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ABSTRACT
Honey bees (*Apis mellifera*) have a peculiar and complex reproductive biology, with queens being polyandrous and mating with several drones during one or more mating (nuptial) flights in so-called drone congregation areas. Observing the virgin queens' and drones' flight behaviour provides data to understand and interpret a portion of the honey bees' complex reproductive process. Observing the behaviour of the virgin queens on the hive entrance also serves to estimate the distance from the mating place or potential drone congregation areas (DCAs) as well as to detect the presence of airborne drones in the area.
In this paper, we provide a detailed description of the methodology used for observing queens' and drones' flights during the period of expected mating. In addition, we provide information about required equipment, tools as well as step by step description of the observation and recordkeeping process.

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Introduction

Honey bees (*Apis mellifera*) have a peculiar and complex reproductive biology, with queens being polyandrous and mating with several drones during one or more mating (nuptial) flights in so-called drone congregation areas (Woyke, 1955; Estoup et al., 1994; Palmer & Oldroyd, 2000; Tarpy et al., 2004; Tarpy et al., 2010). Young virgin queens leave the colony for three different behaviours: orientational flights, nuptial flights, or leaving the colony with an "after-swarm", i.e., a swarm that leaves after the fertile queen has left with a primary swarm (Winston, 1987; Kryger & Moritz, 1997). In parallel, drones also leave the colony to orient themselves, perform cleansing flights or perform nuptial flights (Reyes et al., 2019; Ayup et al., 2021). In case of successful mating, the respective drone dies. However, the sperm he passed to the queen is kept alive within the queen's spermatheca and used for worker or queen production. Unsuccessful drones have a short lifespan, which rarely exceeds six weeks (Page & Peng, 2001). The availability of young queens and drones corresponds to the swarming period, which occurs in the season(s) with abundant food resources, i.e., nectar and pollen. The swarming seasons and in some regions the so-called supersedure seasons vary among regions, reflecting seasonal resource availability.

Observing the virgin queens' flight behaviour provides data to understand and interpret a portion of the honey bees' complex reproductive process. In addition, observing the behaviour of the virgin queens on the hive entrance also serves to estimate the distance from the mating place or potential drone congregation areas

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(DCAs) as well as to detect the presence of airborne drones in the area (Koeniger et al., 2014; Scheiner et al., 2013). The collection of data on the queens' nuptial flights, such as flight frequency, timing, mating success, survivability, spermatheca filling etc., is usually the initial step in assessing the area's suitability as a mating station or for serving other means of mating control such as Delayed Flight Time (Oxley et al., 2010; Musin et al., 2021). Similarly, observing drones' flight activity can also contribute to the understanding of the complex reproductive biology of honey bees and provide information about the suitability of a location to serve as a mating station.

A standardised protocol is needed for the successful monitoring, interpretation, and comparison of data. The execution of the protocol requires a combination of beekeeping and technical skills, equipment, scrupulousness, and patience. The article describes the relevant research aspects for direct observations of honey bee queens' and drones' nuptial flights and expands on information which was shortly described in Büchler et al. (2013) and in Scheiner et al. (2013). Nowadays, besides direct live observations through the human eye, queens' and drones' flight activities can also be monitored by use of Radio-Frequency Identification (RFID), video surveillance (VS), and radar. However, these techniques require significant investment in equipment and infrastructure. In contrast, direct observations allow for instant data collection with the possibility to observe parameters often not accessible through more sophisticated methods.

Observation of honey bee queen nuptial flights

Five to six days post-emergence, the queen becomes sexually mature and starts to leave the hive, being pressed by the worker bees and being attracted to the light (Winston, 1987). A queen performs nuptial flights from noon to around 5 pm, usually on a calm and sunny day (Lensky & Demeter, 1985; Koeniger et al., 1989; Koeniger & Koeniger, 2007; Hayworth et al., 2009). The first flight(s) are short and orientational, followed by actual nuptial flights whose duration depends on drone availability and DCA proximity (Koeniger & Koeniger, 2007). To mate, queens and drones can fly for several km in search of a DCA. Drones at the DCA originate from surrounding colonies (up to 238 colonies reported on a DCA by Baudry et al., 1998) from distances up to 7 km (Ruttner & Ruttner, 1965; Ruttner & Ruttner, 1972, Utaipanon et al., 2019). Drones patrol the DCA and pursue the virgin queens once they arrive in a comet-like formation. The process of mating takes up to 5 s per drone. The queen mates with about 10-20 drones (Adams et al., 1977; Estoup et al., 1994; Neumann et al., 1999; Tarpy et al., 2004; Tarpy et al., 2015; Withrow & Tarpy, 2018). The subsequent drone removes the "mating sign", a structural tissue from the drone reproductive organ, of its predecessor (Koeniger, 1986). The average number of spermatozoa received by the queen is 87 million, the queen's spermatheca has a capacity of up to 6 million spermatozoa only (Baer, 2005). Excess sperm are disposed of, and the remaining spermatozoa migrate into the spermatheca, supported by contractions of the abdomen. The queen might take subsequent nuptial flight(s) on the same day or during the next few days to successfully complete the mating process. Approximately two to 14 days after mating, a successfully mated queen will begin to lay eggs.

There are several approaches a researcher can use to collect data on the queen's mating behaviour (Table 1). Direct observations of the modified mating box entrance provide a fast collection of data. In this method, the observer records the key events of the queen's mating behaviour (Table 1). Some training and practice are needed, but a skilled observer can observe up to 12 mating nuclei at a time. Video recordings do not require significant manpower at the observation site, but the video material needs to be analysed by rewatching, which is time consuming. RFID tags (Heidinger et al., 2014) in combination with video recording may speed up the analytical process; however, to the best of our knowledge, the two methods have not been combined yet. The choice of the method depends on the possibility of engaging trained people or using automated data analysis.

Equipment for conducting observation on queens' nuptial flights

The queens' nuptial flights can be accurately observed using tailored beekeeping equipment to allow for improved insight and control of the queen's activity. Therefore, the observation set (Figure S1) consists of a mating box (Figure S2) and an observation tunnel (Figure S3).

Although the volume and design of the mating boxes for the observation tunnels are flexible, attention should be paid to proper ventilation, feeding and entrance regulation (movable queen excluder or entrance regulator (Figure S4)). In addition, the mating boxes and their observation tunnels should be coloured and numerated distinctively for unambiguous identification.

The observation tunnel consists of three elements: body (Figure 1a), queen excluder (Figure 1b) and transparent lid (Figure 1c), which can be either a piece of fully transparent acrylic Plexiglas, a glass pane, or another piece of queen excluder.

Table 1. Pros and cons of different methods for data collection and observation of queens' nuptial flights.

Method	Number of observable parameters	Workload during observation	Analytical workload	Availability of results	Human resources	Costs	Recheckable observations
Direct observations†	5[1,2,3,4,5,7]	high	low	immediate	high	low	no
Video recording††	6[1,2,3,4,5,6]	low	high	takes time	medium	medium	yes
RFID†††	3[1,2,3]	low	low	immediate	low	medium	no
Video + RFID	6[1,2,3,4,5,6]	low	medium	immediate	low	high	yes

Observable parameters: [1]number of flights, [2]time of departure, [3]time of arrival, [4]mating sign, [5]mating sign removal, [6]behavior prior to departure/at arrival, [7]surroundings. **Sources:** †Scheiner et al., 2013; ††Reyes et al., 2019; †††Heidinger et al., 2014.

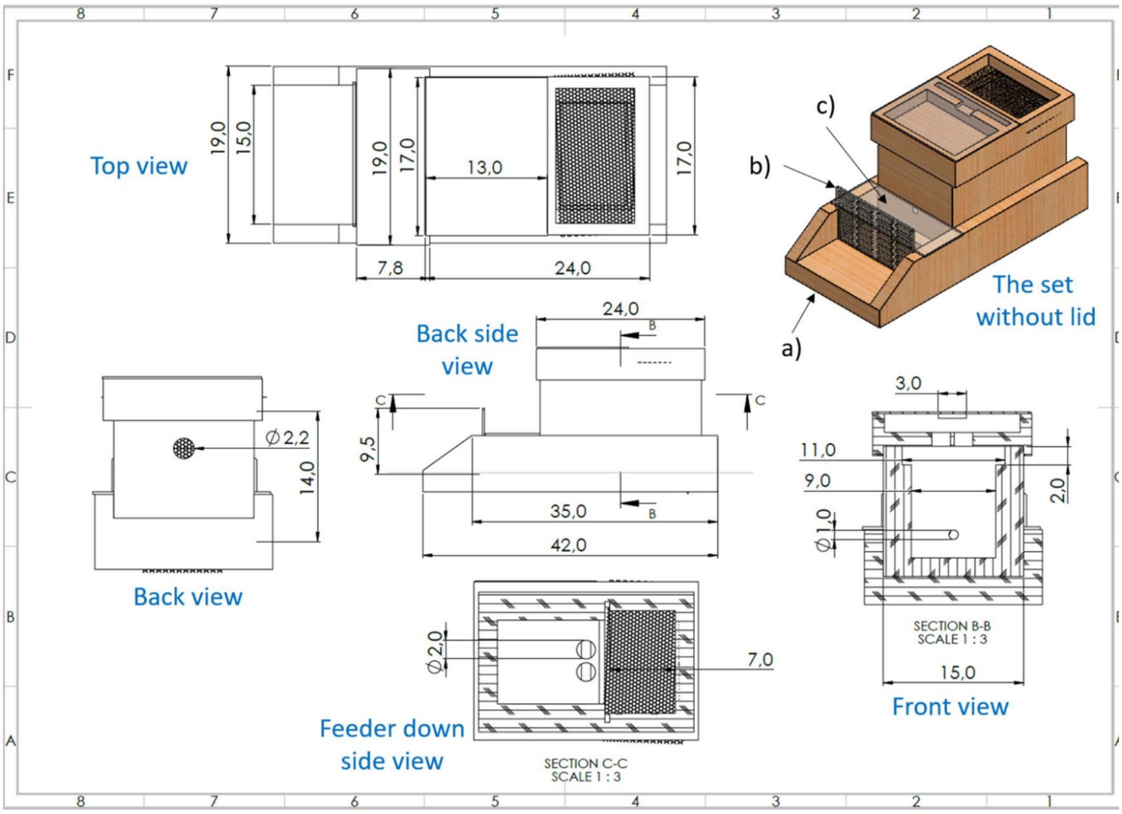


Figure 1. Schematic diagram in metric measurements (cm) of the observation set without the lid.

The body closes the tunnel from below and the sides and can be constructed from wood, waterproof plywood, styrofoam, plastic or other solid materials. The mating box may either be placed in the observation tunnel, or an observation tunnel may be attached to the mating box (Figure S5). To reduce the risk of drifting of queens, the tunnels can be coloured, preferring using different colours for the tunnels of neighbouring boxes. However, the numbering for both elements should be identical. Printing different geometrical forms on the front face of the mating box may also help; however, painting colourful shapes on the observation tunnel reduces the chances of recognizing the queen, particularly during her departure flights.

The transparent lid, which needs to lie on the top of the tunnel's sides, enables a full top view of the queen's activity, such as appearing at the entrance, passing the entrance, or the queen's contact with the lid or the excluder. The lid should be easily detachable from the remaining elements of the observation set.

The excluder, which should preferably be transparent, allows the worker bees to pass out the tunnel but at the same time prevents the queen from exiting or entering. The excluder is vertically held in place by shallow grooves on the tunnel's sides and bottom (Figures S3 and S5). The excluder is needed to enable brief retention of entering and leaving queens and is lifted once these events have been recorded to let the queen pass.

The observation set should be constructed with great precision in order to prevent any unintentional passage of queens or worker bees. The same requirement applies to the mating box. If adequately constructed, the workers can exit or enter the mating box by passing the queen excluder, while the queen may pass only when the observer allows it by removing the transparent lid or the queen excluder.

Queen handling and management of mating boxes

The details of the queen rearing procedure are given in Büchler et al. (2013, 2023 in press). Here we describe additional relevant aspects of the observations. For accurate visibility and more accessible observations, it is recommended to mark queens with coloured and numbered opalite plates (Büchler et al., 2013). Furthermore, the most convenient approach is to use identical numbers for the queens and mating boxes. Painting the queens without numbers is not an alternative, because the number of available colours will normally be too low to unequivocally distinguish each queen and decide whether she is returning to her own or a foreign mating box.

To rationalize the duration of the observation period, it is recommended to start the observations when the queens are six days old and under higher biological time pressure to mate. The observation tunnel with a queen excluder and transparent lid should be installed one or two days before the start of the observation to familiarize the bees with it. This will enable easier observations from the beginning. Since the virgin queens are usually sexually mature at this age, they frequently perform their first flight(s) already on the first observation day.

To observe the virgin queens' mating behaviour, different types of drone-free mating boxes are used, which can be formed either with or without capped worker brood, with a number of worker bees that is adjusted to the size of the mating box (not overcrowded and not under-populated). For an Apidea® type mating box, we recommend between 70 g and 100 g of bees, corresponding to approximately 600 to 900 worker bees. Special attention should be given under cool climates to avoid the under-population of the mating boxes. Broodless mating boxes need to be stored in a cold (approx. 15-18 °C) and dark room for about 72 h before being transported to the mating location and opened (with the use of a queen excluder). In such a way, the foraging bees can leave the mating box, but the queen will be confined for an additional 48 h until the first observation day.

Mating boxes must contain sufficient food supplies, preferably a sugar dough to minimize the possibility of robbery (especially important for mini mating boxes). Proper ventilation is also needed, preferably through the bottom or the top (Figure 1, top view) of the box, in order to avoid drifting of the returning queens to the ventilation openings, following the pheromones of the colony. If a ventilation hole is used on the mating boxes' back or another side (Figure 1, back view), it should be closed using a sponge, for instance, during the observation.

Mini mating boxes, in comparison to larger ones (MiniPlus, whole frames nucs, etc.) are formed with fewer worker bees and are more economical. On the other hand, due to limited space, they are more vulnerable to absconding and high/low temperatures. We recommend renewing the worker bees of the mini mating boxes after every series of mating queens to reduce the risk of absconding and to enhance acceptance of the new queen cells/virgin queens.

One should keep in mind that small queens may pass through the queen excluder undetected. Such queens should be excluded from the observations during the control inspection of the hatching success or subsequently at any given moment. The inspection of the queens should be performed in the evening before the first observation day or in the early morning on the observation day, outside the expected flying period.

Arrangement of the observation site (location)

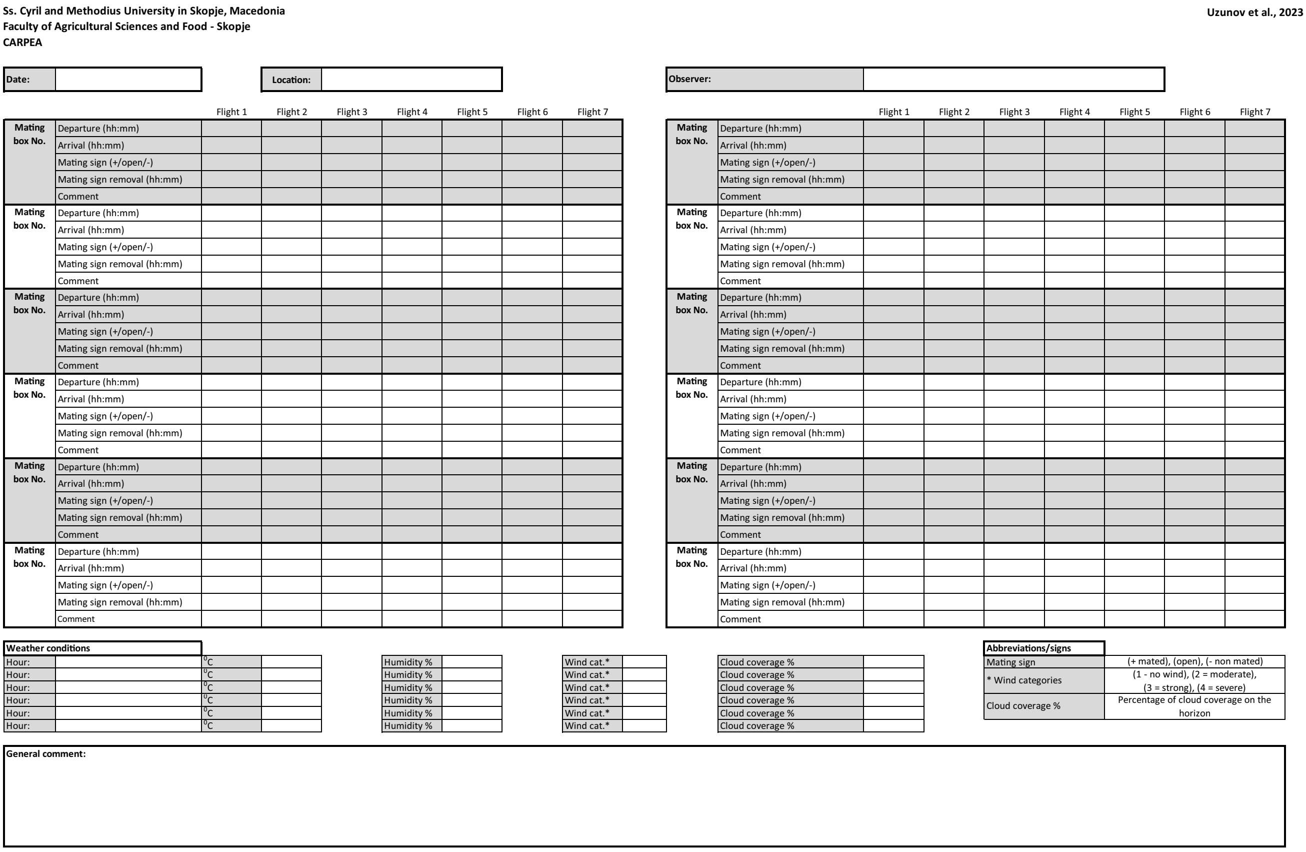
- Place the observation sets on the ground or a proper stand in groups. If the observer does not have much experience, arrange groups of up to five sets in a "U" or semicircle shape (Figure 3). If the observer is experienced, he or she can observe a maximum of 12 sets which should then be arranged in a circle. The entrances of the mating boxes, i.e., the front sides of the sets, should be directed towards the position of the observer, i.e., towards the centre of the circle;
- A distance of one meter between observation sets is suitable to allow for better operational and surveillance position and for placing landmarks to reduce the risk of drifting (Figure 3);
- Take care not to place sets (mating box, tunnel and queens) with the same colours next to each other;
- In the absence of natural landmarks, place some artificial landmarks between the mating boxes, such as big stones, pieces of wood, chairs etc.

Parameters, data collection, data processing and recordkeeping card

The number of parameters for which data are collected is given in Table S1 and consists of general and weather data/information as well as the data on queens' flight activity. The data is recorded on a special recordkeeping card (Figure 2) that each observer keeps a daily record of.

Here is a description of the elements of the recordkeeping card as well as the rational order of the observation and recording procedure.

- In the top field, the observer writes the date **"Date"**, the observation site/location **"Location"**, and its name and surname **"Observer"**.


Ss. Cyril and Methodius University in Skopje, Macedonia
Faculty of Agricultural Sciences and Food - Skopje
CARPEA

Uzunov et al., 2023

Date:		Location:		Observer:	

		Flight 1	Flight 2	Flight 3	Flight 4	Flight 5	Flight 6	Flight 7
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							

		Flight 1	Flight 2	Flight 3	Flight 4	Flight 5	Flight 6	Flight 7
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Mating sign (+/open/-)							
	Mating sign removal (hh:mm)							
	Comment							

Weather conditions

Hour:		°C		Humidity %		Wind cat.*		Cloud coverage %	
Hour:		°C		Humidity %		Wind cat.*		Cloud coverage %	
Hour:		°C		Humidity %		Wind cat.*		Cloud coverage %	
Hour:		°C		Humidity %		Wind cat.*		Cloud coverage %	
Hour:		°C		Humidity %		Wind cat.*		Cloud coverage %	
Hour:		°C		Humidity %		Wind cat.*		Cloud coverage %	

Abbreviations/signs	
Mating sign	(+ mated), (open), (- non mated)
* Wind categories	(1 - no wind), (2 = moderate), (3 = strong), (4 = severe)
Cloud coverage %	Percentage of cloud coverage on the horizon

General comment:

Figure 2. A daily recordkeeping card for recording data from the observation of queens' nuptial flights. Considerations such periods of observations, unusual events and conditions, and other circumstances could be recorded in the section "General comment."

- In "**Mating box No.**", the number of the mating box that the observer monitors should be recorded.
- Each queen flight is recorded separately in the columns "**Flight 1, Flight 2 …** ".
- In the row "**Departure**", the exact time in a format (hh:mm) when the queen is released and takes a flight is recorded.
- In the row "**Arrival**", the exact time is recorded in a format (hh:mm) when the queen returns from the flight and is allowed back into the mating box. Record the moment (time) when the first arrival of that flight attempt is noticed (as sometimes returning queen may circle around the mating box a few minutes before entering)!
- In the row "**Mating sign**", record whether the queen is mated or not (by observing the mating sign) with signs "+" and "- "or "open" if the queen comes with an open abdomen.
- In the row **"Mating sign removal"**, record the exact time in a format (hh:mm) when the removal of the mating sign is recorded (usually a worker bee holds the sign, Figure S7).
- In the row **"Comment"**, note anything which seems relevant for that particular flight.
- In the lower fields in the card, "**Weather conditions**" should be described every hour by recording the temperature (°C), humidity (%), wind (m/s) and cloud coverage on the horizon in the field "**Cloud coverage**" (%).
- In the "**General comment**", the observer may note any additional information that is considered relevant, such as the increased activity of a particular mating box, movement of the queen around the exit etc.

The observation period usually lasts up to 6 days under favourable weather conditions, ending when the queen is 12 days old. Under unfavourable weather conditions, the observation period should be extended to up to 10 days. The obtained data are the basis for the estimation of the basic parameters as well as for generating additional parameters (Table S2). When a high portion of the queens are mated or/and when under favourable weather conditions the queen's flight frequency is low or absent it may indicate the ceasing of the observations.

Procedure for conducting the on-field observations on queens' nuptial flights

Sunny weather with low wind velocity and a temperature above 20 °C is considered suitable for observing the nuptial flights of queens (Ruttner &

Ruttner, 1972). In areas with a colder climate or an expected cold period, observations might even begin at 18 °C (personal observations in Norway). If the prime interest is the assessment of the suitability of a particular location for mating control, observations should be conducted during the queen's naturally expected flying time, which is between 12 and 17 o'clock for Central Europe with most of the flights usually occurring between 13 and 16 o'clock. If the research focuses on studying reproductive biology, one may consider observation during a more extended daily period. However, the determination of the observation periods and consequently data analysis should be done by considering the local solar noon.

The observation site, the mating boxes and the tunnels need to be checked for their functionality and condition.

a. Observer's position, distance and movement

- The inspections start with continuous turns of checks of the front of the observation sets.
- The observer stays or moves at a distance (1 to 2 meters, personal observations) from where he/she has the best view of the observations' sets (Figure 3).

b. Manipulation of the observation sets

- When the virgin queen passes through the entrance of the mating box, the observer removes the lid or/and excluder and allows the queen to get airborne.
- The observer continuously follows the queen with his/her eyes until she flies after which the lid and the excluder are installed back in their positions.
- The queen's departure time (hh:mm) is recorded in the recordkeeping card, and the observer continues to observe the remaining observation sets.
- Once the queen returns and tries to land on any of the elements of the observation set, the observer needs to pay attention to:
  - queen identification (colour and number of plates)
  - time of the queen's arrival
  - presence of mating sign or status of opening of the caudal end of abdomen to determine mating success (Figure S6).
- The observer quickly removes the lid or/and excluder, after which the queen finds the entrance and rushes to get inside the mating box. The mating success can also be observed during the queen's entrance manoeuvre, which is sometimes very fast. The observer should keep the queen in visual contact until she enters the mating box.
- Make sure the transparent lid and the queen excluder are correctly placed back.
- Record the arrival time (hh:mm) and mating success ("+", "open" or "-") on the recordkeeping card.
- Continue to inspect all observation sets regularly.
- The removal of the mating sign might be observed when a worker bee is holding the sign in the mandibles and consequently has difficulties to pass through the queen excluder (Figure S7). The time of the mating sign removal needs to be recorded in the "Mating sign removal".
- At the end of the observation day, remove the transparent lid and the queen excluder and add a piece of queen excluder on the entrance which allows only the worker bees to leave the mating box.

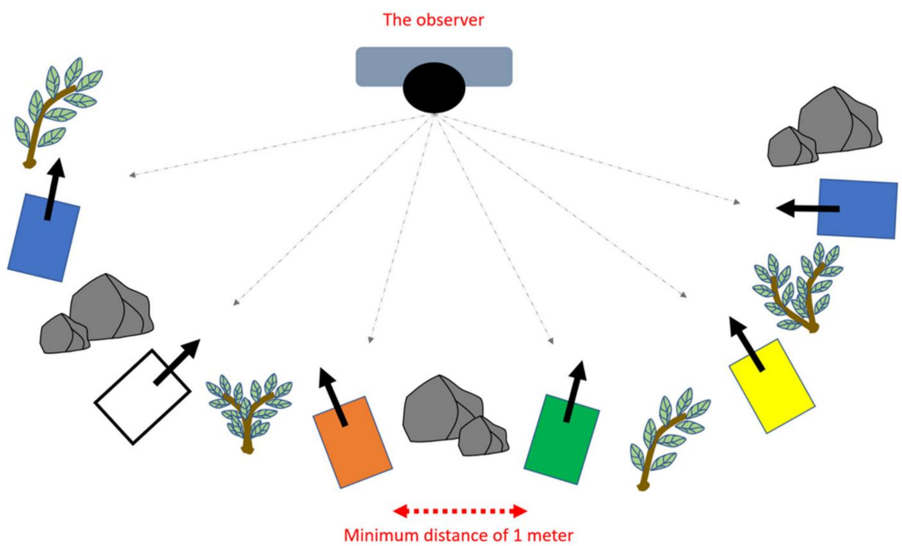


Figure 3. Arrangement of differently coloured mating boxes and adapters, entrance directions, landmarks and observer's position.

Timeline and general recommendations for observations on queens' nuptial flights

Consideration and coordination of different aspects, elements and efforts are required for ensuring an efficient observation. Here we present the timeline of the activities over the entire process as well as recommendations and tips for the execution of a well-organized on-field study.

Timeline

The entire process for observation of one set of queens lasts at least 53 days, from the start of the queen rearing over the on-field data collection to the sampling collection and observation of the queens' laying status (Figure S8).

A deviation of one day occurs when instead of virgin queens, queen cells are used, implying a shortening of the Preparatory period to −7 days and extending the Pre-observation period from −6.

If the primary purpose is to study the reproductive biology of the queens, which implies consideration of broader aspects and parameters, observations should start one day earlier and end 1—2 days later.

If drones from known origins are used in the study, the preparation of the DPCs (Drone-producing colonies) should start at least 45 days before the expected first day of observations, corresponding to an age of the queens of 6 days.

Recommendations

- Training of the observers will significantly improve the work flow and accuracy of data collection.
- The recommended number of observation sets (mating boxes and tunnels) is 1 to 5 for the first-time or non-experienced and 6 to 12 for the experienced observers.
- Time synchronization is required among all observers' timekeeping devices.
- It is necessary to keep focussed through the daily observation period (ca. 5 hours), which is particularly difficult when multiple queens fly at the same time and weather conditions are challenging.
- Poor weather conditions may alter the queens' flight behaviour. If later the weather conditions improve, the queens may extend their period of nuptial flights beyond their regular flying period.
- Keep the queen under observation whenever she is observed outside the mating box, particularly before the departure and after the arrival. However, once airborne, the focus of the observer should be on the entrance.
- Be aware that the first flights may be short and orientational.
- For the sake of simplicity, record all flights shorter than 60 seconds as one-minute-flights.
- Note in "Comment" if flight duration is extended due to the fact that the queen was resting somewhere (leaf, branch or another object) or was disoriented.
- Report in "General comment" incidences like absconding or intensive flight activity in front of the mating box/observation set.
- In case of a massive and rapid worker absconding from the mating box, keep the excluder in place and do not release the queen. This might prevent absconding.

Certainly, some information on mating success can be gained without direct observation, RFID or video surveillance by sampling and dissection of the mated queens (number of sperm in the spermatheca) and by using methods for molecular analysis of sperm (number of patrilines). The latter parameter can also be obtained if a queen is allowed to produce brood which later can be sampled and analysed (DeGrandi-Hoffman et al., 2003).

Observation of drones' nuptial flights

Queens' and drones' flight times significantly overlap under similar weather condition thresholds. However, their activities seem independent (Gary, 1963; Reyes et al., 2019), since both partners will fly regardless of the presence of the opposing sex. The drones start flying about half an hour earlier, visiting DCAs where they perform convoluted flights, waiting for the possible virgin queen's arrival (Woodgate et al., 2021).

Observing drones' flight activity at the hive entrance may have multiple uses. The hive entrance is an appropriate spot for assessing the drones' flight time, flight frequency, and possible drifting from one colony to another. All of this is possible by systematic observation in the front of the drones' colony using a piece of tailor-made simple equipment. In addition, flight activities or even flight patterns can be determined using RFID tags and other high-tech accessories (Heidinger et al., 2014; Reyes et al., 2019; Ayup et al., 2021).

Equipment for conducting observation on drones' flights

A transparent plastic or (plexi) glass lid acting as the ceiling of the tunnel at the height of a single drone (ca. 10 mm, please note that in Figures S9 and S10, the height is more than the recommended height) at the hive's entrance (Figure S10) allows monitoring of inbound and outbound honey bee traffic. The tunnel ceiling needs to be at least 5 cm long to give the observer enough time to recognize drones, particularly the arriving ones. However, avoiding strong traffic and congestion of the drones can be achieved by opening a hole on the closest hive part of the folia or the lid (Figure S10). One should be aware that the length of the hive's landing platform may limit the length of the transparent tunnel.

Management of drone producing colonies

The establishment and maintenance of the DPCs are detailed in Büchler et al. (2013, 2023 in press). However, here we need to stress that the use of the DPCs depends on the study goal and design, which may be the assessment of existing mating stations, identification of new isolated locations or study of the DCA or reproductive biology of the drones and/or queens.

Observation site arrangement

Conducting observation of the drones' activity requires the proper body position of the observers without interruption and disturbance by the flying worker bees. The colonies that are subject to the observation should be distanced from other colonies by at least 2 meters. If the stand of the DPC is elevated slightly, the observer may have an ergonomic position for observation and recording on the card. Sun exposure, reflection on the transparency sheet or lid and other weather conditions need to be taken into consideration. Since the observation of drone activities is intensive and demanding, comfortable working condition needs to be considered (Figure S11 and S12).

Drone marking and data collection

- Mark a minimum of 100 mature drones on their thorax (Figure S13) with an intense colour marker (Human et al., 2013) to allow accurate observation and verification of the drones' affiliation with the observed colony.
- The drones from each colony should be marked with distinct colours for easy identification of drifting, particularly when neighbouring colonies are observed.
- Use various coloured and numbered opalite plates, if the drones' individual flight behaviour is the prime focus of the study.
- The most suitable time for marking the drones is late in the evening a day before the observation or early in the morning on the observation day.
- Gently press the drones against the comb to verify their maturity. Mature drones will vibrate under the finger, whereas immature drones will not.
- For pilot studies, a limited number of drones can be colour marked in front of the entrance for the duration of 5 minutes after which you start observing the return/arrival of the marked drones and record it in intervals of 1 minute. Only the drones arriving within the first 5 minutes are considered as orientational (Figure S14). Due to drones' biological flight duration limit (Ruttner, 1966), the observation period for each colony is limited to 40 minutes.

Here is a description of the elements of the example of a recordkeeping card (Figure S14) as well as the logical order of the recording procedure:

- The observer records site/location **"Location"**, and his/her name and surname **"Observer"**.
- Under the column **"Date"**, **"Colony no."**, **"Time of marking"** and **"Marked drones (N)"** one should record the observation date, colony identification number, time of marking the drones (lasting for a maximum of 5 minutes) and the number of marked drones.
- The number of arriving drones is recorded in the fields of the columns from **"<5"** to **"40"** in an interval of one minute with the exception of the first five minutes.
- The column **"Missing (N)"** stands for the recording of missing drones after an observation time of 40 minutes.
- In the row **"Comment"** note anything which seems relevant for the observation of the hive.
- In the lower fields of the card "**Weather conditions**" should be noted, i.e., the temperature (°C), humidity (%), wind (m/s) and cloud coverage on the horizon in the field "**Cloud coverage**" (%).
- In the "**General comment**" the observer may note any additional information that is considered relevant.

Procedure for conducting the on-field observations on the drones' flights

The drones' activity is associated with similar weather conditions and daytime periods as for the queens. If the observation of drone flight activities is performed together with the queens' observations, then an additional person needs to be involved. Here is a description of the procedure for observation of drone flight activity on the colony level.

- The observation time should start half an hour before the observations of the queens.
- The observer records the arrival of each marked drone as a single slash line in the time interval cell for arrival.
- After a period of 40 minutes, the observer stops the observation, removes the transparent tunnel and moves to another colony, where the transparent tunnel was installed the day before. To avoid bias, each colony should be monitored

several times to cover the early, mid and late flying hours.

- If several colonies are monitored in the same timeslot, the observer also records the arrival of differently coloured drones (from neighbouring colonies) to obtain data on the flight duration of drifting drones and to collect data on drifting among drones. To obtain data on flight activity for each individual drone (flight frequency and duration), the drones should be individually marked with numbered opalite plates.
- For each individually marked drone the time of departure and time of arrival should be recorded throughout the normal flight period of drones.

General recommendations for observations of drones' flights

- Efficient observation and accurate data collection rely on well-trained observers.
- An observer can only collect data from a few colonies per day.
- The use of a counter clicker is recommended due to high drone flight frequency.

Non-direct methods for observation of queens' and drones' nuptial flights

Video surveillance is an alternative to direct observation. While being expensive if the purpose is to record several hives at the same time, the prices of cameras - such as HD webcams - and the availability of inexpensive computers and storage have made the method accessible. While easy on manpower at the time of data collection, the downside is the availability of the results. Without the time points at which to look at the hive, the observer must go through all recordings to find events of interest. For that reason, coupling video recordings to RFID (see below) would probably be best. However, the recordings are available for later check-ups and might also offer information on behaviour around the hive entrance. Also, video recording is the only method that does not interfere with the observed individuals (provided the camera is installed before the onset of flights).

RFID (Radio-Frequency ID tagging) is a method (Heidinger et al., 2014; Reyes et al., 2019) that allows monitoring of passage through a narrow entrance, such as the hive opening. The tag attached to an individual - such as a queen bee - contains a microchip, but no battery. The tag is passively powered by the reader (antenna) which interrogates the microchip at each approach, the length of the antenna, frequency employed and power of the reader defining its operating area/distance. The microchip contains identity information, as each tag has a unique id number, giving exact information on who/what passed the antenna at a certain time. Two sequentially installed readers allow for the identification of the direction of movement (out/in).

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