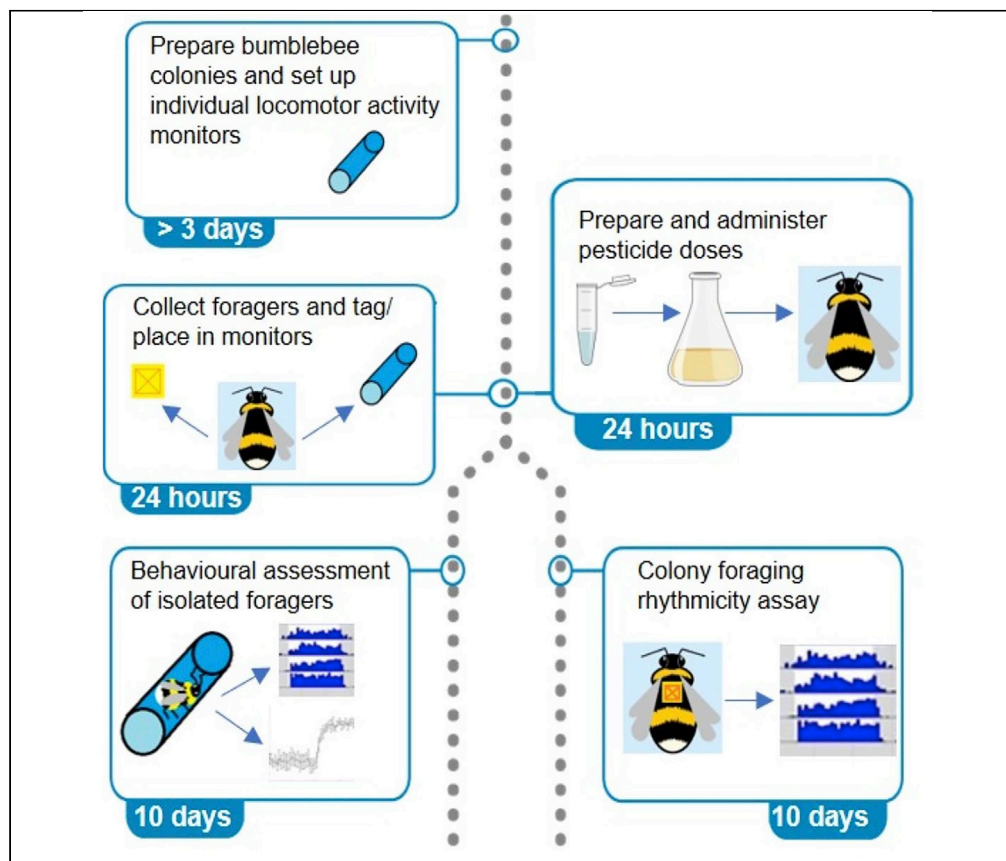## Protocol

Using radio frequency identification and locomotor activity monitoring to assess sleep, locomotor, and foraging rhythmicity in bumblebees

Kiah Tasman, Sean A. Rands, James J.L. Hodge

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Highlights

Easy and reliable way of testing circadian rhythmicity and sleep in invertebrates

Covers colony care, equipment adaptation, and setup and experimental protocol

This protocol can be used to study the effects of any water soluble/liquid insecticide

The multiple ways to adapt the protocol for other organisms are highlighted

Bumblebees are a key pollinator. Understanding the factors that influence the timing of sleep and foraging trips is important for efficient foraging and pollination. Here, we illustrate how individual locomotor activity monitoring and colony-wide radio frequency identification tracking can be combined to analyze the effects of agrochemicals like neonicotinoids on locomotor and foraging rhythmicity and sleep quantity/quality in bumblebees. We also highlight aspects of the design that can be adapted for other invertebrates or agrochemicals, allowing broader application of these techniques.

Protocol

Using radio frequency identification and locomotor activity monitoring to assess sleep, locomotor, and foraging rhythmicity in bumblebees

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SUMMARY

Bumblebees are a key pollinator. Understanding the factors that influence the timing of sleep and foraging trips is important for efficient foraging and pollination. Here, we illustrate how individual locomotor activity monitoring and colony-wide radio frequency identification tracking can be combined to analyze the effects of agrochemicals like neonicotinoids on locomotor and foraging rhythmicity and sleep quantity/quality in bumblebees. We also highlight aspects of the design that can be adapted for other invertebrates or agrochemicals, allowing broader application of these techniques.
For complete details on the use and execution of this protocol, please refer to Tasman et al. (2020).

BEFORE YOU BEGIN

Preparing bumblebee colonies

Bumblebee colonies need to be established in the laboratory before dosing and behavioral assays can occur. Bumblebee colonies can be brought from local suppliers – please check which species and subspecies are appropriate or legal for your country, whether licenses are required to maintain the colonies in the laboratory, and whether action should be taken to stop the bees from escaping into the wild. In this protocol, we use mature colonies of the buff-tailed bumblebee *Bombus terrestris audax* with approximately 80–100 workers. This species was chosen as it is the local sub-species of *B. terrestris*, which is common across Europe, thus giving our results broad applicability. The behavioral assays of individuals and colonies are likely to require separate colonies although the exact number will depend on the experiment being performed. Tasman et al. (2020) used four colonies for the individual forager experiments, and another six for the colony-level experiments. Multiple colonies are required so that several genotypes can be tested, allowing colony level variation to be mitigated. Different colonies were used for individual and colony wide experiments to allow for many foragers to be collected and removed for individual experiments. If an experiment is using a smaller sample, then both experiments could be carried out on the same colonies. Colonies are maintained at 21°C, 12 h:12 h light: dark (LD) with lights on at 9 am and off at 9 pm, with a 30 min dawn/dusk period where lights were at 50%. Ideally, lights should be high frequency with natural UV levels, like the setup described by Whitney et al., (2016). It is standard practice when carrying out invertebrate circadian studies to allow time for entrainment to a new light regime. Chittka et al (2013), used 7 days whilst Tasman et al (2020) used 3 days. **Thus, it is recommended that colonies are given sufficient time (>3 days) to acclimatize, entrain, and settle before experiments begin.**

Colonies are provided *ad libitum* with appropriate food, typically sucrose solution and honeybee collected pollen, (e.g., Agralan #BB008513). In Tasman et al (2020) we used a premixed sugar solution, (Biogluc®, Biobest) but a simpler solution of table sugar dissolved in water can be used, such as the 30% volume/volume mix used previously (Lawson et al., 2016, Lawson et al., 2017) dispensed in the foraging arena *via* a Mikimiqi honeybee entrance feeder. Additionally, pollen should be provided regularly into the nest box. This can be 1–2 teaspoon every 2–5 days (Beer et al., 2016; Tasman et al., 2020), depending on the light regime. Colonies should not be opened during the constant darkness (DD) section of experimentation unless this can be done with no risk of light entering the colony. Alternatively, colonies can be provided with 100 mL of pollen/honey paste, as performed by Chittka et al (2013).

Colonies should have access to an artificial foraging space. Tasman et al. (2020) used a custom-built 1000 × 500 × 500 mm foraging arena that was attached to the nest box *via* a clear plastic tube (15 mm diameter and 200 mm length). The arena was constructed from clear UV-transmitting acrylic. The nest box was kept in the opaque cardboard box it arrived in. An additional wide ramp from the entrance to the foraging arena to the floor of the arena should be built (i.e., from card and duct tape) to ensure that foragers can return to the nest box in darkness, when they cannot fly (Reber et al., 2015). Other bee behavioral studies (Lawson et al., 2016, Lawson et al., 2017, Whitney et al., 2016, Pearce et al., 2017, Avarguès-Weber et al., 2018, Kaczorowski et al., 2012, Muth and Leonard, 2019) have used other dimensions of foraging arena and we advise that protocol users consider their space and equipment requirements before they construct their own arenas.

The protocol below describes the specific steps for assaying the behavioral effects of field-relevant concentrations of different neonicotinoid pesticides administered to bumblebee colonies *via* their food (Biogluc®). We describe the protocol for imidacloprid here (as described in Tasman et al., 2020). The protocol could easily be altered to consider other compounds that are soluble in the bees' food. Similarly, we used variations on the protocol to assay behavior in the fruit fly, *Drosophila melanogaster* in response to imidacloprid, clothianidin, thiamethoxam, and thiacloprid (Tasman et al., 2021a; Tasman et al 2021b) and it could be adapted for use with other invertebrates.

Preparing pesticide doses

⏱ Timing: 24 h

1. Prior to the start of the experiment, prepare a 100 mg/L stock of neonicotinoid such as imidacloprid in ddH2O solution. It is good to do this the day before the experiment as it can take neonicotinoids a few hours to fully dissolve. We recommend placing the imidacloprid into the ddH2O and mixing using a magnetic stirrer and flea overnight. Wrap in tin foil to avoid any UV degradation. Once dissolved, aliquot out 0.1 mL portions and freeze at −18°C.
2. On the day of the experiment use the 100 mg/L stock solution to create the neonicotinoid food. Calculate the dilutions necessary to produce the concentrations needed for the experiment. For example, for a 10 μg/L concentration, mix 0.1 mL of stock solution into 9.9 mL of ddH2O using a vortex, and then add to 990 mL of Biogluc® using a magnetic stirrer and flea. For a 1 μg/L concentration, mix 0.01 mL of stock solution into 9.99 mL of ddH2O using a vortex, and then add to 990 mL of Biogluc® using a magnetic stirrer and flea.

   Note: Quantities should be adjusted based on requirements. The dosed food should be wrapped in aluminum foil to avoid UV degradation and stored in the fridge. Make dosed food up fresh from frozen stock solution each time the experiment is repeated.

Note: The daily quantity of neonicotinoid/ other compound ingested by individual bees can be crudely estimated by measuring the quantity of solution consumed by the individual bee in the Locomotor Activity Monitor (LAM) setup (Tasman et al. 2020). Alternatively, a more precise quantification can be carried out using, for example, the QuEChERS method as done by David et al., (2015).

Preparing the individual forager locomotor activity monitor setup

⊙ Timing: 2 h

3. Before starting the experiment, prepare the locomotor activity setup, as seen in Figure 1. The setup used in Tasman et al., (2020) was created by modifying a LAM setup (LAM16, TriKinetics Inc., USA). This set up consists of glass tubes (16 × 100 mm) placed in a monitor so that the tubes are intersected with an infrared beam. Each beam break is counted as an activity bout, allowing locomotor activity rhythms to be calculated.
    a. For this setup prepare a glass tube for each forager. At one end of the tube, place a disk of thin wire mesh, before attaching the rubber cap provided with the LAM, with a 1 g silica pack glued inside it, to control humidity during the experiment.
    b. At the other end, a 10 mL Falcon tube with a small hole drilled into the tip, should be attached. This should be filled with neonicotinoid/soluble compound dosed Biogluc® and the cap secured, allowing the forager to feed *ad libitum* during the experiment.

Note: This setup can be adjusted to the needs of the experiment. Other variations have been created, e.g. the modified LAM created for studying honeybee rhythmicity in Beer et al. (2016). The silica may not be necessary if using invertebrates which produce less moisture. For example, they were not necessary when using a similar set up for fruit flies (Tasman et al., 2021a).
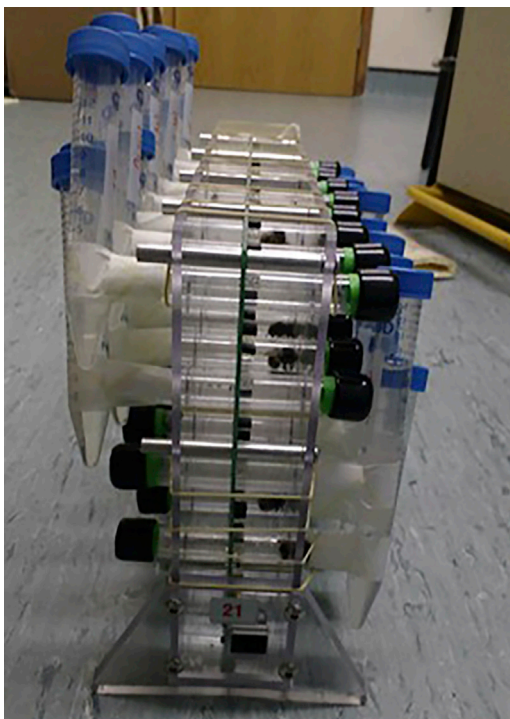
Figure 1. The modified LAM set up used for studying locomotor rhythmicity and sleep in *Bombus terrestris* foragers described in this methodology and used in Tasman et al. (2020)

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Imidacloprid PESTANAL	Sigma-Aldrich	CAT#37894
Biogluc	Agralan	CAT#BB004106
Pollen	Agralan	CAT#BB008513
Experimental models: organisms/strains		
B. terrestris audax research colony, 80–100 workers, with cotton wool	Agralan	CAT#BB121040-CF1
Software and algorithms		
FlyToolBox	Created by Dr Jeffrey C. Hall lab, from paper (Levine et al., 2002)	N/A
Sleep and Circadian Analysis MATLAB Program (SCAMP)	Created by Dr Leslie C. Griffith lab, from paper (Donelson et al., 2012)	N/A
Day/night program	Created by Dr James J. L. Hodge lab, from paper (Julienne et al., 2017)	N/A
MATLAB and Statistics Toolbox	MathWorks	Release 2015b
DAM FileScan 108×	TriKinetics Inc., USA	N/A
Other		
Micro Radio Frequency IDentification (RFID) set up (reader and tags)	Microsensys GmbH	MAJA bundle CAT#2901
Locomotor Activity Monitor	TriKinetics Inc., USA	LAM16
Honeybee Entrance Feeder	Mikimiqi	ASIN#B07SQHPPQF
Deposited data		
Original data of Tasman et al. (2020)	Mendeley Data	https://doi.org/10.17632/m8pykxzkyb.1

Note: Whilst this protocol utilised the RFID tag tool to monitor foragers leaving/entering the colony, we recognize that this is an expensive piece of equipment. A cheaper alternative could be the printable QR code system introduced by Crall et al. (2015), although a good quality camera is needed for this set up to be effective.

STEP-BY-STEP METHOD DETAILS

### Circadian rhythm and sleep analysis for isolated foragers

⊙ Timing: 10 days

Foragers are placed in the modified LAM setup described in the 'before you begin' section and their activity monitored over a ten-day period. Circadian and sleep analysis are then conducted.

1. If focusing on foragers as in Tasman et al. (2020), these can be selected by only using bees seen coming out of the colony and feeding at feeders in the foraging arena, with a thorax diameter of approximately 5 mm (the average size for foragers, (Goulson et al., 2002). In honeybees and bumblebees, nurses show an attenuation of circadian rhythms so experiments should be mindful of which bees are included in their study. Collect an adequate sample size, using several different colonies to control for any colony specific effects. In Tasman et al., (2020) we collected 30 foragers each from 4 colonies, providing a sample size of 40 foragers for each treatment.
2. Load bees into individual tubes in the adapted LAM set up and monitor for five day in 12 h light and 12 h dark (LD) conditions, followed by five days of constant darkness (DD). Activity bouts (recorded via infra-red (IR) beam crosses by the LAM), should be summed into bins for analysis. We used 30 min bins (Tasman et al., 2020), but other studies have used 60 min bins (Chittka et al 2013). Smaller bins will provide more fine scale analysis of activity.

3. Circadian analysis should then be carried out. There are a range of circadian analyses and the specific ones chosen will depend on the focus of the study. We chose to calculate the rhythmicity statistic, period length and total daily activity levels, as these are common measures and provide information on how active the foragers are, how strong their locomotor rhythms are and the length of their free running period. The gives a good basic overview of the function of the clock. Below is a description of how to measure these, with some example data provided in the quantification and statistical analysis section:

   a. The rhythmicity statistic (RS) is a measure of rhythmicity strength. It calculates the auto correlation of locomotor activity on consecutive days. Conventionally, an RS greater than 2 is rhythmic, an RS of 1.5–2 is weakly rhythmic, and an RS less than 1.5 is arrhythmic (Hodge and Stanewsky, 2008).

   The RS is calculated using conventional autocorrelation analysis. First, the data are filtered, using a low-pass Butterworth filter to remove any periodicities of less than 4 h.

   b. The dataset is then paired with itself, gradually moved out of register with itself and the correlation coefficient plotted. At 0 h, the two data sets are identical, and then, for rhythmic data, they return to high correlation approximately every 24 h.

   c. The value of the third peak in the auto correlogram is reported as the rhythmicity index (RI), a statistical representation of the rhythmicity of the bee's activity (example shown in Figure 2). The Rhythmicity Statistic (RS) is calculated as the ratio of this RI to the value of the 95% confidence line (Levine et al., 2002). The period length is also calculated from the auto correlogram, by calculating the time between the peaks in correlation. This analysis can be carried out using *FlyToolBox* (Levine et al., 2002) in MATLAB (MATLAB and Statistics Toolbox Release 2015b).

   d. The day and night activity levels for each bee can be calculated using the *daynight* program (Julienne et al., 2017) in MATLAB. The individual bee's period length is used to split the activity data into subjective days and nights and then the activity counts for each are summed. The mean daily activity in daytime and night-time for the five days are then calculated.

Note: LD and DD data should be split and analyzed separately.
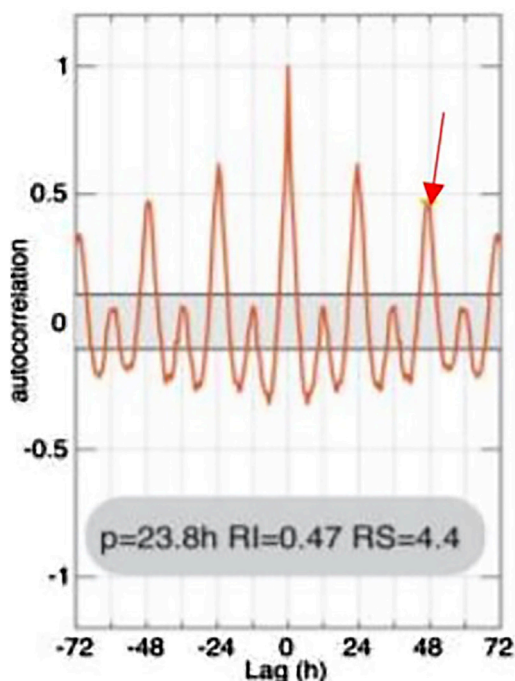
Figure 2. An example of an auto correlogram for locomotor activity, from which period length, RI and RS can be calculated

4. Sleep analysis should be carried out on the five days of LD (Buhl et al., 2019), using activity data that have been summed into both 1 min and 30 min bins. In invertebrates, bouts of inactivity lasting more than 5 min tend to be quantified as sleep (Beyaert et al., 2012, Nagari et al., 2019, Eban-Rothschild and Bloch, 2015). Analysis can be performed using the *Sleep and Circadian Analysis MATLAB Program* (*SCAMP*, Donelson et al., 2012). Again, there are many ways to quantify sleep. We calculated mean total sleep per 30 min bin, as well as mean number and length of sleep episodes per day. This gives a good overview of the quantity and structure of sleep achieved by the individual. An example of the sort of sleep analysis that can be provided by *SCAMP* can be seen in Figure 3.

Note: Measuring sleep under LD is standard practice for this sort of analysis as users can accurately measure what quantity and quality of sleep is occurring in the day compared to the night. It is also more relevant to field conditions. Locomotor activity is measured in DD to give an insight into the function of the endogenous clock when external cues are removed.

⚠ CRITICAL: As mentioned in the 'before you begin' section, it is essential that foragers have been given at least 3 days in the colony to entrain to the 12 h: 12 h LD schedule before they are collected and used in the LAM.

Note: Other values can be extracted from the *SCAMP* program, such as sleep latency (the time after lights turn off before first sleep bout).

Note: Only bees that showed activity bouts up until the end of the experiment were included in analysis, to remove any inactive or dead bees.

⚠ CRITICAL: Data from the LAM must be processed by the DAMFileScan 108× (free to download from www.trikinetics.com) and then the '.txt' extension has to be removed from the file name before it can be used by *FlyToolBox*. For a full description of how to use the *FlyToolBox* see Levine et al, 2002.

⚠ CRITICAL: When setting up the LAM, make sure to set the program to collect data every 1 min, so that it can be summed into both 1 m and 30 m bins (or whatever size bin is being used for the activity data), allowing the data to be used for both circadian and sleep analysis.

Foraging rhythmicity assay for colonies

⏱ Timing: 10 days
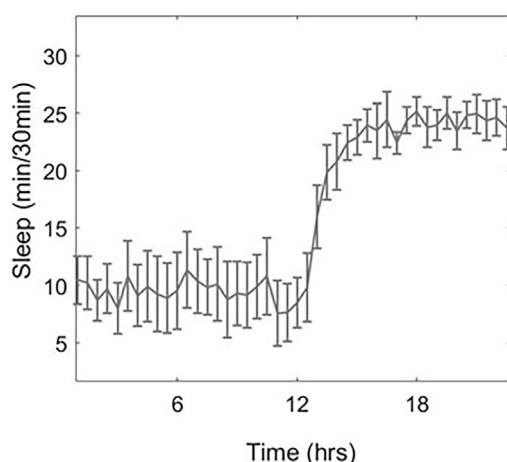
Figure 3. An example sleep plot for a control bee, taken from Tasman et al. (2020)
The plot shows the mean quantity of sleep (± SEM) achieved every 30 m of the 24 h period in LD conditions.

Circadian rhythmicity within the colony can be assayed using a micro-RFID setup (Microsensys GmbH), (Stelzer and Chittka, 2010). This is used to monitor the movement of foragers in and out of the colony and to estimate their foraging activity and rhythmicity.

5. Approximately 40 foragers should be collected from the foraging arena, in the same way as previously described in the LAM set up. They should be anesthetized using CO_2, and an RFID tag (Microsensys GmbH mic3-TAG) stuck to the center of their thorax with superglue (Loctite) as in Figure 4. Foragers should then be returned to the colony nest box.

6. After a day for acclimatization and recovery from the CO_2 exposure, recording can begin. An RFID reader (Microsensys GmbH iID®MAJAreadermodule4.1) is placed at the entrance to the foraging arena so that foragers pass through the reader to enter the arena. The data from the readers are collected on a host (microsensys GmbH iID®HOSTtypeMAJA4.1), for a 10-day period; 5-day LD and 5 day DD, as in the LAM experiments above.

7. The data are then summed into bins, 30 min or other, as required by the experiment, with each pass through the reader being counted as a single activity bout. These data can then be analyzed in the same way as the individual locomotor data above, to provide in and out bout rhythmicity data for each tagged forager.

Note: Sometimes foragers sit in the reader, causing continuous reading of their RFID tag by the reader- this is discussed in the trouble shooting section.

⚠ CRITICAL: As mentioned in the 'before you begin' section, it is essential that foragers have been given at least 3 days to entrain to the 12:12 LD schedule before recording begins.

Note: Only those that showed foraging activity every day of the study period were used for analysis, ensuring that only foragers were assessed. For control groups in our study (Tasman et al., 2020) this was 24, 27 and 29 bees per colony. For the treatment group this was 10, 17 and 21 bees per colony.

Note: For these experiments, passing through the reader was taken as a single in/out activity bout and the number of bouts in total was taken as a measure of foraging activity. This is like other rhythmicity analysis carried out with bumblebees using RFID tags (Stelzer and Chittka, 2010). However, if the experimenter wants to also look at the length of foraging bouts etc. then two readers could be used, one at the entrance to the colony and one at the entrance to the foraging arena, to determine directionality of the foragers. This is discussed further in the limitations section.
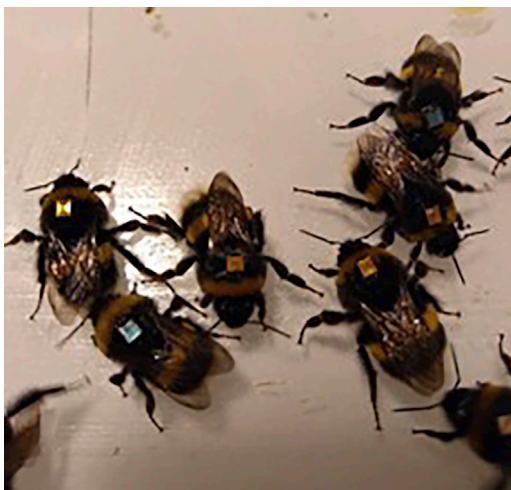
Figure 4. An example of RFID-tagged *Bombus terrestris* foragers

Table 1. Example of raw activity data for individual *Bombus terrestris* foragers in the LAM setup with each column showing the number of activity bouts per minute for individual bees

Line No.	Date	Time	Lights	Bee 1	Bee 2	Bee 3	Bee 4	Bee 5	Bee 6
19817	6 Dec 18	09:16:00	1	24	0	3	9	0	4
19818	6 Dec 18	09:17:00	1	5	0	9	4	13	0
19819	6 Dec 18	09:18:00	1	0	0	10	0	38	0
19820	6 Dec 18	09:19:00	1	17	1	0	0	19	0
19821	6 Dec 18	09:20:00	1	2	6	0	0	13	0
19822	6 Dec 18	09:21:00	1	6	0	0	0	9	0
19823	6 Dec 18	09:22:00	1	17	0	0	0	7	3

EXPECTED OUTCOMES

For both the individual (as seen in Table 1) and in colony experiments (as seen in Table 2), the outcome will be a log of activity bouts, for each 1- or 30-min period (or other bin length), for a 10 day stretch. This can be used to produce an actogram and to extract the RS and the activity levels of individuals in both LD and DD.

For control bees, you would expect them to successfully entrain to the light cycle, showing more activity in the daylight segment of the 24 h period. This should continue in constant darkness, showing a functional circadian clock that, once entrained, can maintain rhythmicity in the absence of continued environmental signals. Additionally, analysis should show them to have an RS above 1.5, indicating rhythmic activity. An example actogram, for a control bee taken from data from Tasman et al. (2020) can be seen in Figure 5. For neonicotinoid treated bees, this rhythmicity will likely reduce. This is what we observed, with activity during the subjective night increasing and activity during the subjective day decreasing (Tasman et al., 2020).

For individual control foragers in the LAM set up, sleep data were also collected. You would expect control bees to sleep about twice as much during the night as in the day and for night-time sleep episodes to be longer. An example of sleep data for a control bee, taken from Tasman et al., (2020) can be seen in Figure 3. For neonicotinoid treated bees, the quantity and structure of sleep will likely change. We saw an increase in daytime sleep quantity and bout length (Tasman et al., 2020).

Further examples of the results for circadian rhythmicity and sleep for bumblebees, both control bees and those exposed to imidacloprid, can be observed in our paper (Tasman et al., 2020).

Example data

Table 2. Example raw activity data output for RFID-tagged *Bombus terrestris* foragers, where Reader ID records which of the RFID readers is registering an activity, and Forager ID records the code of the unique pre-registered tag number attached to each individual bee

| Date/Time	Reader ID	Forager ID
01/03/2005 01:35:13.552	122116	5E 9C 1B 06 08 00 12 E0
01/03/2005 01:37:51.978	122115	9C 9D 1B 06 08 00 12 E0
01/03/2005 01:39:20.151	122115	F9 9A 1B 06 08 00 12 E0
01/03/2005 01:39:22.112	122116	5E 9C 1B 06 08 00 12 E0
01/03/2005 01:39:59.159	122115	9C 9D 1B 06 08 00 12 E0
01/03/2005 01:50:43.627	122116	5E 9C 1B 06 08 00 12 E0
01/03/2005 01:51:22.289	122115	9C 9D 1B 06 08 00 12 E0
01/03/2005 01:54:39.033	122115	F9 9A 1B 06 08 00 12 E0
01/03/2005 01:57:12.442	122116	5E 9C 1B 06 08 00 12 E0
01/03/2005 01:35:13.552	122116	5E 9C 1B 06 08 00 12 E0
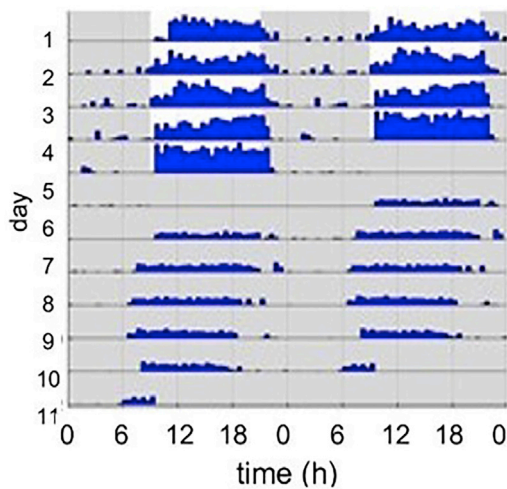
Figure 5. An example actogram for a control bee, taken from Tasman et al. (2020)
The blue bars show activity bouts, the white background shows lights on and the gray background indicates lights off. Shown is the 5 days of LD, followed by 5 days of DD. The actogram is double-plotted to assist visualization of the rhythm.

QUANTIFICATION AND STATISTICAL ANALYSIS

For the circadian data, analysis can be carried out using the *FlyToolBox* (Levine et al., 2002) in MAT-LAB (MATLAB and Statistics Toolbox Release 2015b). Raw data, as seen in Figure 6, can be loaded into the DAMFileScan 108, outputting an individual text file showing a list of activity bouts per summed bin for each individual, as seen in Figure 7. The .txt should be removed from these files and they can then be loaded into the *FlyToolBox* on MATLAB using the 'dam_load' code in the toolbox, as seen in Figure 8. Actograms for each individual can then produced, using the 'ralf' code in the toolbox. These can be used to identify and disregard any individuals that weren't active till the end of the experiment. The parameters for analysis are then set and the data analyzed using
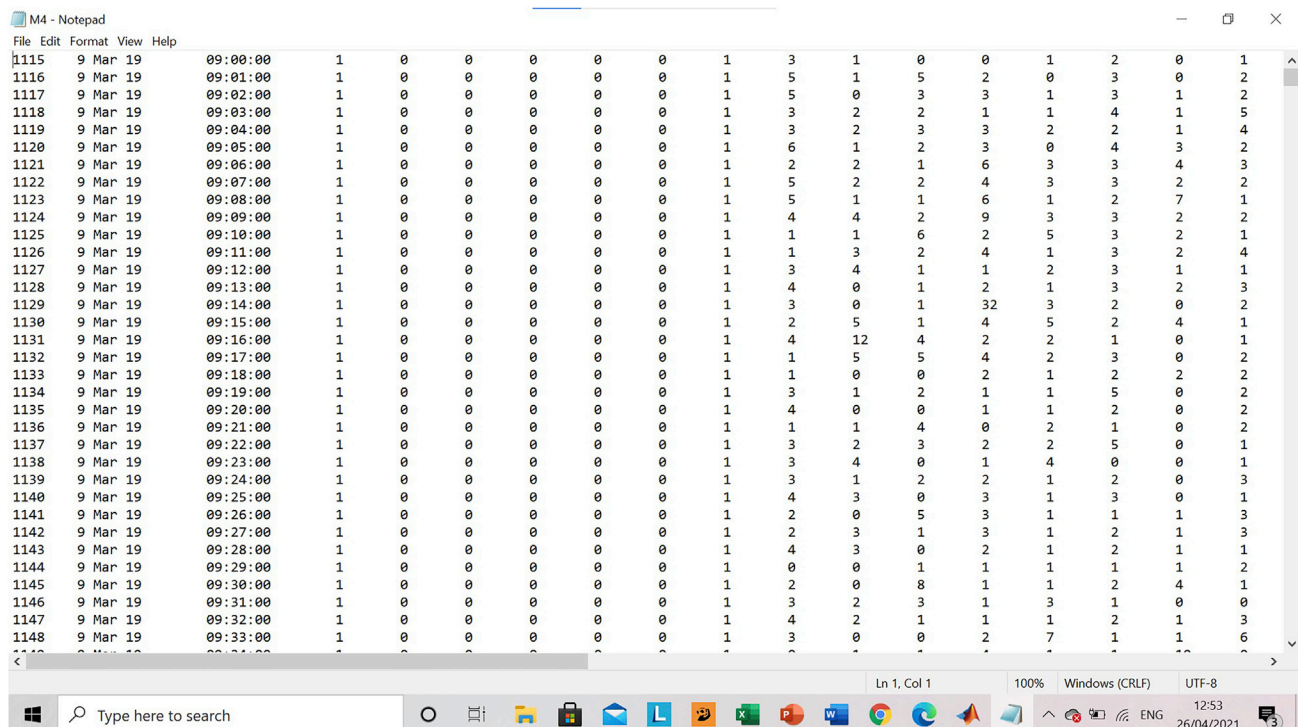
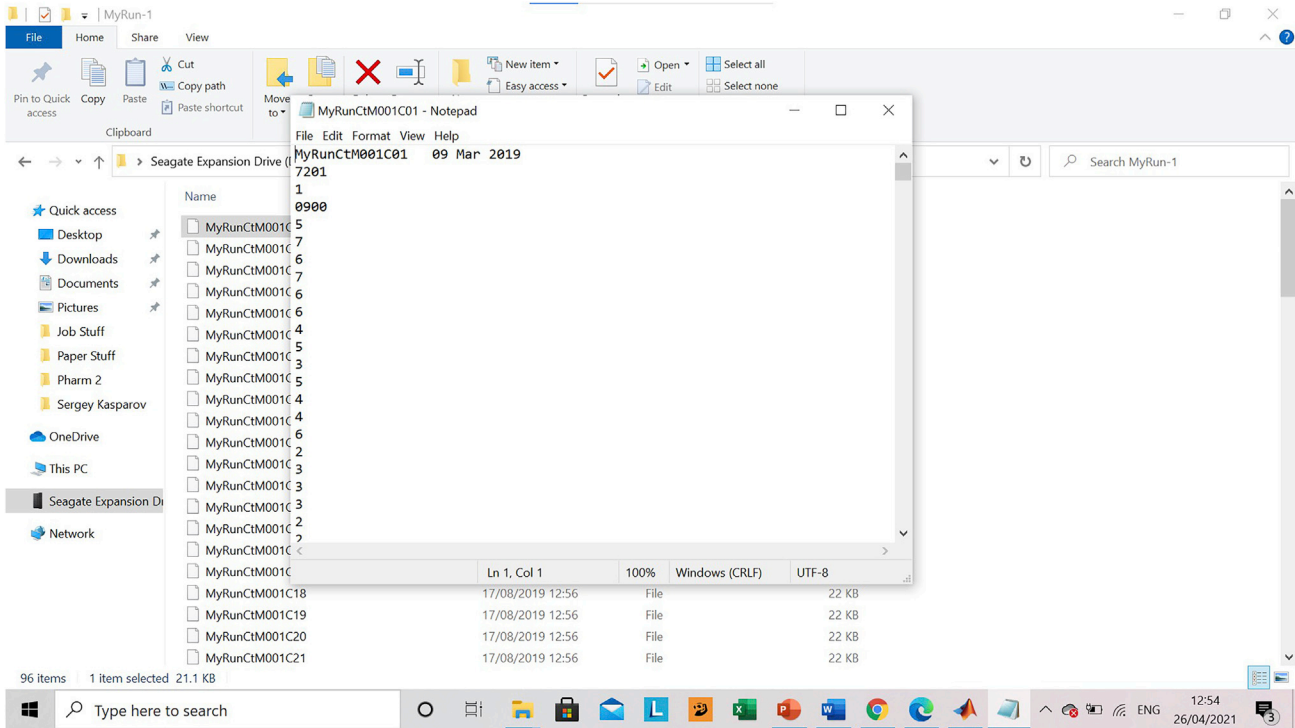**Figure 6. An example of the raw data file outputted from the LAM setup**

Figure 7. An example of a scanned data file, showing the activity of an individual forager, summed into bins
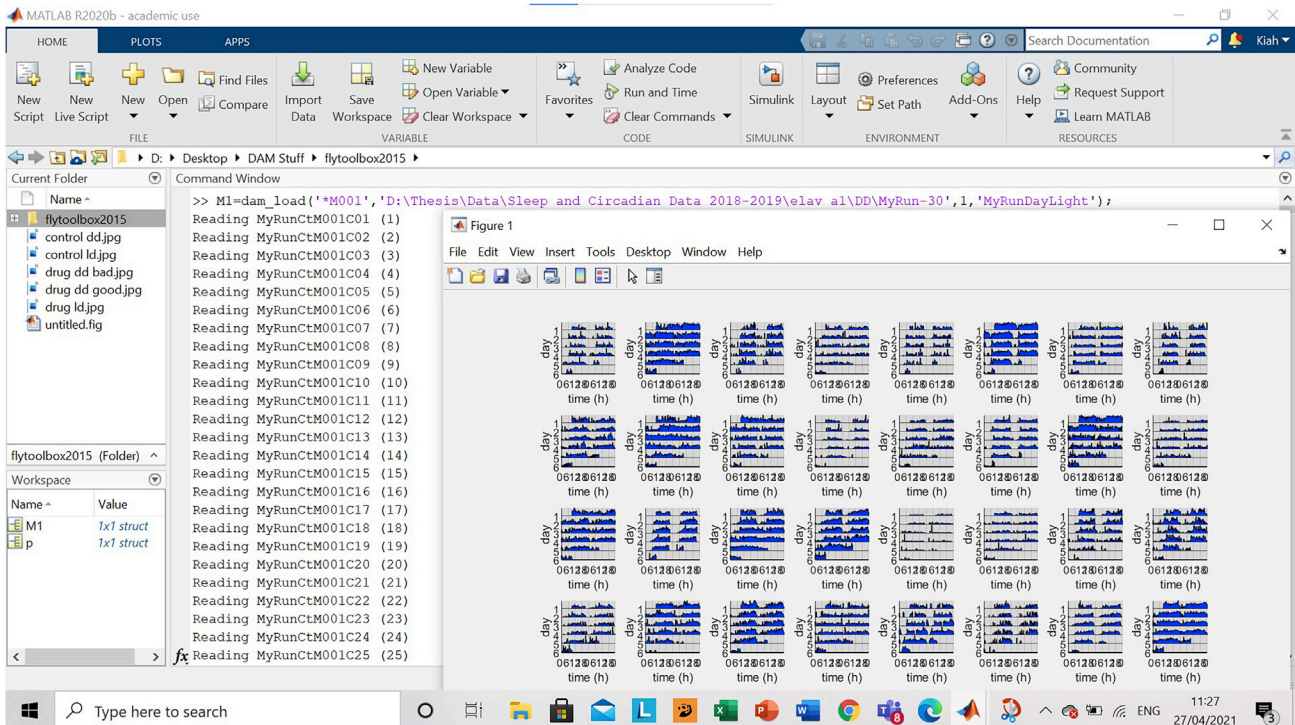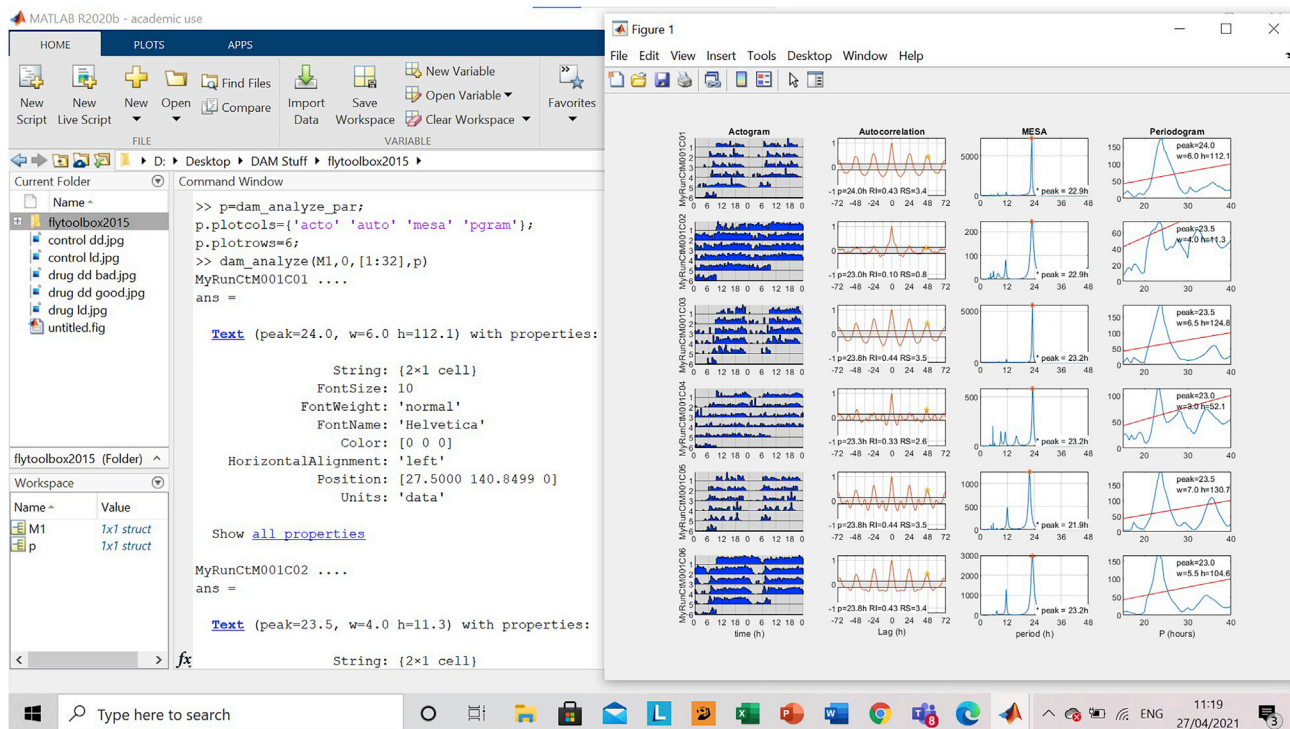

Figure 8. A screenshot showing how data is loaded into the *FlyToolBox* on MATLAB using the 'dam_load' code and the actograms produced by the 'ralf' code

Figure 9. A screenshot showing how the parameters can be set for analysis of activity and how the *FlyToolBox* can be used to produce actograms, autocorrelation, MESA and periodograms for individual foragers

the 'dam_analyze' code in the toolbox as seen in Figure 9. This produces a series of figures for each individual, including an actogram, an autocorrelation, a maximum spectral entropy analysis (MESA) and a periodogram (Figure 10). The readouts for the actogram and autocorrelation are then commonly used, to give a visual representation of activity, a rhythmicity statistic and period length for each individual (highlighted in Figure 10).

For the sleep data, analysis can be carried out in the *Sleep and Circadian Analysis MATLAB Program* (*SCAMP*, Donelson et al., 2012) in MATLAB (MATLAB and Statistics Toolbox Release 2015b). Again, run the raw data through DAMFileScan 108× (Trikinetics Inc., USA) and used the scanned files for analysis. Start *SCAMP* in MATLAB and select the 1 min and 30 min activity bins for analysis. Select the monitor for analysis and click 'load individual sleep plots' (as in Figure 11). This will show the sleep plots for each individual, allowing any individuals who were inactive the entire time to be discounted before carrying out analysis. Deselect any individuals that aren't being included and then click 'ANALYZE selected data'. Choose which sleep data and graphs to generate from the data (as in Figure 12).
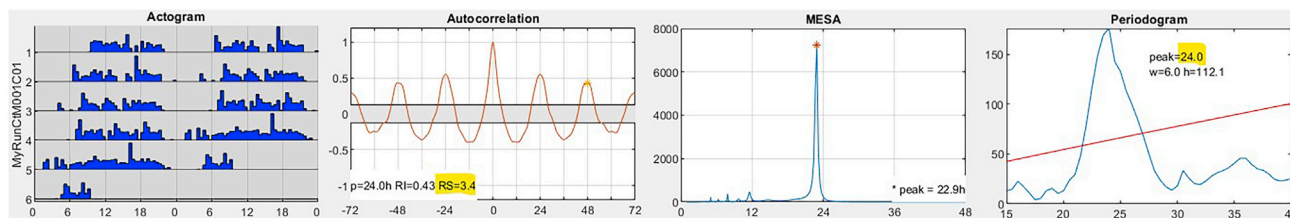
Figure 10. An example of the graphical output of the 'dam_analyze' code in *FlyToolBox*, with the rhythmicity statistic (RS) and period length (p or peak) highlighted
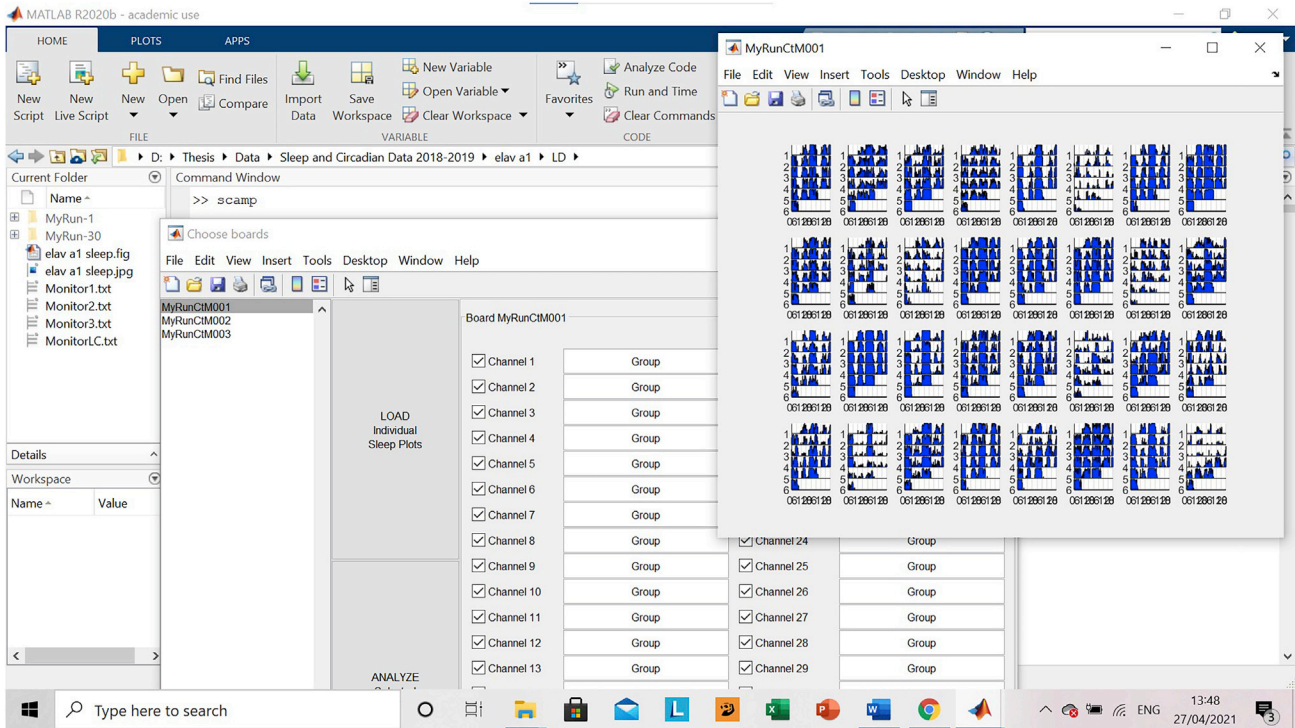
Figure 11. A screenshot showing how *SCAMP* can be initiated in MATLAB and sleep activity plotted for individual foragers
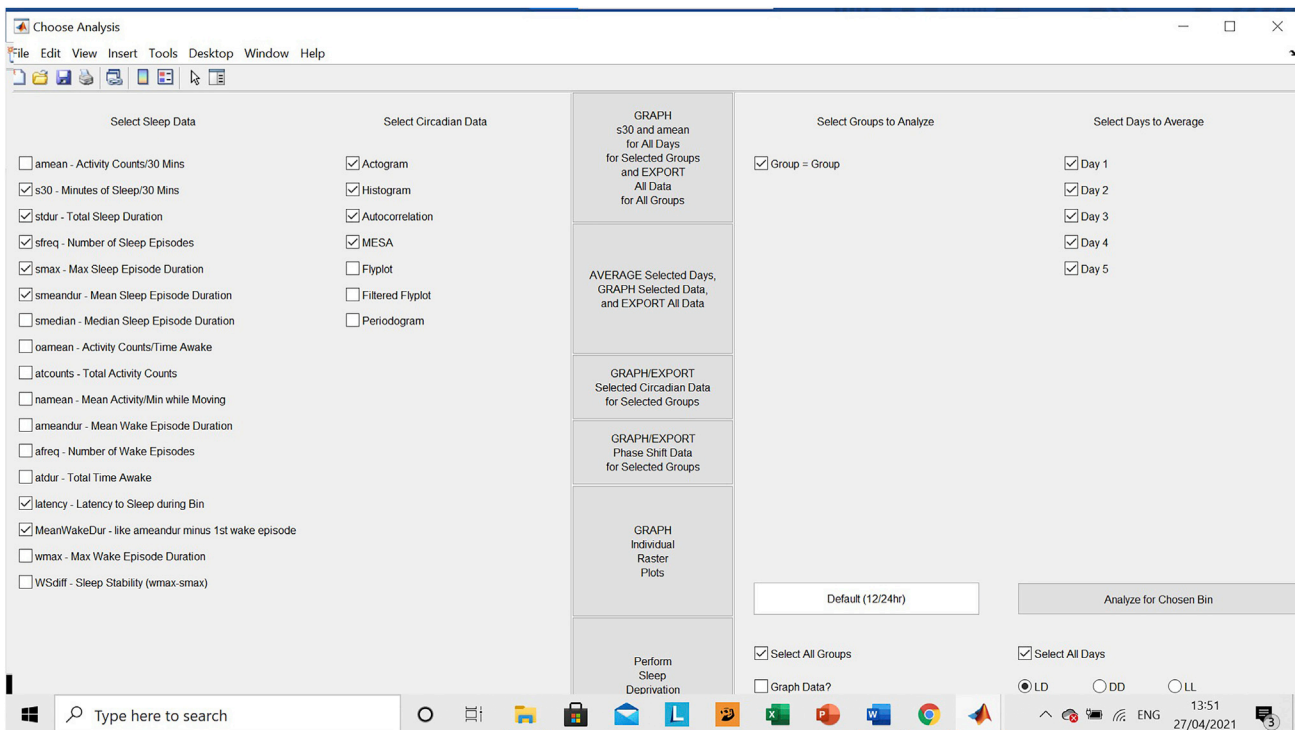

Figure 12. A screenshot showing the kinds of graphical and analytical outputs that *SCAMP* can produce for sleep data

A suitable statistical test should then be chosen to analyze the data collected. We used a two-way ANOVA to quantify the effect of both treatment and colony. Once colony effects were shown to be nonsignificant, colony was removed as a factor and a one-way ANOVA was carried out with *post hoc* pairwise comparisons using Tukey's multiple comparisons test (Tasman et al., 2020).

LIMITATIONS

One limitation of this methodology is that due to the 5 days in constant darkness, when the colony cannot be accessed, colonies can only be fed with pollen every 5 days during the experiment. Usually, colonies would be fed with pollen more frequently than this. However, due to the large, established nature of the colonies used (80+ workers) and the *ad libitum* supply of pollen provided before the experiment, this should not be detrimental to the colony over the experimental time period (10 days).

Another limitation is that in this methodology, a single RFID reader is used at the entrance to the colony, and each time a forager crosses the reader, this is counted as one activity bout. This provides no information about whether the bee was entering or leaving the foraging arena and does not allow us to collect more in-depth information about foraging e.g., the length of foraging trips carried out. This sort of foraging data was not the focus of our experiment and we wanted a simple measure of activity that was comparable to the beam crossing method of counting activity used for the individual foragers in the LAM. However, if someone wanted to collect this sort of behavioral data, it would be easy to adjust the methodology to allow this, by placing a reader at both the entrance to the colony and at the entrance to the foraging arena.

Another limitation is that this methodology considers bumblebees kept in a highly constrained artificial system with constant environmental conditions, easy access to food (with virtually no travel time), and no predators. All of these could have an effect on the routines and amount of sleep required by individual bees, when compared to wild foragers in a naturally fluctuating environment (where even the availability of food may be entrained to natural rhythms that lab-based bees do not experience, e.g., Bloch et al., 2017). Care therefore needs to be taken in extrapolating effects of the lab-based treatments onto wild-living bees.

One further limitation to this methodology is that the LAM set up cannot differentiate between inactivity and sleep. Previous studies (Sauer et al., 2003) have shown that in honeybees, 5 min of inactivity is a good indicator for sleep and this methodology has also been used successfully in bumblebees (Nagari et al. 2019). Thus, in most instances this method will work well for identifying and analyzing sleep. However, it may not be suitable for analyzing the effects on sleep of substances known to cause inactivity or immobilization in bees, or those that may change the distribution of sleep bout length. In these circumstances, other measures of sleep could be utilized, such as changes in antennal movement (Nagari et al., 2019) as has previously been used in bumblebees, or changes in arousal threshold as has been done in fruit flies (Faville et al., 2015).

TROUBLESHOOTING
Problem 1
There is the potential to tag drones (males), nurse bees, or bees who spend most of their time acting as nurses who happen to be in the foraging arena when foragers are collected. This will not provide useful data on foraging rhythmicity (steps 1 and 5).

Potential solution
Carefully choosing bees that are seen entering and feeding in the foraging arena and with a thorax width of approximately 5 mm should ensure that only foragers are used. Additionally, only those bees that show foraging activity every day of the study period should be used for analysis, ensuring that only foragers were assessed.

Problem 2

Foragers sometimes sit in the reader, or pass through it slowly, causing continuous reading of their tag by the reader. This could be misidentified as a large volume of activity if not recognized (step 7).

Potential solution

Readings for an individual that occur within 10 s of each other without another bee being read between them should be counted as a single reading.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. James Hodge (james.hodge@bristol.ac.uk).

Materials availability

No novel materials or reagents were used for these experiments.

Data and code availability

Original data have been deposited to Mendeley Data: https://doi.org/10.17632/m8pykxzkyb.1.

AUTHOR CONTRIBUTIONS

All experiments were carried out by K.T., who wrote the first draft of the paper. The project was supervised by J.J.L.H. and S.A.R., who secured funding and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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