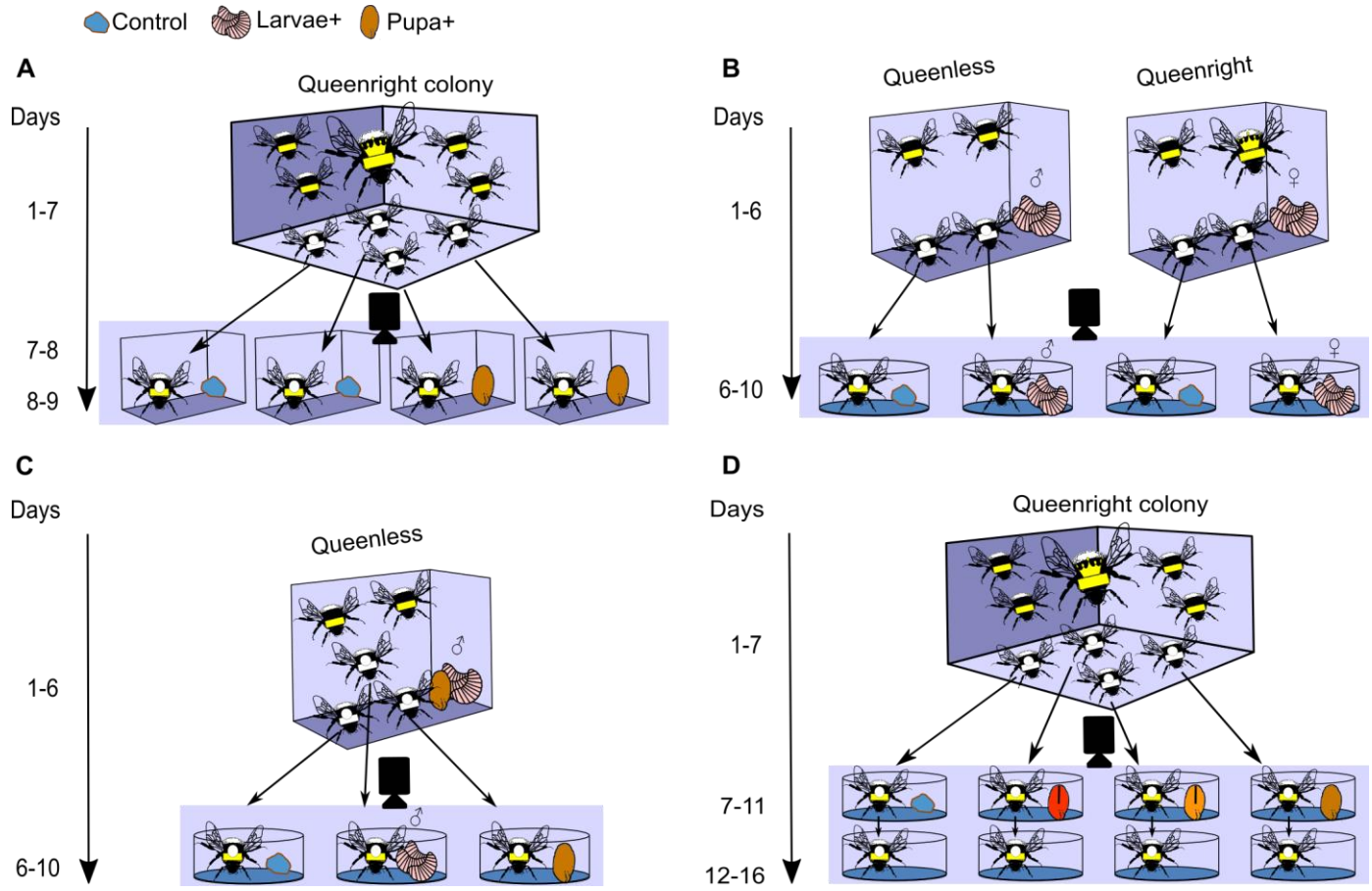

Figure S1. Experimental setups for experiments 1 and 4—6. Related to STAR methods. (**A**) Experiment 1. Paint marked sister callow bees were introduced into queenright colonies on Day-1 (top panel). On Day-3, we switched the illumination regime to 12h Light: 12h Dark (LD) illumination regime. On Day-7, we transferred four bees into individual cages with either a piece of wax ('*Control*') or a single live pupa ('*Pupa+*') and placed the cages under constant darkness (DD). We then video recorded the four bees on Days 8—9 for two hours during the subjective day (starting at 11:00 and starting at 17:00) and two during the subjective night (23:00 and 5:00). We repeated this procedure with three sets of four bees each. All 12 bees were 7—8 days of age at the beginning of video recordings. (**B**) Experiment 4. Pairs of sister callow bees (white thorax color and a white dot) were introduced into queenless (top left) or queenright (top right) 'mini colonies" on Day-1 or 2 and kept in constant darkness and temperature. On Day-6 (bottom panels), each focal bee was isolated individually with either a piece of wax ('*Control*') or with ~8 male or female larvae ('*Larvae+*') from her queenless or queenright cage, respectively. (**C**) Experiment 5. Trios of sister callow worker bees were introduced into queenless "mini colonies" on Day-1 or 2. On Day-5, a pupa was introduced into each cage. On Day-6, each focal bee was individually isolated with a '*Control*', a 'Larvae+' or a single live pupa ('*Pupa+*') from her source cage. **(D)** Experiment 6. Paint marked sister callow bees were introduced into queenright colonies on Day-1 or 2 (top panel). On Day-7, each bee was transferred into an individual cage with one of the following treatments (middle panel): '*Control*', an empty cocoon from which the pupa was removed ('*Empty*'), a sham treated cocoon containing a live pupa ('*Sham*'), or *'Pupa+*'. After five days of monitoring, the treatment stimuli were removed and activity was monitored for additional 5 days (bottom panel). Timeline for each experiment is shown to the left.

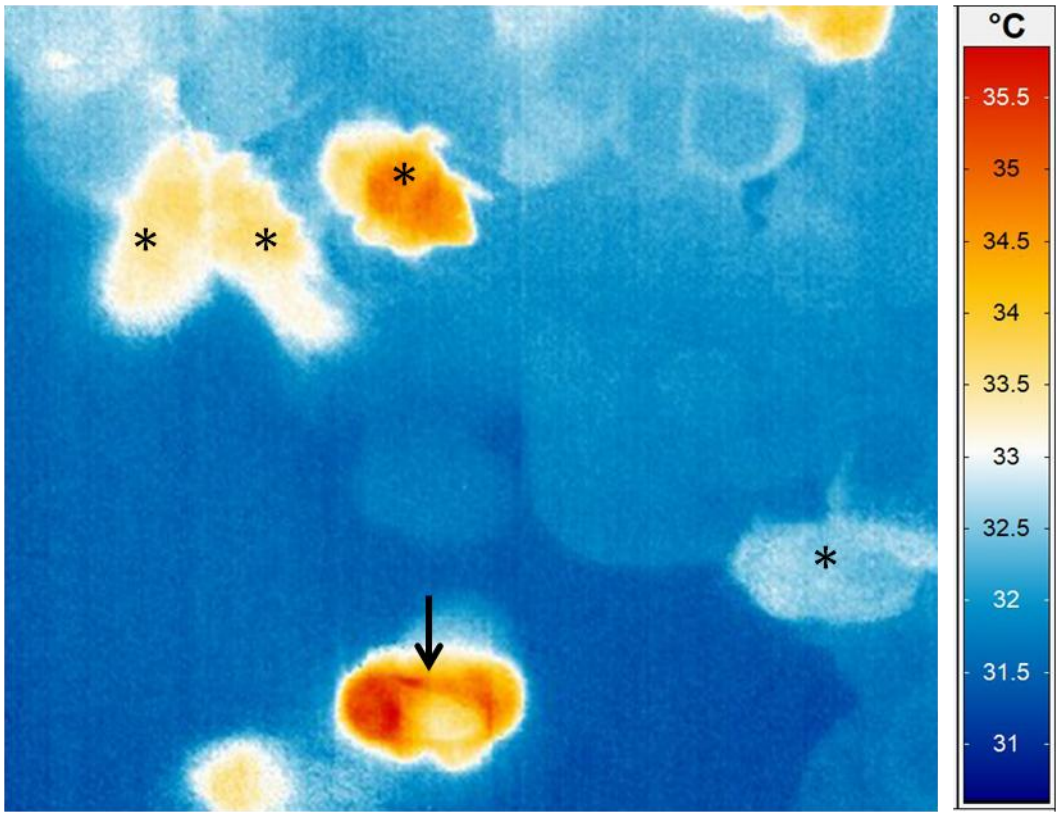

Figure S2. Thermal image of a bumble bee colony. Related to Figure 1. The bee that is marked with an arrow is incubating a pupa, and both her thorax (left part) and abdomen (right part; see the lighter colored wings facing downward) are relatively hot (indicated by the warm colors). The bees that are marked with asterisks are performing other behaviors and their abdomens or whole bodies are colder (cold colors). The scale on the right is relative and not absolute because we did not correct for the transmissivity of the plastic wrap through which the colony was filmed. The image was taken with a thermographic camera (InfraTec Infrared LLC; Plano, Texas, USA). Photo credit, Barrett Klein.

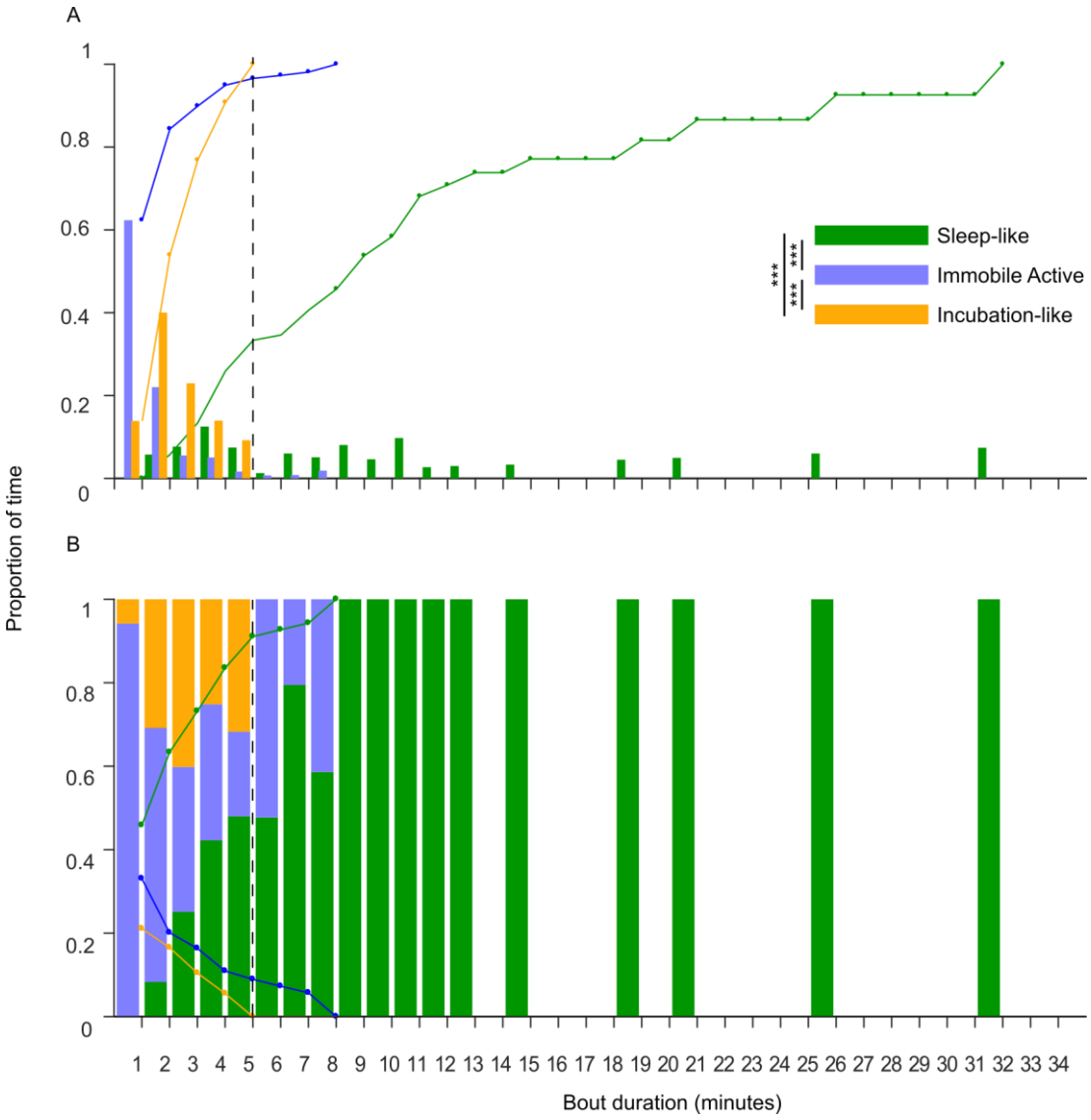

Figure S3. Bout duration for immobile bees showing different behaviors. Related to Figure 1. The color legend is shown in panel **A**, and the behaviors' descriptions are provided in the text. (**A**) The frequency distribution (proportion of time) of bout duration shown by immobile bees performing different behaviors. The colored lines show the cumulative proportion of time. The asterisks next to the legend summarize the results of two-sample Kolmogorov-Smirnov test (***- $p < 0.001$). (**B**) The proportion of time relative to other stationary behaviors for a given bout duration. The lines show the accumulative proportions (≥). The vertical dashed black lines in (**A**) and (**B**) outline the 5' of immobility bout-length threshold.

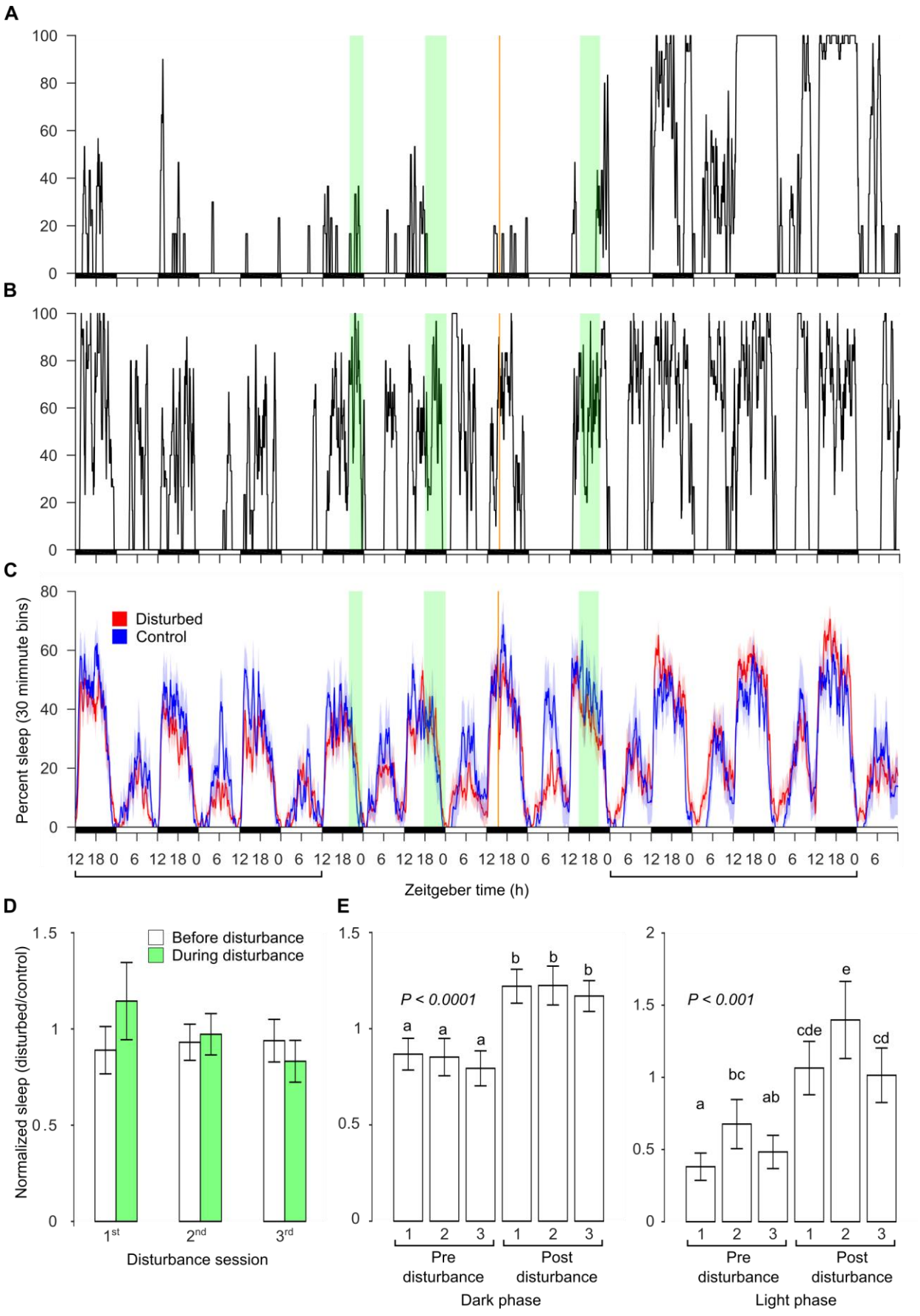
A
B
C
Disturbed
Control
Percent sleep (30 mimnute bins)
Zeitgeber time (h)
D
Before disturbance
During disturbance
Normalized sleep (disturbed/control)
Disturbance session
1st
2nd
3rd
E
P < 0.0001
P < 0.001
Pre disturbance
Post disturbance
Dark phase
Light phase

Figure S4. Bumble bee workers disturbed by means of substrate-borne vibrations show an increase in sleep amount during the following days. **Related to Figure 1**. (**A, B)** Individual variation in the response of bumble bee workers to substrate vibrations. The plots show the percent of sleep using 30-minute sliding window, for 2 representative individual bees that were subjected to substrate vibrations ('*Vibration'* treatment). The blank and filled bars at the bottom show the 12h:12h light: dark phases of the illumination regime, respectively. Green shadings outline the vibration sessions. The vertical orange line on the 6th night shows the time when experimenters entered the room to observe bees while experiencing strong vibrations. (**A**) A bee showing reduced sleep during the 2nd and 3rd vibration sessions and an increase in sleep amount on subsequent days. (**B**) A bee that did not show reduced sleep during the vibration sessions but showed an increase in sleep amount on subsequent days. (**C**) A summary of the percent of sleep (means ± s.e.m) in bees subjected to substrate vibrations ('*Vibrations*', red, n = 25), and control bees ('*Control*', blue, n = 14). The horizontal brackets below the X-axis depict the days used for the analyses shown in (**E**)**.** All other details as in (**A, B)**. (**D**) The amount of sleep shown by bees experiencing vibrations normalized relative to the mean sleep of '*Control'* bees, during 3 hours before (white) and 3 hours after (green) the onset of each vibration session (paired t-tests: *P = 0.07, P = 0.54* and *P = 0.27,* for the 1st, 2nd, and 3rd session, respectively). (**E**) The normalized amount of sleep of bees subjected to vibrations during the three dark phases (left panel) and light phases (right panel) before and after the three disturbance sessions. The bars show the means ± s.e.m. *P-values* were obtained from repeated measures ANOVA followed by LSD *Post-Hoc* tests.

Behavior	Description
On pupa/wax	The bee is on the pupa or piece of wax for ≥ 5 consecutive seconds.
Mobile active	The bee changes position for ≥ 5 consecutive seconds.
Immobile active*	The bee does not change her position for ≥ 5 consecutive seconds. Antennae are raised at an angle of > 90° between scape and flagellum. Antennae or head move at a frequency ≥ 20 sec^{-1}. Abdomen shows continuous pumping movements.
Immobile unknown*	The bee does not change its position for ≥ 5 consecutive seconds, but behavior cannot be unmistakably determined because she is partially hidden.
Sleep-like*	The bee does not change her position for ≥ 5 consecutive seconds. Antennae form an angle of ≤ 90° between frons and scape. Antennae move at a frequency of < 20 sec^{-1}. Abdominal pumping is discontinuous. Relaxed body posture.
Incubation-like*	The bee does not change her position for ≥ 5 consecutive seconds. The abdomen is extended over the pupa, and vibrates with vigorous pumping movements. The bee's legs are wrapped around the pupa. The antennae are raised at an angle of > 90° between falgellum and scape and are constantly moving. Head and antennae do not touch the substrate.
Total activity	All the activities other than sleep and Immobile unknown summed together.

* Behaviors in which the bee is stationary

Table S1. Behavioral categories used to classify behaviors in video records in Experiment 1. Related to STAR methods.