1   **Colony volatiles and substrate-borne vibrations entrain circadian rhythms and**

2   **are potential mediators of social synchronization in honey bee colonies**

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18   honeybees

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21   non-photic entrainment, self-organization, vibrations, olfactory signals,

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Abstract

Internal circadian clocks organize animal behavior and physiology and are entrained by ecologically-relevant external time-givers such as light and temperature cycles. In the highly social honey bee, social time-givers are important and can override photic entrainment, but the cues mediating social synchronization are unknown. Here we tested whether substrate-borne vibrations and hive volatiles can mediate social synchronization in honey bees. We first placed newly-emerged worker bees on the same or on a different substrate on which we placed cages with foragers entrained to ambient day- night cycles, while minimizing transfer of volatiles between cages. In the second experiment, we exposed young bees to constant airflow coming from either a free-foraging colony or a similar size control hive containing only empty combs, while minimizing transfer of substrate-borne vibrations between cages. After five days, we individually isolated each focal bee in an individual cage in an environmental chamber, and monitored locomotor activity. We repeated each experiment five times, each trail with bees from a different source colony, monitoring a total of more than 1000 bees representing diverse genotypes. We found that bees placed on the same substrate as foragers showed a stronger phase coherence; and in 3 of 5 trials their phase was more similar to that of foragers, compared to bees placed on a different substrate. In the second experiment, bees exposed to air from a colony showed a stronger phase coherence, and in 4 out of 5 trial their phase was more similar to that of foragers, compared to control bees exposed to air from an empty hive. These findings lend credence to the hypothesis that surrogates of activity such as substrate-borne vibrations, and volatile cues entrain circadian rhythms in natural free-foraging honey bee colonies.

Introduction

52   Circadian clocks are endogenous pacemakers generating rhythms of about 24 hrs

53   that organize animal physiology and behavior. The ubiquity of circadian rhythms in

54   animals is consistent with the notion that circadian clocks are functionally significant

55   because they allow animals to adjust their physiology and behavior to predicted

56   changes in their environment (Dunlap et al., 2004; Yerushalmi and Green, 2009; Helm

57   et al., 2017; Kumar, 2017). These internal clocks are entrained by ecologically-relevant

58   environmental cues (known as "Zeitgebers" or "time-givers") that are detected by

59   sensory systems, converted to neuronal information, and transmitted to set the phase

60   of central pacemakers. Output pathways carry temporal signals from the entrained

61   clock to various downstream processes (Dunlap et al., 2004).

62   Most studies on the entrainment of circadian clocks have focused on photic

63   entrainment which is considered evolutionary ancient and the most important time-

64   giver to circadian clocks. In addition to light, the sun also produces daily fluctuations in

65   ambient temperature that can effectively entrain the circadian clocks of many

66   organisms (Sharma and Chandrashekaran, 2005; Albrecht, 2012). Non-photic, non-

67   thermal, time-givers have received little attention. However, recent studies have

68   highlighted the importance of entrainment by feeding time that may act on clock in

69   tissues other the central light entrained clock (e.g., the SCN in mammals; Mendoza,

70   2007).

71   A somewhat more controversial line of research relates to the importance of social

72   entrainment. Although observations consistent with entrainment by social interactions

73   have been reported for diverse animal species, there is no clear relationship between

75   the level of sociality and the efficacy of social entrainment (reviewed in Favreau et al.,

76   2009; Castillo-Ruiz et al., 2012; Eban-Rothschild and Bloch, 2012b; Bloch et al., 2013).

77   Some social animals such as Mongolian gerbils, sugar gliders, and common

78   marmosets could not be effectively entrained by even intensive social interactions such

79   as contacts with receptive females or aggressive opponents. On the other hand, social

80   influences on activity rhythms have been reported for species which are not considered

81   social, such as the fruit fly *Drosophila melanogaster* (Levine et al., 2002; Lone and

82   Sharma, 2011; Bloch et al., 2013; Castillo-Ruiz et al., 2012). An additional difficulty is

83   that no neuronal or molecular pathway transmitting social stimuli to the core clock

84   system has been described to date. Rather, it has been suggested that social

85   influences on the mammalian clock are mediated by changes in arousal state, by

86   learning processes associating circadian time with specific actions, or by social

87   gating of the time or pattern of exposure to photic time-givers (Reebs, 1989; Amir

88   and Stewart, 1996; Mistlberger et al., 2004). The best evidence for social

89   entrainment is found in cavity-dwelling social animals such as bats, ants, and bees

90   in which at least some individuals do not experience ambient conditions directly, but

91   rather rely on information received from group mates that forage outside (Regal and

92   Connolly, 1980; Favreau et al., 2009; Eban-Rothschild and Bloch 2012b; Bloch et

93   al., 2013; Mildner and Roces, 2017). In social insects it is thought that social

94   synchronization of activity time is essential for temporal coordination and efficient

95   colony-level performance. For example, honey bee nectar receivers and foragers need

96   to act in a concerted manner and this can be achieved by means of social

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97   synchronization (reviewed in Sharma, 2003; Bloch et al. 2013; Eban-Rothschild and

98   Bloch, 2012b).

99   We study the highly social ("eusocial") honey bee (*Apis mellifera*), that has been

100   relatively well-studied both in terms of social behavior and circadian rhythms. The

101   organization of work in honey bee colonies relates to the age of worker bees. Young

102   workers typically stay in the constantly dark and tightly thermoregulated cavity of the

103   hive in which they typically care for ("nurse") the brood. Older workers typically forage

104   outside the hive for food and other resources (reviewed in Robinson, 1992). Foragers

105   are diurnal and show a sleep-like behavior at night, whereas nurses typically tend

106   brood around-the-clock (Eban-Rothschild and Bloch, 2012a; Bloch, 2010). Although

107   around-the-clock active nurses typically do not show overt circadian rhythms in

108   locomotor activity or whole brain clock-gene transcript abundance, there is evidence

109   that they do have functional and entrainable circadian clocks. Firstly, nurses that are

110   removed from the hive and isolated individually in a cage, rapidly switch to activity and

111   brain gene expression with robust circadian rhythms that are in phase with ambient

112   day-night cycles (Shemesh et. al., 2007; 2010). Secondly, the expression levels of

113   more than 150 brain transcripts vary with a 24 h cycle (Rodriguez-Zas et al., 2012).

114   Thirdly, recent immunocytochemical studies show that the levels of the clock protein

115   *amPeriod* and the circadian neuropeptide *Pigment Dispersal Factor* (PDF) cycle with

116   a similar phase and amplitude in brain circadian neurons of foragers that show, and

117   nurses that do not show, circadian rhythms in locomotor activity (Fuchikawa et al.,

118   2017; Beer et al., 2018). Here we ask what are the time-givers that entrain the clocks

119   of nurses that dwell inside the constantly dark and tightly thermoregulated hive?

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120   There is good evidence that the clocks of nurses are entrained by social time givers

121   (reviewed in Bloch, 2010; Eban-Rothschild and Bloch, 2012b; Bloch et al., 2013).

122   Moreover, Fuchikawa et al., (2016) showed that nest bees as young as two days of

123   age can be socially entrained to the colony cycle. Entrainment was effective for

124   caged bees deprived of access to the hive entrance and direct contact with other

125   individuals in the colony. Thus, social entrainment cannot be explained by gating the

126   time of exposure to light. Remarkably, social entrainment was potent enough to

127   override photic entrainment, because forager activity stably entrained the clock even

128   for nest bees confined to the inner dark nest cavity and experiencing conflicting

129   photic and social environmental cycles. This evidence for the power of social

130   entrainment highlights the need to know what are the signals and interactions

131   mediating social entrainment in honey bees.

132   We hypothesize that surrogates of forager activity, such as humidity, CO_2 levels,

133   volatile pheromones or comb vibrations, act to synchronize circadian rhythms of

134   individual bees and entrain the colony to a common phase (Bloch et al., 2013). Here

135   we focused on two important surrogates of bee activity, substrate-borne vibrations and

136   hive volatiles. Honey bees, as well as other social insects, use vibrations for

137   intracolony communication (reviewed Hunt and Richard, 2013). Volatile pheromones

138   and other hive odours are also well established for their pivotal and diverse

139   communication functions in insect colonies (Wilson, 1971; Conte and Hefetz, 2008;

140   Leonhardt et al., 2016). The hypothesis that chemical cues affect social

141   synchronization was supported by a study showing that allowing airflow between small

142   groups of bees facilitates their synchronization to a common circadian phase (Moritz

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143   and Kryger, 1994). There is also evidence that hive CO_2 -levels vary over the day

144   (Ohashi et al., 2009; Murphy et al., 2015; Edward-Murphy et al., 2016), and in other

145   insects there is evidence consistent with the premise that CO_2 can entrain circadian

146   rhythms (Nicolas and Sillans,1989).

147   To test the hypothesis that substrate-borne vibrations generated by forager activity

148   synchronize circadian rhythms in locomotor activity we analyzed the circadian phase

149   of newly-emerged bees placed on the same or on different substrate as foragers. To

150   test the second hypothesis that volatile cues from a free-foraging colony can mediate

151   social synchronization, we exposed newly-emerged bees to a constant air flow coming

152   out from either a hive housing a free foraging colony or a control empty hive. Our

153   findings show that both vibrations generated by foragers activity and volatile cues from

154   a free-foraging colony can entrain circadian rhythms in locomotor activity in young

155   worker bees. These findings lend credence to the hypothesis that surrogates of forager

156   activity mediate social synchronization in honey bees.

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159   **Material and Methods**

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161   Bees

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163   Honey bees were maintained according to standard beekeeping techniques at the

164   Bee Research Facility at the Edmond J. Safra campus of the Hebrew University of

165   Jerusalem, Givat-Ram, Jerusalem, Israel. The honey bees in our apiary are derived

166   from a mixture of subspecies typical to Israel. To obtain newly-emerged bees, we

167   removed honeycomb frames with emerging worker pupae, brushed-off all adult bees,

168   and immediately transferred each frame into a separate lightproof container. We

169   placed the frames in an incubator (33ºC ±1 ºC, 60%RH ±5%) for the bees to emerge.

170   The emerging bees were collected from the comb within two hours post emergence

171   under dim red light (DD; using Edison Federal EFEE 1AE1 Deep Red LED; mean

172   wavelength = 660 nm, maximum and minimum wavelengths = 670 and 650,

173   respectively) light to avoid influences of light on the circadian system (e.g., photic

174   entrainment).

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176   General procedure

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178   Fig. 1A summarizes the general experimental outline. On Day-1, we collected

179   newly-emerged worker bees and transferred them to Libfield wooden cages (12 x 8 x

180   4.5cm) with glass covers in which they were exposed to the tested environmental cues.

181   On Day-6, we collected samples of bees experiencing the various treatments, as well

182   as foragers that served as a control group. We isolated each focal bee in a monitoring

183   cage and monitored locomotor activity for at least seven successive days under dim

184   red light and constant lab conditions (Fig. 1A). We used the locomotor activity data to

185   determine the circadian phase of each bee and assess social synchronization among

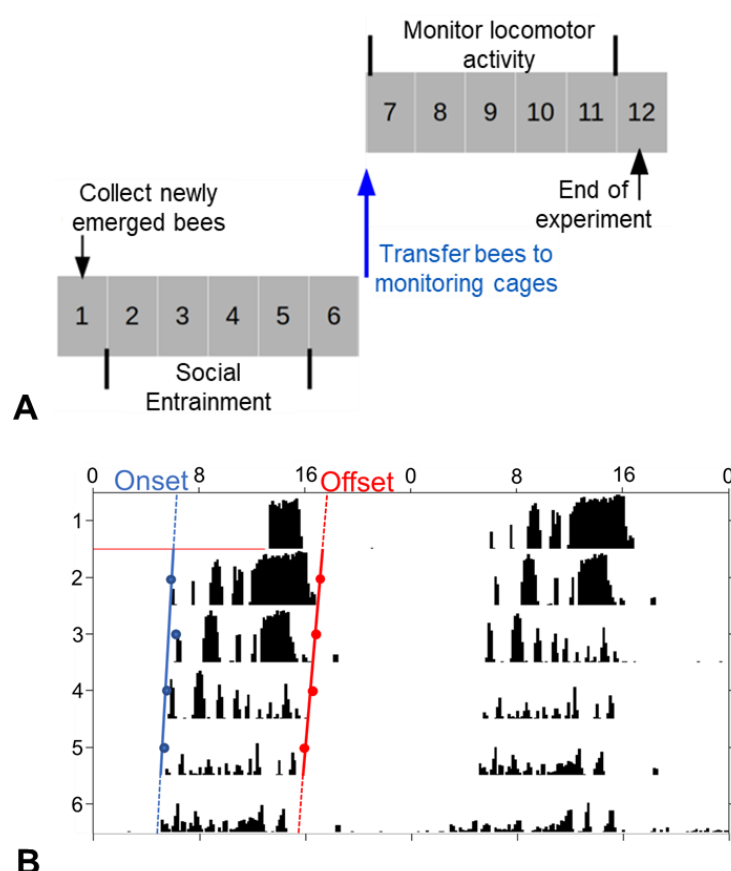186   bees subjected to the same treatment (e.g., colony odors or vibrations).

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Figure 1. (A) General experimental outline. The numbers depict the days of the experiment and the grey filling indicate that the bees were kept under dim red light. On Day-1 (1), we collected the bees and transferred them to cages in the lab, in which they were exposed to the tested signals. On Day-6, each focal bee was collected and transferred to an individual monitoring cage in which her locomotor activity was automatically monitored for at least seven successive days. **(B) Representative double-plotted actogram of an individually isolated bee.** The y-axis shows days in the monitoring cage, and the X-axis the time of day, double plotted for easier visual detection of rhythms. The height of the black bars within each day corresponds to the level of locomotor activity in a 10 min. bin, determined by distance moved in pixels (translated to millimeters) [mm] in 1-min. The blue and red dots show the estimated

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200   times for the onset and offset of activity for each day, respectively. Linear regression

201   models are fitted to these points and the phase is determined based on the

202   extrapolation of the regression lines on Day-1.

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204   Monitoring and analyzing locomotor activity

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206   We placed each bee individually in a monitoring cage made of a modified Petri dish

207   (diameter = 90mm) provisioned with *ad libitum* sugar syrup (50% w/w) and pollen. The

208   monitoring cages with the bees were placed in a tightly regulated environmental

209   chamber (29 ±°1C, 55 ±5% RH). The chamber was illuminated with dim red light

210   (Edison Federal EFEF 1AE1 Far (Cherry) Red LED; mean wavelength = 740 nm,

211   maximum and minimum wavelengths were 750 and 730, respectively). Locomotor

212   activity (measured as number of pixels traveled over a time unite on the camera field

213   of view and transformed to millimeters) was recorded automatically at a frequency of

214   1 Hz with the ClockLab data acquisition system (Actimetrics Inc., Evanston, IL, USA).

215   Our monitoring system is composed of four infra-red light-sensitive black and white

216   cameras (Panasonic WV-BP334, 0.08 lux CCD video cameras or Sentech STC-

217   MB33USB mini USB video cameras with Computer TZ32910CS-IR lenses) and a high-

218   quality monochrome image acquisition board (IMAQ 1409, National Instruments) or a

219   National Instruments USB-6501 interface. Each camera records the activity of 30

220   cages placed on a single tray. Thus, in each trial we could monitor up to 116 bees;

221   additional 4 empty cages, one on each tray, were used as a control recording

222   background noise.

223   For the analyses of circadian rhythms, we used the ClockLab circadian analyses

224   software package (Actimetrics, USA). We used the χ^2 periodogram analysis with 10-

225   minute bins to generate actograms for the analyses of circadian rhythms. As a proxy

226   for the strength of circadian rhythms we used the *Power* which was calculated as the

227   height of the periodogram peak above the P = 0.01 significance threshold line (for more

228   details see Yerushalmi et al., 2006). For each day we calculated the onset and offset

229   of the daily bout of activity. The precise timing of the onset or offset were defined as at

230   least three consecutive 10-min bins each with activity reaching at least 10% of the

231   maximal activity per bin during this day. In addition, there was a period of at least 5 hrs

232   of reduced activity between the offset and the following onset (as described in

233   Fuchikawa et al., 2016). We used the ClockLab software to fit linear regression models

234   passing through the onset or the offset points of at least four consecutive days. The

235   extrapolations of these regression lines on the first day in which the bees were

236   transferred to the monitoring system were used for estimating the timing of onset and

237   offset of activity (Fig. 1B). We included in our analyses only bees with statistically

238   significant circadian rhythms (χ^2 periodogram analysis; P < 0.01, period between 20-

239   28 hrs.) for which we could unambiguously determine the onset/offset of activity.

240   We used the Oriana circular statistics software package (KCS, USA) to determine

241   the degree of synchronization and the phase coherence among bees within each

242   treatment group. We used the time of onset, offset, and median (which was defined as

243   the midpoint between the times of onset and offset) as indices for the phase. The

244   Rayleigh test was used to determine if phase synchronization is significantly different

245   from random distribution, and the mean length of the Rayleigh vector was used as an

246   index for phase coherence. We used the Watson Williams F-test to compare the

247   phases of two groups.

248

249   **The influence of substrate-borne vibrations on social synchronization of**

250   **circadian rhythms in locomotor activity**

251   This experiment was designed to examine the hypothesis that substrate-borne

252   vibrations can mediate social synchronization among honey bee workers. The

253   experimental design is summarized in Fig. 2. On the first day, we caged groups of ~30

254   callow (i.e., newly emerged) bees or foragers in Libfield wooden cages (12 x 8 x 4.5cm)

255   with glass covers, provisioned each cage with ad libitum sugar syrup (50% w/w and

256   pollen, collected by honey bees). Foragers were collected at the hive entrance and

257   identified as bees returning to the hive with pollen loads conspicuously attached to

258   their hind legs. They were collected on the same day and from the same source colony

259   as the callow bees. The foragers were then entrained to a light-dark cycle that was 5

260   hours advanced relative to the phase of the colony in the first trial, and to the natural

261   day-night cycle in all other trials. We placed the cages containing groups of callow bees

262   either on the same substrate (a canvas frame placed on a table) as the foragers

263   ("*Same Substrate"*) or on a different substrate (*"Different Substrate"*). In each trial we

264   used two cages for each treatment (see Fig. 2). We placed all the cages in the same

265   environmental chamber (30 °C ±1°C, 55% RH ±5%, illuminated with dim red Edison

266   Federal EFEE 1AE1 Deep Red LED lights, mean wavelength = 660 nm, maximum and

267   minimum wavelengths = 670 and 650, respectively). The distance between the cages

268   of foragers to those with callows on the *Same Substrate-* and *Different Substrate-* was

269   identical (Figure 2). We sucked out volatiles (that may mediate social entrainment)

270   using an exhaust air unit with its opeining (diameter 10cm) placed above the foragers

271   cages. We repeated this experiment five times between May to August 2017, each trail

272   with bees from a different source colony. The survival rate was 45% (range 30% - 70%)

273   for foragers and 87% (range 61% - 100%) for young bees. Such differences in survival

274   are common because foragers are older and foraging activity is a physically

275   demanding activity.

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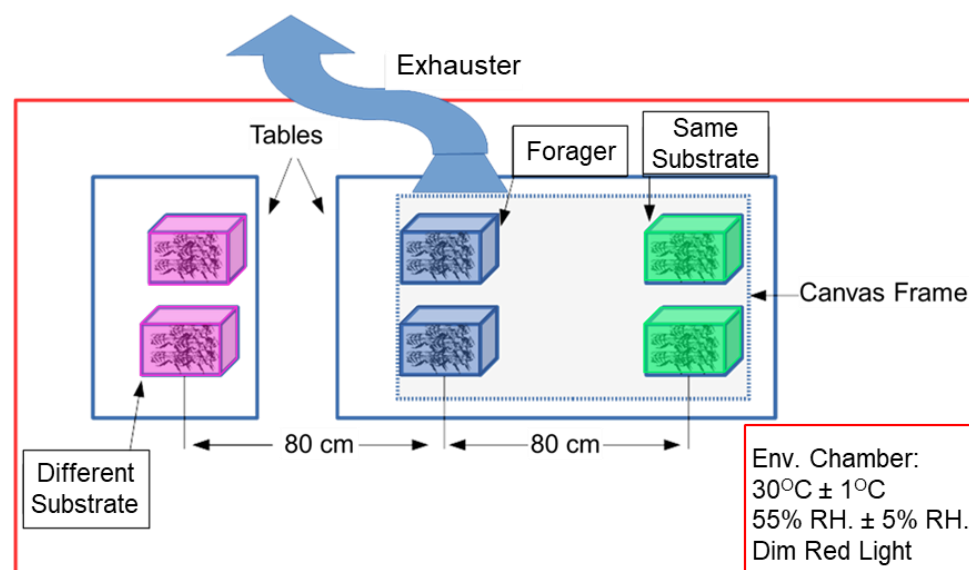
278   **Figure 2: Setup for the experiment testing whether substrate-borne vibrations**

279   **entrain circadian rhythms in locomotor activity.** We placed newly emerged bees in

280   two small cages (30 bees/cage) on the same (*'Same Substrate'*) or on a different

281   substrate (*'Different Substrate'*) on which we placed two cages with foragers. We

282   sucked out the air above the foragers to prevent the spread of forager emitted odorants

283   that could influence circadian rhythms of young bees.

284

The influence of colony volatiles on social synchronization of circadian rhythms in locomotor activity

To test the hypothesis that volatile chemicals mediate social synchronization of honey bee workers, we exposed callow bees to volatiles pulled out from a regular hive housing a free-foraging colony ("*Colony*"), or from a control empty hive (*"Empty Hive"*). The empty hive was of a similar size and contained the same number of empty combs. To keep the temperature in the *Empty Hive* constant at a similar level as in the *Colony*, we heated it with a "Reptile-heating mat" (20W- 16.5x11 in). Both hives were prepared with a similar hole in the back board where an exhauster tube (diameter 25mm) was installed (Figure 3).

We pumped the air out of the hive boxes using two aquarium pumps (Atman, Aquarium Air Pump AS-1063). The air from each hive was flowed into two Libfield cages, each containing 30 callow bees (Fig. 3). The callow bees in these cages were provisioned with ad libitum sugar-syrup (50% w/w) and pollen. We placed the cages on vibration-absorbing bases to minimize possible transfer of vibrations. We placed a tube (diameter 10cm) from the exhaust-air unit above the cages of the *Colony-group* in order to prevent unintended transfer of volatiles to the Control cages. The pump and the cages were housed inside a tightly regulated environmental chamber (30 ±1°C, 55 ±5% RH) constantly illuminated with dim red light (as in the first experiment above). The populated and empty hives were placed outside the building. The colony in the populated hive was self-sustained (Fig. 3). We repeated this experiment five times between September to November 2017, each trail with bees from a different source

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307   colony. The average survival rate was 79% for foragers (range 70 - 100%) and 67%

308   for the young bees (51 - 84%).
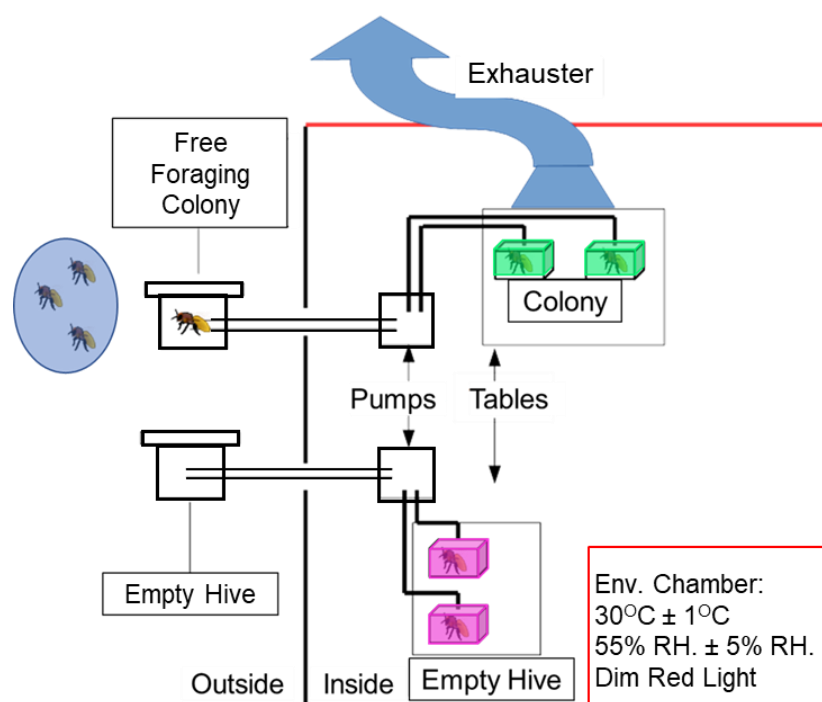
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311   **Figure 3. Setup for the experiment testing whether hive volatiles entrain**

312   **circadian rhythms in locomotor activity.** Cages, each with 30 callow bees, were

313   exposed to a constant air flow sucked out from either a hive housing a free-foraging

314   colony (*'Colony'*) or a similar control empty hive containing the same number of empty

315   combs (*'Empty Hive'*). We placed all the cages on vibration-absorbing bases to

316   minimize possible transfer of vibrations. We sucked the air above the *Colony-group* to

317   prevent unintended release of volatiles that could affect the behavior of other bees.

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Results

The influence of substrate-borne vibrations on social synchronization of circadian rhythms in locomotor activity

We repeated this experiment five times, each trail with bees from a different source colony, and altogether monitored 454 bees. The experimental groups differed in the strength of circadian rhythms. The foragers had stronger rhythms (Power = 222.76 ±160.09 SD) compared to the two groups of young bees, that were overall similar ("*Same Substrate":* 128.89 ±119.81, and *"Different Substrate":* 122.52 ±115.04). The proportion of bees showing statistically significant circadian rhythms was 91% (range 70-100%) for *Foragers,* 71% (range 58-85%) for young bees on the *Same Substrate,* and 68% (range 50-70%) for the *Different Substrate-group* (data not shown).

The phase of *Same Substrate* bees was overall more similar to that of the foragers compared to that of the *Different Substrate* treatment. In 4 out of 5 trails, the phase of the *Different Substrate-group* was significantly different from that of the foragers (Watson Williams F test < 0.05; Fig. 4A). The young bees on the same substrate also showed stronger phase coherence (longer Rayleigh vectors) compared to those placed on a substrate different from that of the foragers (Fig. 4B; Kruskal-Wallis test p = 0.007; Steel-Dwass-Critchlow-Fligner *post hoc* test: *Forager – Same Substrate* p = 0.417; *Forager - Different Substrate, p* = 0.024; *Same Substrate – Different Substrate* = 0.116). We further compared the phase-difference (differences in minutes between the mean vectors) relative to the foragers. In all five trials, the phase difference

343   between the *Foragers-* and the *Different Substrate-group* was larger than compared to

344   the *Same Substrate-group* (Binomial test, n = 5, p = 0.031) (Fig. 4C).

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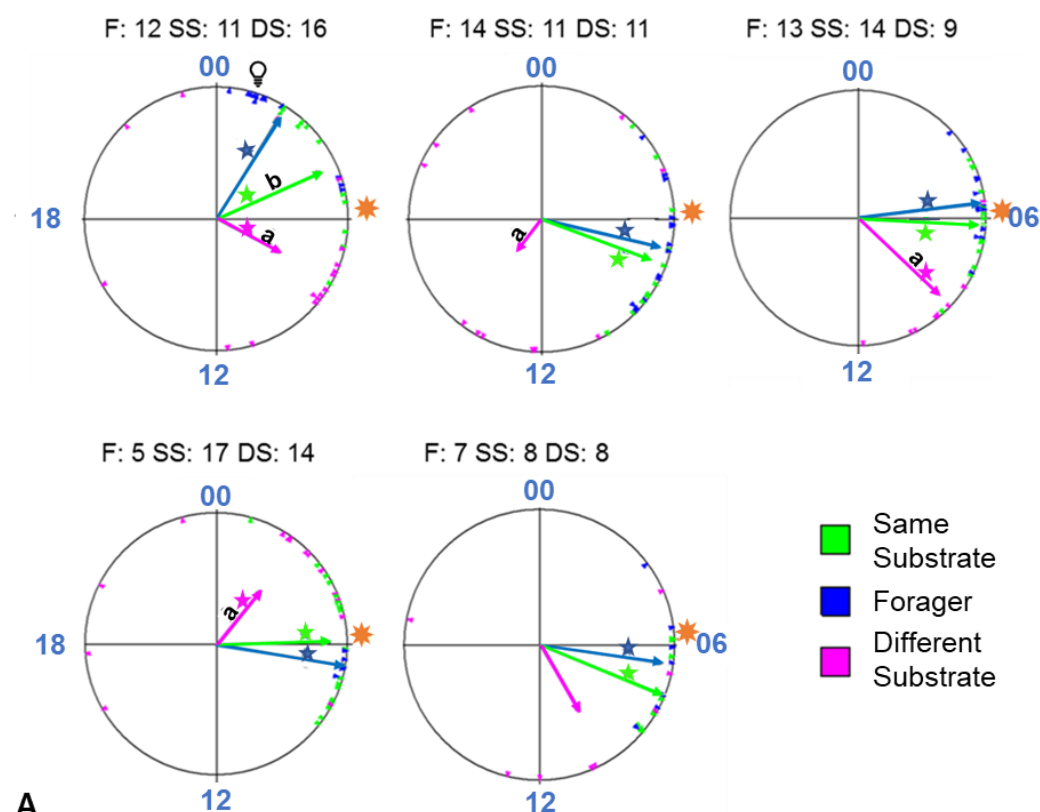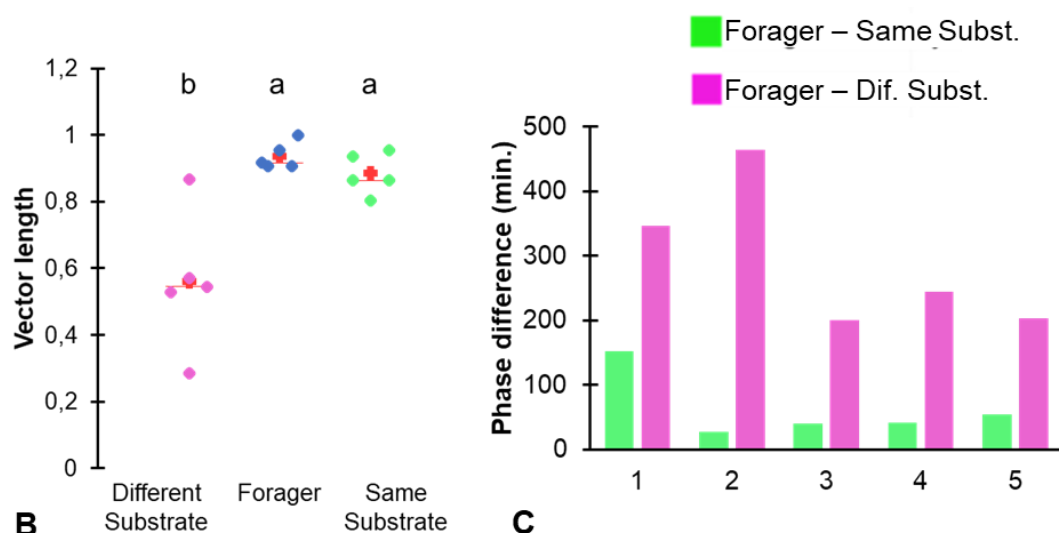
347 **Figure 4. Substrate-borne vibrations entrain circadian rhythms in honey bee**

348 **workers. (A)** Circular plots comparing the phases of bees from the different treatment

349 groups. Each plot summarizes a different trial. Sample sizes (number of bees with a

350 clear onset) are shown above each plot. F= foragers; SS = callows on the same

351 substrate; DS = callows on a different substrate. The time of day is depicted on the

352 circular plot perimeter. Each triangle depicts the onset of an individual bee. The vectors

353 point to the average onset time, and their length corresponds to the degree of phase

354 coherence. Asterisks indicate the p-value obtained from Rayleigh test is significant (*-

355 α < 0.05). Vectors marked with different letters differ in phase in a Watson-Williams F-

356 test. The foragers were entrained to a phase 5 hours advanced (illustrated by the light

357 bulb symbol) relative to the phase of the colony (illustrated by the sun symbol) in the

358 first trial, and to the natural sunrise in all other trials (symbol). **(B)** A Scatter plot

359 summary of the degree of synchronization (length of the Rayleigh vector) across the

360 different treatments. The red crosses correspond to the means and the central

361 horizontal bars to the medians.   Treatments marked with different letters are

362 significantly different in a Kruskal-Wallis Test (P<0.05) followed by Steel-Dwass-

363 Critchlow-Fligner *post hoc* test. **(C)** The phase-difference between each of the two

364 groups of young bees relative to the foragers.

365

366 **The influence of hive volatiles on social synchronization of circadian rhythms**

367 **in locomotor activity**

368 We performed five repetitions of this experiment, each with bees from a different

369 source colony. In total, we monitored the locomotor activity of 580 bees. As in the first

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370 experiment, the foragers had stronger circadian rhythms (mean Power = 234.43

371 ±119.48 SD) than the two groups of young bees which were overall similar (*Colony-*

372 166.93 ±115.44; *Empty hive-group-* 158.94 ±127.31). Almost all the foragers (98%;

373 range 95-100%) showed statistically significant circadian rhythms, whereas only 75%

374 (range 62-89%) in the *Colony-* and 70% (range 58-84%) in the *Empty hive-group*

375 showed significant circadian rhythms (Data not shown).

376   The phase of the young bees that were exposed to air from a hive housing a colony

377 ("*Colony*") was more similar to that of the foragers (collected from the same colony

378 from which we sucked the air) compared to that of the callow bees exposed to air

379 sucked from a similar hive with no bees. In 3 out of 5 trails, the phase of the *Empty*

380 *hive bees* was significantly different from that of the *foragers* (Watson Williams F-Test,

381 $p < 0.05$; Fig. 5A). Bees from the *Colony* treatment showed stronger phase coherence

382 (longer Rayleigh vector) compared to the Empty hive treatment (Kruskal-Wallis test, p

383 < 0.0001; Steel-Dwass-Critchlow-Fligner *post hoc* test: *Forager-Colony*, p = 0.043;

384 *Forager-Empty hive*, p = 0.024; *Colony-Empty hive* p = 0.043) (Fig. 5B). In a pooled

385 analysis across all five trials, the phase-difference relative to the *Foragers*, was similar

386 for the two groups of young bees (Binomial test p = 0.31; Figure 5C). It is notable

387 however, that in the first two trials the phase difference was much larger for the bees

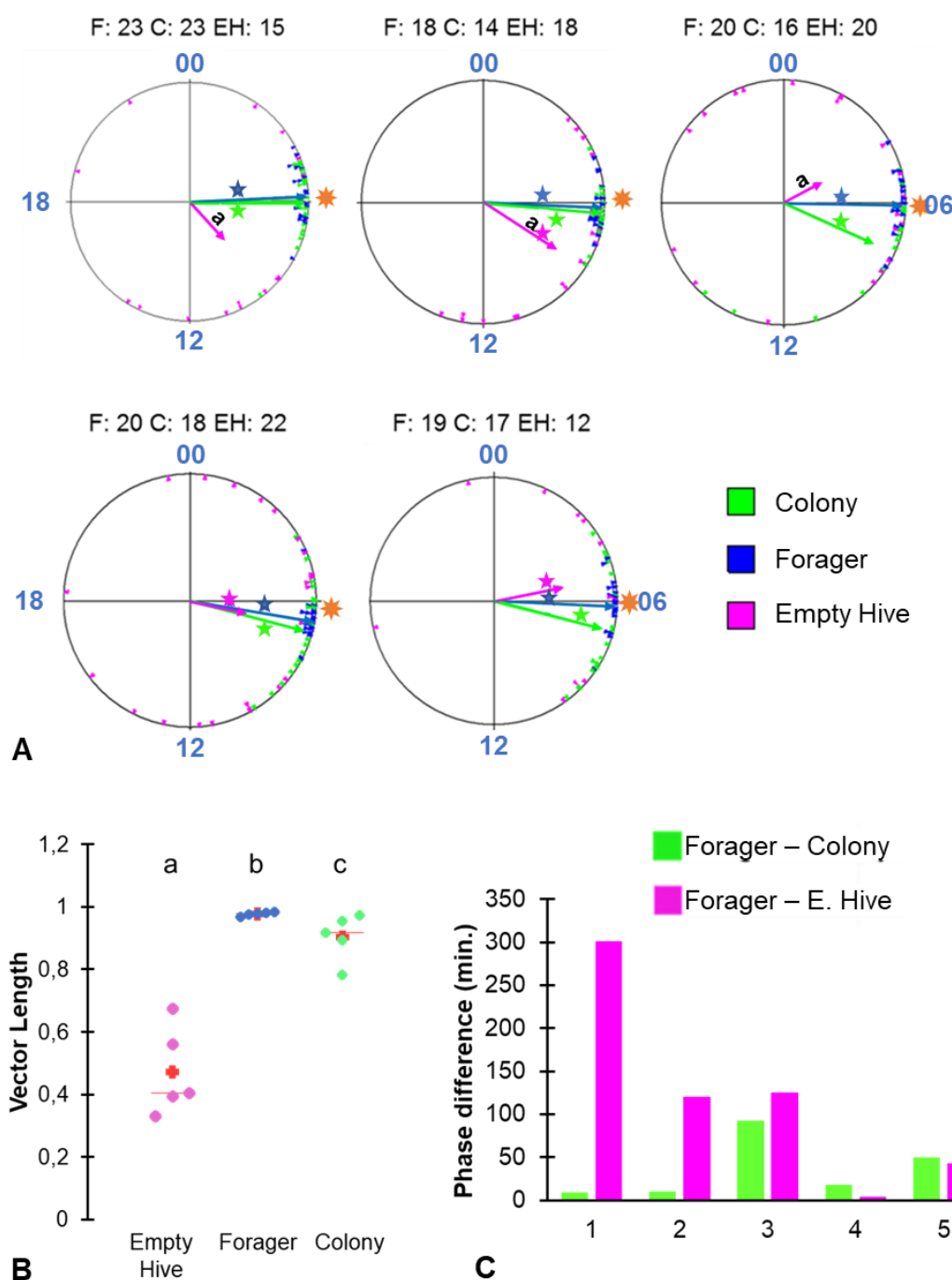388 exposed to the empty hive.

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Figure 5. Hive volatiles mediates social synchronization: (A) Circular plots comparing the phases of bees from the different treatment groups. Each plot summarizes a different trial. Sample sizes are shown above each plot (number of bees with a clear onset) F= foragers; C = callows exposed to air from a colony; EH = callows

395   exposed to air from an empty hive). The time of day is depicted on the circular plot

396   perimeter. Each triangle depicts the onset of an individual bee. The vectors point to the

397   average onset time, and their length corresponds to the degree of phase coherence.

398   Asterisks indicate the p-value obtained from Rayleigh test (*- α < 0.05). Vectors marked

399   with letters have a significantly different phase in a Watson-Williams F-test. The

400   foragers were entrained by the natural sunrise (illustrated by the sun symbol) in all

401   trials. **(B)** A Scatter plot summary of the degree of synchronization (length of the

402   Rayleigh vector) across different treatments. The red crosses correspond to the means

403   and the central horizontal bars are the medians. Treatments marked with different

404   letters are significantly different in a Kruskal-Wallis Test (P<0.05) Steel-Dwass-

405   Critchlow-Fligner *post hoc* test, *Forager-Colony* p = 0.043; *Forager-Empty hive* p =

406   0.024; *Colony-Empty hive* p = 0.043. **(C)** The phase-difference in minutes between

407   each of the two groups of young bees relative to the foragers.

408

409

410   **Discussion**

411

412   Our experiments show that both substrate-borne vibrations generated by forager

413   activity, and volatile cues emitted by a free-foraging colony can entrain circadian

414   rhythms in locomotor activity of young honey bees. These results were obtained using

415   a robust dataset composed of more than 1000 bees that were each monitored

416   individually. We repeated each experiment five times, each trial with bees from a

417   different source colony. Given that bees in each colony are the offspring of a different

21

418   queen and drones, our findings are therefore not limited to certain genotypes or

419   laboratory lines. It is also important to note that given that we determined the circadian

420   parameters for bees after removing them from the environment in which they were

421   entrained, our measurements reflect properties of the internal clock rather than

422   environmental (e.g. social) factors that could mask the clock effect on locomotor

423   activity. Our experimental approach allowed us to uncouple the effects of substrate-

424   borne vibrations and volatile colony cues. In the experiment testing the influence of

425   substrate vibrations, we minimized the transfer of volatiles by sucking out the air above

426   the forager cages. In the experiment testing the influence of hive volatiles, we

427   minimized possible effects of vibrations by placing the cages on vibration-absorbing

428   bases. The conclusive evidence for synchronization in both experiments suggest that

429   both vibrations and volatile cues are potent enough to mediate social synchronization.

430   Taken together, these findings lend credence to the hypothesis that vibrations and

431   volatile olfactory cues, that are generated by active bees, mediate social

432   synchronization among worker bees in a honey bee colony.

433   These findings are consistent with the hypothesis that volatile cues in the hive

434   mediate social synchronization in honey bee colonies (Moritz and Kryger, 1994; Bloch

435   et al., 2013; Fuchikawa et al., 2016). We show that young bees exposed to volatiles

436   from a hive housing a free-foraging colony are better synchronized with each other and

437   overall have a phase more similar to that of the free-foraging colony, compared with

438   similar bees that were exposed to air coming from a similar hive with no bees (Fig. 5).

439   These findings are consistent with earlier studies showing that young bees that were

440   caged inside double mesh enclosures were similarly synchronized with the ambient

441   day- night cycles as bees freely moving in the hive, indicating that direct contact is not

442   necessary for social synchronization in honey bees (Fuchikawa et al., 2016). Similarly,

443   Moritz and Kryger (1994), showed that the synchronization among bees in two small

444   groups was improved when they allowed air to flow between the two groups. What are

445   the colony volatiles mediating this effect? We propose that the two most likely factors

446   are volatile pheromones or fluctuations in CO_2 concentrations. The CO_2 concentration

447   within the hive shows a clear diurnal cycle (Murphy et al., 2015; Edward-Murphy et al.,

448   2016; Ohashi et al., 2009). Typically, lower levels are recorded during daytime when

449   the forager are outside the hive, and high levels at the morning and the late afternoon.

450   These two CO_2 peaks apparently correspond to the time when a large cohort of

451   foragers leave the nest for their first morning foraging trips, and then the evening peak

452   is when all (or most) of them return (Ohashi et al., 2009).

453   Honey bees act to homeostatically regulate their nest microenvironment. To

454   regulate CO_2 levels, workers stand near the hive entrance and fan with their wings

455   ventilating the nest cavity. There is a positive correlation between CO_2 levels inside the

456   hive and the number of fanning bees (Seeley, 1974; reviewed in Guerenstein and

457   Hildebrand, 2008). Thus, we assume that CO_2 levels can entrain the circadian clock of

458   bees by two non-mutually exclusive mechanisms: First, elevated in CO_2 levels lead to

459   an increase in the number of bees fanning and this activity entrains their circadian

460   clocks. Second, fluctuation in CO_2 levels act as a zeitgeber setting the phase of

461   circadian clocks affecting locomotor activity. Although we are aware of only a few

462   evidences that CO_2 can entrain circadian rhythms in insects (Nicolas and Sillans,

463   1989), there is better evidence for such effects for mammals. Adamovich et al., (2019)

464 reported that changes in carbon dioxide concentration act at the cellular level and can

465 phase shift oscillations in clock gene expression in a cell culture. Similar cellular

466 mechanisms may be also found in honey bee and other insects.

467 Honey bees use a wide variety of vibrational signals to communicate and

468 coordinate colony-level activities. For example, both the "waggle dance" with which

469 foragers recruit followers to a rewarding flowering patch, and the "tremble dance" with

470 which they inhibit further recruitment, are at least partially mediated by comb vibrations

471 (reviewed in Hrncir et al., 2005; Hunt and Richards, 2013). The hypothesis that

472 substrate-borne vibrations can mediate social synchronization is also consistent with

473 findings that in the fruit fly *Drosophila melanogaster* circadian rhythms of locomotor

474 activity can be entrained by oscillations in vibration intensity (Simoni et al., 2014). We

475 do not know yet of any study recording comb vibrations over several days in honey

476 bee colonies. Therefore, we can only speculate that forager activity which changes

477 substantially during the day (Moore et al., 1998; Bloch and Robinson, 2001), generate

478 comb vibrations that are sensed by nestmates and can mediate clock entrainment.

479 Our experiments show that comb vibrations and colony odors can synchronize

480 circadian rhythms in honey bee workers. These findings lend credence to the

481 hypothesis that surrogates of worker activity mediate social synchronization of

482 circadian rhythms in honey bee colonies. The link between worker activity and comb

483 vibration is quite straightforward, although the repertoire in the hive is broad (Hrncir et

484 al., 2005; Hrncir et al., 2019; Hunt and Richards, 2013) and additional work is needed

485 for identifying the specific vibrations that entrains the clock. As for colony odors,

486 additional information on the specific of volatile chemicals that meditate this effect is

487 clearly needed. Changes in carbon dioxide and oxygen concentration inside the hive

488 are influenced by worker activity and metabolism. However, given that active bees

489 typically increase their body temp', their level of activity may also affect the release

490 (e.g. evaporation) of additional chemicals such as cuticular pheromones from their

491 body surfaces may be also important. We suggest that in natural colonies, these two

492 factors, and perhaps additional surrogates of worker activity, act together to create

493 oscillations in the nest microenvironment. This idea is consistent with self-organization

494 models in which the sum activity of individual workers produces oscillations in the

495 microenvironment of the hive which in turn entrain the circadian clocks of other

496 individuals in the colony (Moritz and Fuchs, 1998; Camazine et al., 2003).

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506 Conflict of interest statement

507 The author(s) have no potential conflicts of interest with respect to research,

508 authorship, and / or publication of this article.

509

References

Adamovich Y, Ladeuix B, Sobel J, Manella G, Neufeld-Cohen A, Assadi MH, ... and Asher G (2019) Oxygen and carbon dioxide rhythms are circadian clock controlled and differentially directed by behavioral signals. Cell Metab *29*(5):1092-1103.

Albrecht U (2012) Timing to perfection: the biology of central and peripheral circadian clocks. Neuron 74:246–260.

Amir S and Stewart J (1996) Resetting of the circadian clock by a conditioned stimulus. Nature 379:542–545.

Beer K, Kolbe E, Kahana NB, Yayon N, Weiss R, Menegazzi P, Bloch G and Helfrich-Förster C (2018) Pigment-Dispersing Factor-expressing neurons convey circadian information in the honey bee brain. Open Biol 8(1):170224.

Bloch G and Robinson GE (2001) Chronobiology: reversal of honeybee behavioural rhythms. Nature 410(6832):1048-1049.

Bloch G (2010) The social clock of the honeybee. J Biol Rhythms 25:307-317.

Bloch G, Herzog ED, Levine JD and Schwartz WJ (2013) Socially synchronized circadian oscillators. Proc R Soc B 280:20130035.

Camazine S, Deneubourg JL, Franks NR, Sneyd J, Bonabeau E and Theraula G (2003) Self-organization in biological systems. Princeton university press.

Castillo-Ruiz A, Paul MJ and Schwartz WJ (2012) In search of a temporal niche: social interactions. In Progress in brain research. (Vol. 199, pp. 267-280) Elsevier.

Conte YL and Hefetz A (2008) Primer pheromones in social hymenoptera. Annu Rev Entomol 53:523-542.

Dunlap JC, Loros JJ and Decoursey PJ (2004) Chronobiology: Biological timekeeping. Sunderland, MA: Sinauer Associates, Inc.

546 Eban-Rothschild A and Bloch G (2012a) Circadian Rhythms and Sleep in Honey Bees.
547 In: Galizia GC, Eisenhardt D, Giurfa M, Honeybee Neurobiology and Behavior: A
548 Tribute to Randolf Menzel. Dordrecht: Springer Netherlands; pp. 31–45.
549
550 Eban-Rothschild A and Bloch G (2012b) Social influences on circadian rhythms and
551 sleep in insects. In Advances in genetics (Vol. 77, pp. 1-32). Academic Press.
552
553 Edwards-Murphy F, Magno M, Whelan PM, O'Halloran J and Popovici EM (2016) b+
554 WSN: Smart beehive with preliminary decision tree analysis for agriculture and honey
555 bee health monitoring. Comput Electron Agric 124:211-219.
556
557 Favreau A, Richard-Yris MA, Bertin A, Houdelier C and Lumineau S (2009) Social
558 influences on circadian behavioural rhythms in vertebrates. Anim Behav 77:983-989.
559
560 Fuchikawa T, Eban-Rothschild A, Nagari M, Shemesh Y and Bloch G (2016) Potent
561 social synchronization can override photic entrainment of circadian rhythms. Nat
562 Commun 7:11662.
563
564 Fuchikawa T, Beer K, Linke-Winnebeck C, Ben-David R, Kotowoy A, Tsang VWK,
565 Warman GR, Winnebeck EC, Helfrich-Förster C and Bloch G (2017) Neuronal
566 circadian clock protein oscillations are similar in behaviourally rhythmic forager
567 honeybees and in arrhythmic nurses. Open Biol *7*(6):170047.
568
569 Guerenstein PG and Hildebrand JG (2008) Roles and effects of environmental
570 carbon dioxide in insect life. Annu Rev Entomol 53:161-178.
571
572 Helm B, Visser ME, Schwartz W, Kronfeld-Schor N, Gerkema M, Piersma T and
573 Bloch G (2017) Two sides of a coin: ecological and chronobiological perspectives of
574 timing in the wild. Philos Trans R Soc, B Biol Sci, 372(1734):20160246.
575
576 Hrncir M, Barth FG and Tautz J (2005) Vibratory and airborne sound-signals in bee
577 communication (hymenoptera). In Drosopoulos S. and Claridge M. Insect Sounds
578 and Communication: Physiology, Behaviour, Ecology, and Evolution pp. 421– 436.
579
580 Hrncir M, Maia-Silva C and Farina WM (2019) Honey bee workers generate low-
581 frequency vibrations that are reliable indicators of their activity level. J Comp Physiol
582 205(1):79-86.
583

584 Hunt JH and Richard FJ (2013) Intracolony vibroacoustic communication in social
585 insects. Insectes Soc 60(4):403-417.
586
587 Kumar V (Ed) (2017) Biological timekeeping: clocks, rhythms and behaviour.
588 Springer.
589
590 Leonhardt SD, Menzel F, Nehring V and Schmitt T (2016) Ecology and evolution of
591 communication in social insects. Cell 164(6):1277-1287.
592
593 Levine JD, Funes P, Dowse HB and Hall JC (2002) Resetting the circadian clock by
594 social experience in *Drosophila melanogaster*. Science *298*(5600):2010-2012.
595
596 Lone SR and Sharma VK (2011) Social synchronization of circadian locomotor activity
597 rhythm in the fruit fly *Drosophila melanogaster*. J Exp Biol 214(22):3742-3750.
598
599 Mendoza J (2007) Circadian clocks: setting time by food. J Neuroendocrinol
600 19(2):127-137.
601
602 Mildner S and Roces F (2017) Plasticity of daily behavioral rhythms in foragers and
603 nurses of the ant *Camponotus rufipes*: influence of social context and feeding times.
604 PloS one 12(1):e0169244.
605
606 Mistlberger RE and Skene DJ (2004) Social influences on mammalian circadian
607 rhythms: animal and human studies. Biol Rev 79(3):533-556.
608
609 Moore D, Angel JE, Cheeseman IM, Fahrbach SE and Robinson GE (1998)
610 Timekeeping in the honey bee colony: integration of circadian rhythms and division of
611 labor. Behav Ecol Sociobiol 43(3):147-160.
612
613 Moritz RFA and Kryger P (1994) Self-organization of circadian rhythms in groups of
614 honeybees (*Apis mellifera L*). Behav Ecol Sociobiol 34:211–215.
615
616 Moritz RF and Fuchs S (1998) Organization of honeybee colonies: characteristics
617 and consequences of a superorganism concept. Apidologie 29(1-2):7-21.
618
619 Murphy FE, Popovici E, Whelan P and Magno M (2015) Development of an
620 heterogeneous wireless sensor network for instrumentation and analysis of beehives.
621 In Instrumentation and Measurement Technology Conference (I2MTC), 2015 IEEE
622 International (pp. 346-351) IEEE.
623

624 Nicolas G and Sillans D (1989) Immediate and latent effects of carbon dioxide on
625 insects. Annu Rev Entomol 34(1):97-116.
626
627 Ohashi M, Okada R, Kimura T and Ikeno H (2009) Observation system for the control
628 of the hive environment by the honeybee (*Apis mellifera*). Behav Res Meth 41(3):782-
629 786.
630
631 Reebs SG (1989) Acoustical entrainment of circadian activity rhythms in house
632 sparrows—constant light is not necessary. Ethology 80:172–181.
633
634 Regal PJ and Connolly MS (1980) Social influences on biological rhythms. Behaviour
635 72:171–199.
636
637 Robinson GE (1992) Regulation of division of labor in insect societies. Annu Rev
638 Entomol 37(1):637-665*.*
639
640 Rodriguez-Zas SL, Southey BR, Shemesh Y, Rubin EB, Cohen M, Robinson GE and
641 Bloch G (2012) Microarray analysis of natural socially regulated plasticity in circadian
642 rhythms of honey bees. J Biol Rhythms *27*(1):12-24.
643
644 Seeley TD (1974) Atmospheric carbon dioxide regulation in honey-bee (*Apis
645 mellifera*) colonies. J Insect Physiol *20*(11):2301-2305.
646
647 Sharma VK (2003) On the significance of circadian clocks for insects. J Indian I Sci.
648 83(1&2), 3.
649
650 Sharma VK and Chandrashekaran MK (2005) Zeitgebers (time cues) for biological
651 clocks. Curr Sci 89(7):1136 – 1146.
652
653 Shemesh Y, Cohen M and Bloch G (2007) Natural plasticity in circadian rhythms is
654 mediated by reorganization in the molecular clockwork in honeybees. FASEB J
655 21:2304-2311.
656
657 Shemesh Y, Eban-Rothschild A, Cohen M and Bloch G (2010) Molecular
658 dynamics and social regulation of context-dependent plasticity in the circadian
659 clockwork of the honey bee. J Neurosci 30:12517-12525.
660
661 Simoni A, Wolfgang W, Topping WP, Kavlie RG, Stanewsky R and Albert JT (2014) A
662 mechanosensory pathway to the *Drosophila* circadian clock. Science 343(6170):525-
663 528.

664
665 Wilson EO (1971) The insect societies. The insect societies.
666
667 Yerushalmi S, Bodenhaimer S and Bloch G (2006) Developmentally determined
668 attenuation in circadian rhythms links chronobiology to social organization in bees. J
669 Exp Biol 209(6):1044-1051.
670
671 Yerushalmi S and Green RM (2009) Evidence for the adaptive significance of
672 circadian rhythms. Ecol 12(9):970-981.