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REVIEW ARTICLE

Standard methods for chemical ecology research in *Apis mellifera*

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Summary

This paper describes basic methods essential in elucidating chemically-mediated behavioural interactions among honey bees, and between honey bees and other arthropods. These range from bioassay methods used to demonstrate the role of specific behaviours, techniques and equipment used to collect and analyse semiochemicals (both volatiles and non-volatiles e.g. cuticular hydrocarbons) from individual honey bees, groups of bees or an entire colony in its native environments. This paper covers: collection and analysis of honey bee volatiles in the natural environment, collection and analysis of bee volatiles out of their natural environment and their antennal detection, collection and analysis of non-volatile cuticular hydrocarbons, bioassays with queen pheromone and finally a section focusing on *in vitro* bioassays as a tool for elucidation of mechanisms regulating pheromone gland activity.

Métodos estándar para la investigación en la ecología química en *Apis mellifera*

Resumen

Este artículo describe los métodos esenciales básicos para dilucidar las interacciones de comportamiento mediadas por la química entre las abejas y entre éstas y otros artrópodos. Estos van desde los métodos de bioensayo usados para demostrar el rol de comportamientos específicos, hasta las técnicas y equipamientos usados para colectar y analizar semioquímicos (tanto volátiles como no volátiles, por ejemplo hidrocarburos cuticulares) en abejas al nivel individual, en grupos de abejas o en una colonia entera en su ambiente natural. Este artículo engloba: colección y análisis de los volátiles de la abeja de la miel en su ambiente natural, colección y análisis de los volátiles de la abeja fuera de su ambiente natural y con su detección por las antenas, colección y análisis de los hidrocarburos cuticulares no volátiles, bioensayos con la feromona real y finalmente una sección enfocada a los bioensayos *in vitro* como herramienta para dilucidar los mecanismos que regulan la actividad de la glándula que produce feromonas.

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西方蜜蜂化学生态学研究的标准方法

摘要

本文介绍用于研究蜜蜂之间以及蜜蜂与其它节肢动物间化学介导的交互行为作用的基本方法。涵盖用于论证特定行为的作用的生物测定方法，以及从蜜蜂个体、群体或整个蜂群自然环境中收集和分析化学信息素的技术和装备，包括挥发性和非挥发性成分，如表皮烃类。本文包含：在自然条件下收集和分析蜜蜂挥发物；在非自然条件下收集和分析蜜蜂挥发物及这些挥发物的触角探测；收集和分析非挥发性表皮烃类；生物测定蜂王信息素；最后集中介绍用于阐明信息素腺体活性调控机理的体外生物测定方法。

Keywords: COLOSS *BEEBOOK*, semiochemicals, headspace, EAG, exocrine gland, *in vitro*, QMP, honey bee

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1. Introduction

Maintenance of integrity of a honey bee colony is highly dependent on a sophisticated communication system that is largely dependent on chemical cues. Due to the crowded environment within the hive, bees appear to communicate to large extent through chemicals, of low volatility, vibrations, and other near-contact or contact modalities (Blum and Fales, 1988; Breed *et al.*, 1988; Naumann *et al.*, 1991; Breed, 1998; Slessor *et al.*, 2005). However, volatiles form a small but important part of the signalling chemicals (semiochemicals), that mediate interactions between colony members (for reviews, see Free, 1987; Pankiw, 2004; Slessor *et al.*, 2005). Some volatiles are used as releaser pheromones to rapidly communicate information to the rest of the colony (Pankiw, 2004), including alarm pheromones for defence (Boch *et al.*, 1962; Hunt *et al.*, 2003) and Nasanov pheromones for colony cohesion (Boch and Shearer, 1964; Pickett *et al.*, 1980). Other volatile pheromones, such as (*Z*)-β-ocimene, have long-term primer effects on physiology, development and fertility (Maisonnasse *et al.*, 2009; Maisonnasse *et al.*, 2010). A few volatile pheromones, such as the alarm pheromone component isoamyl acetate (IAA), have both releaser effects on behaviour and primer effects on physiology (Alaux and Robinson, 2007). On a more localized scale, both volatiles and non-volatile contact cues (e.g. cuticular hydrocarbons) serve as signalling cues for intimate interactions of workers with their immediate colony environment such as hygienic behaviour (Masterman *et al.*, 2001; Gramacho and Spivak, 2003; Swanson *et al.*, 2009; Schöning *et al.*, 2012). Volatiles also mediate interactions of bees with non-nestmates.

Like many social hymenopterans, honey bees use non-volatile to volatile acquired colony chemicals to distinguish between nestmates and non-nestmates (Breed, 1998). Natural enemies of the honey bee such as the small hive beetle *Aethina tumida* and the parasitic mite *Varroa destructor* also use colony odours as kairomone cues (Nazzi *et al.*, 2004; Torto *et al.*, 2005; Torto *et al.*, 2007a; Nazzi *et al.*, 2009; see the *BEEBOOK* papers on small hive beetles (Neumann *et al.*, 2013) and varroa mites (Dietemann *et al.*, 2013) for more details on these organisms).

This paper describes basic methods essential in elucidating chemically-mediated behavioural interactions among honey bees, and between honey bees and other arthropods. These range from bioassay methods used to demonstrate the role of specific behaviours, techniques and equipment used to collect and analyse semiochemicals (both volatiles and non-volatiles) from individual honey bees, groups of bees or an entire colony in its native environments. This paper is subdivided into 5 main sections; collection and analysis of honey bee volatiles in the natural environment (Section 2), collection and analysis of bee volatiles out of their natural environment and their antennal detection (Section 3), collection and analysis of non-volatile semiochemicals (Section 4), bioassays with queen pheromone (Section 5) and the *in vitro* bioassays as a tool for elucidation of mechanisms regulating pheromone gland activity (Section 6). While this paper intends to provide simple easy to follow and replicate guidelines when working on the semiochemically-mediated interactions of honey bees, readers must bear in mind that chemical ecology requires basic understanding of behavioural biology and analytical chemistry, which are two very broad fields of study, that cannot be exhaustively dealt with in this *BEEBOOK* paper. Therefore, before embarking on any chemical ecology experiments, it is advised to consult a chemist (if a biologist) or a biologist (if a chemist) and the other *BEEBOOK* papers (e.g. for behaviour, Scheiner *et al.*, 2013).

2. *In situ* volatile collection of odours in the colony environment

2.1. Introduction

Semiochemicals play a vital role within and among colony interactions, and they also mediate interactions between honey bees and their parasites and predators. In this section, we describe methods used to collect and analyse volatiles from honey bees in their native environments. The discussion focuses on *in situ* collection of volatiles because natural emission rates are the most biologically relevant metric of volatile characterization. While volatile collection and analysis techniques are described broadly, these methods are specifically discussed in the context of working with a hive and its inhabitants. Given the complexity of honey bee chemical interactions, the authors strongly emphasize that detection of a compound in the gaseous phase does not confirm activity of the compound in that phase (see Keeling *et al.*, 2003, on contact activity of volatile queen retinue pheromone components). Researchers use bioassays to determine the mode of biological activity for volatiles collected from honey bees (Torto *et al.*, 2007b). *In situ* analytical methods are most informative when used in tandem with other methods to characterize volatiles. In particular, *ex situ* volatile collection (see section 3 of this paper) and chemical analysis of tissue extracts (see sections 4 and 6) can be used to specifically identify the odour source in the colony and within the bee itself.

2.2. Collection and analyses of honey bee volatiles

In situ volatile collection and analytical methods can be conceptually divided into four basic sections: headspace environment, volatile collection, volatile separation, and volatile detection and analysis (see Table 1).The reader should be aware that no one method provides all the information required to identify or characterize volatiles. Different methods are better suited for elucidation of compound identity, mass, quantification and sensitivity to trace volatiles (D'Alessandro and Turlings, 2006). In the following sections, the advantages and disadvantages of methods commonly used at each step of volatile collection and analysis are briefly discussed.

2.2.1. Volatiles in the headspace environment

Headspace environment refers to the techniques and devices used to manipulate the headspace, or air surrounding an odour source where volatiles are actively emitted. The volatile profile obtained from an odour source strongly depends on whether the headspace volatiles are contained (concentrated) or actively relayed to the collection device (via air flow) (for examples, see Heath and Manukian, 1994; Tholl *et al.*, 2006; Carroll and Duehl, 2012). Volatiles can be collected from an open or closed air space. Open sampling schemes are simple to carry out but result in variable losses of target emissions and contamination by background odours. Partially or completely enclosed air systems surround the odour source with a containment system (containers built of glass, metal and other odourless materials) to concentrate volatile emissions and control the sampling rate. For longer collections in an enclosed system, an air flow system is required to ventilate the bees.

Collection techniques can also be classified as dynamic or static. Dynamic flow systems use active air flow (push), vacuum flow (pull), or combined air and vacuum flow (push-pull) systems to move headspace volatiles through a filter containing an adsorbent material volatile trap. The benefit of dynamic collections is that quantification is easier because volatiles are trapped at a known rate. Static flow systems have little or no flow through the headspace. In a static airspace, there will be equilibrium between volatile compounds present in the solid or liquid source and the same volatiles in the gas phase. Changes in any volatile component will affect the equilibrium for all other components in the airspace blend. The main benefit of static technique is that it is very easy to use and does not require any expensive equipment or instrument modifications.

2.2.1.1. Volatile sampling in the headspace environment

The complete flow path for a closed push-pull flow system (described without connecting lines) is 1) air source, 2) bubbler humidifier, 3) air flowmeter array, 4) air line, 5) air port connector, 6) port connector extension tubes, 7) observation frame, 8) vacuum port connector, 9) absorbent filter volatile trap and 10) vacuum line (see Fig. 1). Each setup may not necessarily have all these features. A step-by-step guideline to the selection, assembly and sampling of headspace volatile is provided below.

- Collect volatiles in a headspace environment that mimics core colony conditions (Winston, 1987; Kraus and Velthuis, 1997).
- Use incubators and glass in-line bubblers to control temperature and humidity.
- To minimize contaminants, construct all enclosures, lines, and tubing before the volatile trap out of odourless glass, Teflon/ PFTE (polytetrafluoroethylene), copper, or stainless steel. All line materials under significant pressure or vacuum should be constructed of metal tubing. Materials after the volatile trap can be made of "dirty" (e.g. odiferous) materials such as Tygon plastics.
- Control the air and vacuum flow rates in closed and partially-closed collection systems with flowmeters. Flowmeters are necessary for quantitative comparisons of volatile emissions.
- Filter the main air source with gas filters (molecular sieve and activated charcoal) to eliminate water and oil contaminants from air pumps. Clean air can also be purchased in pressurized tanks.

Table 1. Headspace environments and methods commonly used for the collection and analysis of volatiles. Several of these methods have been well developed in other systems (e.g. plant-insect, microbial) but not applied to honey bee volatiles yet. Not all headspace environments and methods can be used interchangeably.

Headspace environment	**Volatile collection**	**Volatile separation**	**Volatile detection**
Enclosure type Open Partially-closed (excess air) Closed (balanced flow) ***Flow system*** *Static flow* *Dynamic flow* Push (air only) Pull (vacuum only) Push-pull (air and vacuum)	***Adsorption method*** *Thermal desorption* SPME Tenax TA *Solvent desorption* Porapak HayeSepQ (SuperQ) Tenax TA Activated charcoal	***Injector type*** Split Splitless On-column SPME (insert) Tenax TA (trap) ***Column type*** HP-1 (non-polar) DB-1 (non-polar) DB-35 (polar) Affinity Chiral	***Detector type*** *Mass spectrum* Electronic ionization (EI – compound identity) Chemical ionization (CI – compound mass) *Ionization (detection/quantification)* Flame ionization (FID - organic compounds) Photoionization (PID - heteroatoms) Flame thermionic detector (FTD - heteroatoms) Electron capture (ECD - nitrogenous compounds) Flame photometric (FPD - sulfides) *Thermal conductivity (detection/quantification)* Thermal conductivity (TCD) *Electrophysiological response* GC-EAD (electrophysiology)

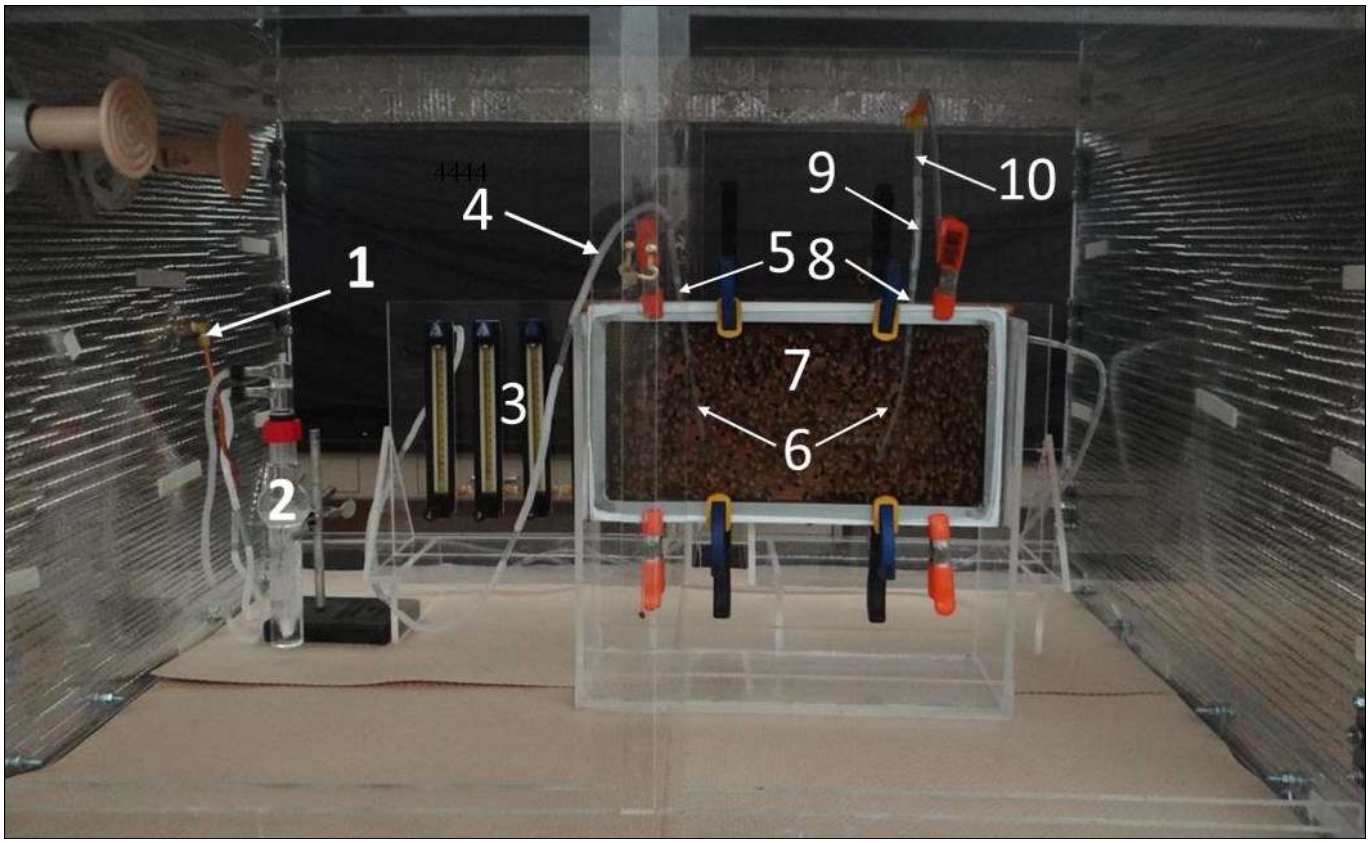

Fig. 1. *In situ* collection from a whole frame face with an observation frame containing pupae and adult workers. Air flows sequentially from the **(1)** air source through the **(2)** bubbler humidifier, **(3)** air flowmeter array, **(4)** air line, **(5)** air port connector, **(6)** port connector extension tubes into the **(7)** observation frame. Sample headspace is pulled through the **(6)** extension tubes through the **(8)** vacuum port connector, **(9)** adsorbent filter volatile trap, by the **(10)** vacuum line which is regulated by the vacuum flowmeters (hidden by the observation frame). The collection equipment is kept in a temperature controlled environmental chamber to keep the frame near colony temperature (32°C). Some flow system features such as the air source pressure reducer, air filter, air heater, vacuum pressure regulator and the vacuum source are not visible in this picture.

- Provide airflow to ventilate subjects without creating disturbance. A flow rate that exchanges the air in the container about once every 20 minutes is sufficient.
- Protect SPME fibres, adsorbent traps, and other sensitive objects from direct contact with bees by shielding the devices with a screen or Teflon shield.
- Collect appropriate odour "blanks" to account for background odours originating from the equipment, the air supply, and the surrounding environment. Use hive equipment of similar age (preferably less than two years old) whenever possible.
- Limit background odours by eliminating unnecessary odour sources. Avoid hive materials containing dead or diseased bees, rotten food stores, active small hive beetle infestations, or odiferous treatments such as essential oil patties. Limit the use of smoke. If use of smoke is unavoidable, pass clean air through the collection system for 30 minutes before volatile collection, or collect appropriate background samples.
- If needed, scale up the amount of volatiles sampled either by extending the sampling period or increasing the sample size by forming homogenous patches (e.g. single age brood cohorts, pollen patches, and simultaneous infection of brood).
- Avoid accidental contamination of collection equipment by household cleaners and detergents. Use GC grade solvents (ethanol, methanol, hexanes, and acetone), water, or unscented detergent to clean equipment. Equipment can be baked at 100°C for 1 hour to remove most volatile contaminants.

2.2.2. Collection and recovery (desorption) of volatiles

Volatiles are collected by trapping sample odours on exposed inert polymer matrix (adsorbent packing materials) which vary in their adsorbent properties and method of analysis. The trapped volatiles are then removed by using either heat (thermal desorption) or solvent (solvent desorption) to strip (desorb) the compounds from the packing material. Collected volatiles can be desorbed either directly into the analytical instrument or into a solvent that is later analysed. Each approach has distinct advantages and disadvantages in terms of information generated, ease-of-use, and cost to the researcher and are described below.

2.2.2.1. Solvent desorption of volatiles

The most common method for dynamic headspace collection is to use adsorbent materials that require a solvent wash to remove trapped volatiles (Heath and Manukian, 1994; D'Alessandro and Turlings, 2006; Tholl *et al.*, 2006; Carroll and Duehl, 2012). In general, adsorbent techniques that employ solvent extraction are best adapted for large scale sampling over longer periods of time. Solvent-based adsorbent techniques can also be used to quantify volatiles in closed airspace systems or when the relative capture rate can be calculated. Sample headspace air is actively pulled through an adsorbent filter trap where the volatiles adhere onto the adsorbent matrix. Commonly used adsorbent materials include Super Q, Hayesep Q, Porapak Q, Tenax TA, and speciality adsorbents that target specific chemical groups (Núñez *et al.*, 1984; D'Alessandro and Turlings, 2006). Most of these adsorbents trap large quantities of volatiles without much bias toward specific chemical classes. The captured volatiles are desorbed off the matrix by a solvent (chosen on the basis of the polarity of the target compounds i.e. polar solvent for polar volatiles, non-polar solvents for non-polar volatile) rinse. An active vacuum source is required to draw the sample air through the tightly-packed adsorbent material. Refer to paragraphs 2.2.2.3. and 2.2.2.4. for application examples.

Pros: A distinct feature of solvent desorption is that only a small fraction of the sample solution is normally used during analysis. Thus, a single volatile sample can be analysed separately by different instruments and also tested for bioactivity.

Cons: On the negative side, analysing just a small fraction of the sample also decreases sample sensitivity. Another disadvantage of solvent desorption is that highly volatile compounds may co-elute with the solvent peak during separation (Núñez *et al.*, 1984).

2.2.2.2. Thermal desorption of volatiles

Thermal desorption is an approach that uses heat rather than solvents to remove trapped volatiles from the adsorbent packing material. During analysis, the packing material is heated in a temperature-controlled environment in the analytical instrument and the volatiles desorb off the collection surface into the column flow. Thermal desorption techniques are particularly advantageous for: 1) sampling volatiles emitted at very low concentrations, 2) identifying highly volatile chemicals that would co-elute with solvent during separation, 3) obtaining a rapid profile of the volatiles associated with an odour source, or 4) collection of volatiles in a static system with very limited or no air exchange. Thermal desorption also has certain disadvantages compared to solvent desorption. Because the entire sample is desorbed on column during GC analysis, each sample can be analysed only once. For various reasons, most thermal desorption techniques are not particularly well suited to quantification of volatile compounds. Methods of thermal desorption using SPME and Tenax cartridges are described in the following sections.

2.2.2.2.1. Thermal desorption of static headspace volatiles by SPME

SPME (solid phase microextraction), is the most common thermal desorption method (Augusto and Valente, 2002). The SPME technique includes exposing a fibre to odour source headspace in a static environment with little or no air flow. SPME is ideal for rapid analysis

of volatiles emitted by small and strong odour sources, preferably in a closed container. Because of its ease of use, many researchers have begun to use SPME fibres in honey bee systems (Gilley *et al.* 2006; Schmitt *et al.*, 2007; Maisonnasse *et al.*, 2010). SPME has rather uniquely been used repeatedly to sample odours *in situ* from single bees in the open comb environment (Thom *et al.*, 2007).

Procedure:

1. Insert the SPME holder in the container with the sample. Volatiles should reach equilibrium (which should be in trial sampling and analysis) in the closed container before the SPME fibre is exposed to the headspace.
2. Expose the SPME fibre to the headspace without the fibre touching the sample or container.

The fibre should ideally be allowed to reach equilibrium with headspace volatiles – typical adsorption times ranging from a few seconds to 30 minutes.

3. Retract the fibre into the protective sheath.
4. Inject the fibre into a splitless GC injection port for analysis (see section 2.2.3.)

Trapped volatiles must be desorbed rapidly after collection since the trapped volatiles are exposed to heat and carrier gas flow and desorb off the fibre onto the column head as the fibre is heated.

The selection of SPME fibre type influences the sensitivity toward specific compound groups as well as the exposure time required to reach sorption equilibrium (Augusto and Valente, 2002). Polydimethylsiloxane (PDMS) fibres have been used to sample less polar volatiles and Carbowax/PDMS to capture more polar volatiles (Zabaras and Wyllie, 2002). Researchers should try a variety of related fibres to determine what works best for their system. For queen and worker volatiles, a number of fibre compositions were tested and PDMS/divinylbenzene was selected as the best (Gilley *et al.*, 2006). Consider the following guidelines for use of SPME:

- Use enclosed or partially enclosed static systems to limit dissipation of headspace volatiles (see Augusto and Valente, 2002 for a design). In general, the more static and concentrated the headspace volatiles, the more rapidly equilibrium is achieved.
- To limit background contaminants, only expose the fibre from its sheath when you are actively collecting volatiles. Protect the exposed fibre from the bees and hive materials with a Teflon jacket perforated with holes.
- Before sampling, bake the fibre in the GC port to remove residual volatiles left on the fibre. Follow the guidelines in the instructions that come with the SPME fibre.
- If unable to detect compounds of interest, try a longer equilibrium and exposure times first and different fibre materials next.
- Once specific chemicals of interest have been identified, optimize detection with chemical standards. Test equilibration time and sensitivity by exposing the fibre to each standard's headspace for different periods of time. Fibres have reached minimal equilibrium time when the volatile capture no longer increases with exposure time.

Pros: SPME is easy to use

Cons: Collected compounds cannot be stored on the fibre for longer period before analysis. Researchers should take caution in over-interpreting the volatile profiles obtained with SPME. Fibres are easily contaminated by background volatiles that may not be present in the target odour source. SPME is also poorly suited for volatile quantification because the fibres have different affinities for different chemical classes (Agelopoulos and Pickett, 1998). Unfortunately, the adsorbance rate of each volatile can be significantly influenced by the other compounds present in the headspace (Romeo, 2009). There are methods to quantify SPME samples, but given the variable chemical affinities, it is difficult to calculate amounts with confidence (Augusto and Valente, 2002). Volatile emission rates are often expressed as relative emission ratios rather than absolute amounts. For these reasons, researchers should use other methods to quantify volatile emission rates (see sections 2.2.2.3 and 2.2.2.4).

2.2.2.2.2. Thermal desorption of dynamic headspace volatiles by Tenax

A second set of thermal desorption techniques combines the sensitivity of thermal desorption with the controlled sampling of dynamic headspace collections. Collection of sample volatiles is similar to other dynamic headspace collection techniques. Unlike SPME, sample peaks collected and separated by thermal desorption can be readily quantified using techniques for solvent-desorbed collection systems (see section 2.2.2.3.).

Sample headspace is actively drawn by vacuum through a cartridge where volatiles are trapped in the adsorbent packing material. The packing material most commonly used is Tenax TA (replacing Tenax GC), which can be combined with activated carbon to increase capture of both non-polar and polar certain chemicals (Raguso and Pellmyr, 1998). Thermally-desorbed Tenax has a long history of use in honey bee systems and is still used today (Moritz and Crewe, 1991; Schöning *et al.*, 2012). The cartridge is later (immediately after volatile sampling) inserted into a modified GC injection port (which is larger than the normal syringe needle injection port to accommodate the Tenax filter) and rapidly heated to desorb all of the trapped volatiles from the packing material onto the column (see section 2.2.3.). Refer to sections 2.2.2.3. and 2.2.2.4. for application examples.

Pros: Like SPME, Tenax thermal desorption has a distinct advantage over solvent-desorbed samples in the absence of solvent peaks that may obscure highly-volatile compounds. Typically, samples are analysed immediately after volatile collection.

Cons: One major disadvantage of Tenax is that these thermal desorption methods require significant equipment and expertise compared with SPME and solvent desorption methods. Tenax collections make use of the volatile collection infrastructure used with solvent-extracted adsorbents as well as a modified injector port. Compared to solvent desorption, thermal desorption is a relatively slow sample injection technique that will lead to peak broadening for very volatile compounds during GC separations. These problems are partially corrected by cryofocusing techniques that use an automatic thermal inject system to rapidly heat and inject sample chemicals onto the column. Because cryotrap methods are advanced techniques, new researchers should use other collection techniques to initially sample volatiles (see sections 2.2.2.2.1 and 2.2.2.3).

2.2.2.3. Sampling odours at the whole colony scale

Whole colony volatiles can be collected from colonies using the hive equipment itself as a partial enclosure to concentrate colony volatiles. The colony must be well sealed to capture colony odours before volatiles escape to the outside atmosphere. Either replace leaky hive components or plug the gaps with wax. Collect a sample from air outside the colony as a control since this air replaces the colony headspace.

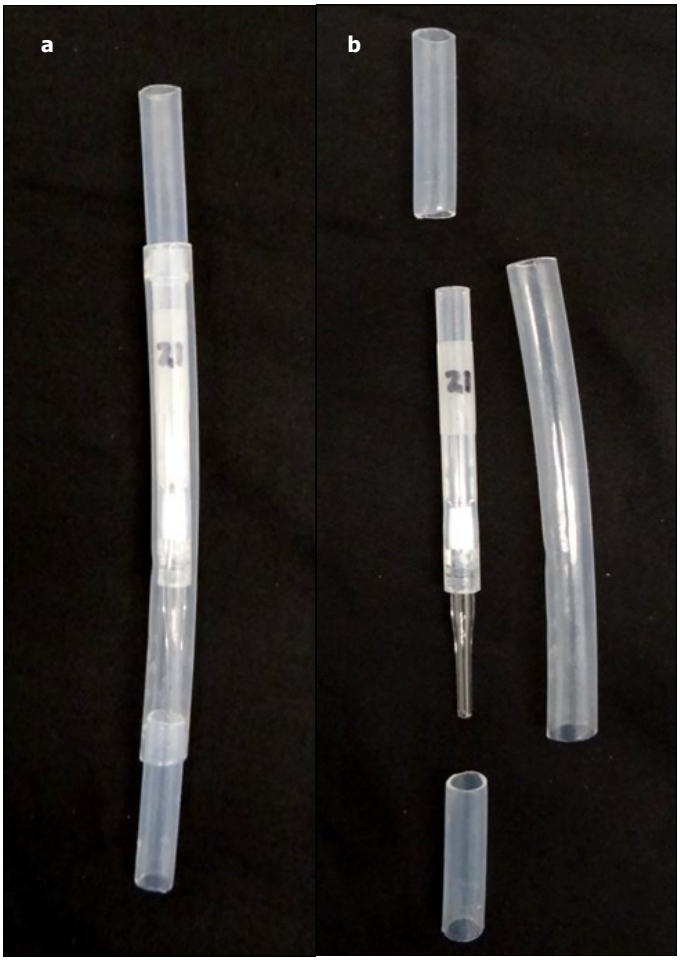

Fig. 2. **(a)** Assembled and **(b)** unassembled views of a SuperQ adsorbent filter enclosed in a protective Teflon tube jacket made of interlocking sections of tube.

1. Add 200 µl of the elution solvent (dichloromethane or hexane) to the filter solvent reservoir (just above the adsorbent packing material).
2. Gently push the solvent through the packing material with a clean air or nitrogen flow.
3. Repeat steps 1 and 2 two times.

This procedure rinses residual contaminants from the adsorbent filter to prepare the adsorbent filter trap for volatile collection.

4. Place the filter into a sleeve jacket made of short interlocking sections of rigid Teflon tubing (0.635 cm OD, 0.794 cm OD, 0.952 cm OD) (Fig. 2) to protect the filter from the bees.

The top of the filter needs to attach tightly to the jacket tubing; otherwise, air will flow around, instead of through, the filter.

5. Construct a sampling tube out of 0.64 cm OD Teflon tubing that reaches from outside the colony to the centre of the colony (~ 30 cm for a Langstroth deep).

Cover the end with metal screen to prevent bees from entering the tube.

6. Carefully insert the sampling tube into the colony either through the entrance or a small hole in the equipment to the centre of the colony.
7. Attach the adsorbent filter in-line between the sampling tube and a flowmeter-regulated vacuum line leading to the vacuum pump.
8. Collect colony volatiles by pulling colony headspace through the filter at 600 ml/min to 3l / min with flowmeter-regulated vacuum (exchange at least one volume of colony airspace every 20 minutes).

Most volatile collections require 3 to 12 hours to collect sufficient material for GC analysis.

9. End the volatile collection by removing the filter trap from the vacuum line.
10. Carefully remove the filter from its protective plastic jacket.

Secure the filter in a holder.

11. Add 5 µl of an internal standard solution (80 ng nonyl acetate/ µl) directly to the top of the adsorbent packing material with a syringe.

Avoid touching the packing material with the syringe.

12. Place a GC vial or vial with a glass insert directly underneath the tip of the filter.

13. Add 200 µl solvent (dichloromethane or hexane) to the solvent reservoir above the packing material to extract the trapped volatiles from the filter packing material.
14. Gently push the solvent through the packing material at a steady drip with clean air or nitrogen flow.
15. Cap and store the sample vials in a -80°C freezer until GC analysis (see section 2.2.3.).
16. Volatile emissions can be calculated by comparing compound peak areas to the known amount of internal standard added to the sample:

$$\text{Sample compound amount (ng)} = \frac{\text{Area of compound peak}}{\text{Area of internal standard peak (area counts)}} \text{ x internal standard amount (ng)}$$

Note that quantification may be difficult if large amounts of the whole colony volatiles escaped the colony headspace before collection due to a slow sampling rate (colony volumes/h) or poor seals between hive equipment.

2.2.2.4. Sampling odours at a whole frame scale

Volatiles can be sampled from bees enclosed on a single frame face with a partially-enclosed push-pull airflow system (Fig. 1; Carroll and Duehl, 2012). A metal and glass observation frame is pushed into the wax comb of a colony frame to enclose the bees and materials on the frame face inside. Controlled airflow from the enclosed headspace through a filter trap allows for ventilation of the bees and recovery of most volatile emissions. This approach provides a much more targeted method for *in situ* sampling of colony volatiles since background odours from most hive materials and outside air are excluded from the collection.

1. Weld together the observation frame from metal L-bar material. This rectangular frame should precisely fit the inside the inner perimeter of the wooden bars of the colony frame (23.2 cm x 51.1 cm for a standard Langstroth deep) out of 1.91 cm stainless steel or aluminium angle L-bar. The two edges of the L-bar extend perpendicularly down toward the wax comb and outward horizontally as a flat phalange.
2. To provide access for air and vacuum flow into the enclosure, drill two 0.65 cm diameter port holes through the top edge of the metal frame.

Drill the holes about 7.70 cm in from the ends of the frame. Position the holes as close to the angle of the metal frame as possible.

3. Provide a junction (port connector) through the port holes with 4 cm pieces of 0.635 cm outer diameter (OD) Teflon tubing. Air and vacuum lines attach to the outside of the port connector and port connector extension tubes attach to the inside of the port connector.
4. To enclose the bees in the metal frame, cut a piece of 0.47 cm thick piece of rectangular plate glass that extends to the outer edges of the metal frame.
5. Form a partial gasket between the glass plate and metal frame of the observation frame by wrapping the perimeter of the glass plate 3 times with 0.635 cm wide Teflon tape. Secure the glass plate to the phalanges of the metal frame with four small (3.75 cm) spring clamps.
6. Connect air and vacuum lines consisting of 0.635 cm OD flexible Teflon tubing to the outside of the port connectors with slightly larger diameter pieces of Teflon tubing.
7. To direct flow into the centre of the frame airspace, attach an 11.6 cm long piece of 0.48 cm OD Teflon tubing to the inside of each port connector.

Orient these slightly curved port connector extension tubes toward the plate glass to avoid contact with the comb (Fig. 3).

8. The volatile collection system flow rates must be adjusted to final rates with all of the components in place except for the enclosed frame.

Removal of any part, especially the adsorbent filter traps, alters the resistance of the system to air flow. Likewise, changes in flow in one sampling line affects other sampling lines. Provide a slight excess of air flow (680 ml/min) to vacuum flow (515 ml/min) to ensure that no outside air enters the enclosed frame headspace. Check air and vacuum flow rates against a calibrated flowmeter placed in line after the air flowmeter and between the filter trap and the vacuum flowmeter.

9. Remove the adsorbent filter traps
10. Rinse adsorbent filter traps of residual contaminants 3 times with solvent as previously described in section 2.2.2.3.
11. Place the cleaned filters into protective tube jackets as previously described in section 2.2.2.3 (Fig. 2).
12. Select the frame face to be sampled.

The frame should have completely drawn comb without any major holes or gaps. Small cracks and gaps can be plugged with wax from the colony.

13. Carefully push the metal frame into the wax comb along the inner perimeter of the wooden colony frame.

If bees are present, move slowly to avoid crushing them.

14. Secure the observation frame to the colony frame with four C-clamps.
15. Remove any bees remaining on the outside of the frames.

Keep the enclosure out of direct sunlight to avoid overheating the bees.

16. Transfer the frame quickly to the volatile collection system site (Fig 3) and place the frame on a frame stand to hold the frame upright.
17. To ventilate the bees, connect one observation frame port connector to the air flow.
18. To collect volatiles, attach the filter trap in-line to the vacuum line and the other port connector. Check the air and vacuum

flow rates to see that they match the previous settings. Sampling times run from 30 minutes to 12 hours, with 3 hours being sufficient for most odour sources.

19. End the collection by detaching the filter trap from the vacuum line.

Maintain an air flow through the observation frame headspace to keep the bees ventilated until you return them to their colony.

20. Extract the trapped volatiles from the filter trap with solvent and analyse the samples as previously described in section 2.2.2.1.

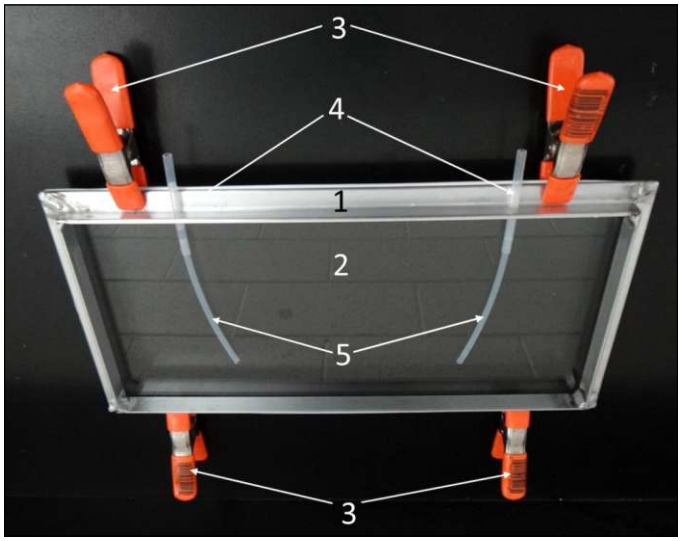

Fig. 3. Top inside view of assembled observation frame consisting of a **(1)** metal frame attached to a **(2)** glass plate by **(3)** four spring clamps and a Teflon tape gasket (hidden on other side). Air and vacuum enter across the frame through **(4)** two port connectors and **(5)** two port connector extension tubes.

2.2.3. Separation and analysis of volatiles by gas chromatography (GC)

Sample volatiles are separated by gas chromatography due to the greater sensitivity, easy reproducibility, and lower operating costs of this method (Handley and Adlard, 2005). Volatiles can be reliably separated under different chromatographic conditions by varying the injection conditions, the column, and the run parameters (temperature and flow). Here is a set of sample methods with comments on how certain parameters can be changed to evaluate chemicals with different volatilities.

2.2.3.1. Injector

GC injection ports are used to desorb trapped volatiles from the sample (solvent, surface, or adsorbent matrix) and channel sample volatiles into the column. Injectors differ in their temperature profiles and the proportion of sample volatiles directed to the column head. The following tips should serve as a guide when deciding on what kind of analysis and injector type to use.

1. For solvent desorption methods, the injection port temperature should be at least 10°C warmer than the maximum oven temperature used in the analysis. For thermal desorption methods such as SPME or Tenax, a slightly cooler injector temperature of 200°C or less is sufficient to desorb all of the absorbed volatiles.
2. The injection split refers to the proportion of the volatilized sample channelled onto the column. To maximize the amount of material injected on the column, use splitless injection.
3. Purge the injector at 1 minute to keep additional less volatile compounds from loading onto the column and degrading the column chromatography.
4. For thermal desorption methods such as Tenax TA, a modified injector improves the chromatography. By contrast, SPME thermal desorption and solvent desorption methods use an unmodified injection inlet with a special inner glass liner.
5. A serious drawback of most GC methods is that the extremely high temperatures commonly used in injector ports can thermally degrade unstable compounds. Destruction of these thermolabile compounds can be minimized through the use of Cool-on-column (COC) injector ports that allow sample volatiles to enter the column at low injector temperatures before the system is heated up.

2.2.3.2. Column

GC columns vary considerably in their polarity and affinity for different chemical functional groups. Compound separation is largely dependent on the affinity each sample compound has for the stationary phase (column coating material) relative to the mobile phase (nitrogen, hydrogen or helium carrier gas). A standard column is a DB-1 column (30 m long x 0.25 mm ID x 0.25 µm coating thickness), a column with a nonpolar stationary phase (a column whose inner surface is lined with a nonpolar matrix such as dimethylpolysiloxane) which is an excellent general purpose column that separates nonpolar and slightly polar compounds very well. Polar compounds such as organic acids chromatograph poorly on this column. Similar nonpolar dimethylpolysiloxane (PDMS) columns, such as HP-1 and SPB-1, also separate most volatiles associated with honey bees quite well. For polar compounds, a better alternative is the DB-35 ((35 % Phenyl)-methylpolysiloxane) column.

2.2.3.3. Run parameters:

2.2.3.3.1. Column flow parameters

Helium or hydrogen can be used as a carrier gas at a constant velocity of 20 cm/sec. The GC holds the carrier gas velocity constant as the temperature and pressure change. Check the system for leaks by placing small amounts of a column-compatible solvent (i.e. hexane, a mixture of isopropanol and water) around fittings, applying gas pressure to the system and observing for tiny bubbles. This should be

done routinely especially after the installation of a new column to avoid chromatography degradation and accidental explosions (with hydrogen).

2.2.3.3.2. Oven temperature ramp

The oven temperature affects how compounds interact with the stationary phase (column coating). Generally, more volatile compounds with lower column coating affinities pass through the column before less volatile compounds with higher affinities for the column coating. Choosing the right temperature regime is important for GC analysis. The points below are basic guidelines which should the selection of the right temperature conditions for any given analysis.

- A standard method for solvent desorption starts at 35°C, holds for 2 minutes at this temperature, and then increases at 10°C per minute to 230°C, followed by a final temperature hold for 5 minutes.
- To separate highly volatile compounds, start the oven lower (30°C) and hold temperature for 5 minutes before starting the temperature ramp. A slower ramp (5°C per minute to 75°C, followed by 10°C per minute to 230°C) can also help to separate highly volatile compounds that would otherwise elute together early in the run.
- For thermal desorption, start oven temperatures at 30°C with similar hold and ramp parameters.
- To separate compounds with lower volatility, a higher maximum oven temperature with a longer final temperature hold is required. Increase the maximum oven temperature to 280°C and then hold the maximum temperature for 10 minutes.

2.2.3.3.3. MS detector parameters

The MS detector provides useful information about the chemical structure of compounds which is vital to their identification. When using a MS connected in tandem to a GC, ensure that:

1. The transfer line is generally held at the same temperature as the injector and should be a section of column that extends between the GC oven and the MS detector..
2. The ion source should also be held at a high temperature (220°C).
3. Scan parameters define the m/z (mass/charge) range of fragmented ions scanned by the detector. This range needs to contain the ion fragments of the compounds of interest while avoiding major low mass background ions such as nitrogen gas. Use a m/z range of 35 to 40 minimum to 400 to 600 maximum.

2.2.4. Detection and analysis of volatiles

The various types of GC detectors provide different information about volatile compound identity, emission rates, and activity (Table 1; Tholl *et al.*, 2006; D'Alessandro and Turlings, 2006; Skoog *et al.*, 2007).

- Mass spectrometry (MS) detection is used to provide information about peak identity and molecular mass.
- Electron ionization (EI) mass spectrometry is used to identify unknown compounds based on the comparison of their mass spectral fragment patterns with the fragment patterns of chemical standards found in mass spectra software libraries (i.e. NIST, WILEY, and other commercial libraries).
- Chemical ionization (CI) mass spectrometry is a much milder ionization technique than EI for mass spectrometry that provides information about the molecular mass of sample compounds. It is an excellent technique to quantify known compounds in complex mixtures, especially when combined with selected ion monitoring (SIM) to filter out all ions (m/z) except the selected ions from the chromatogram.
- SIM can also be used with EI ionization if characteristic mass fragments are known for the compounds of interest (Tholl *et al.*, 2006).

Unfortunately, EI-MS is less accurate for quantification due to differences in ionization rates between compounds. This is even more pronounced with SIM and CI where quantification is impossible unless standard curves are generated separately for each compound. For accurate compound quantification, detection methods are available that combine great sensitivity with linear responses over a wide range of concentrations (Table 1; Skoog *et al.*, 2007). Detectors differ considerably in their sensitivities and biases toward various compounds (Núñez *et al.*, 1984). Flame ionization detection (FID) is a technique commonly used for quantification because of its sensitive and relatively unbiased detection of organic compounds. However, FID often displays notably less sensitivity to some oxygen, nitrogen, and sulphur-containing compounds. Other less common, specialized ionization detectors have been developed to provide sensitive detection of these heteroatom-containing compounds. Researchers should generate standard curves of synthetic chemical standards to test the response of detectors to their compounds of interest over a range of concentrations.

2.2.4.1. Identification of volatile and non-volatile compounds

With advances in analytical techniques, the minimal criteria for conclusive identification of natural products have substantially increased. Compounds can no longer be identified solely on the basis of a retention time match with a synthetic chemical standard or a single mass spectrum library match. It is strongly suggested that the reader consult guidelines for natural product identification (Ducret *et al.*, 2008). These guidelines outline how confirmation of compound identity should involve multiple methods (mass spectral matches by GC-MS EI, molecular mass determination by GC-MS CI, structure elucidation by NMR) and comparison with synthetic chemical standards whenever possible. These guidelines may appear stringent to someone not familiar with chemical ecology but are important to assure the quality of chemical identifications.

2.2.4.1.1. Using GC-MS to identify sample peaks

1. Run the sample on EI GC-MS (section 2.2.4.). Examine a peak of interest and make sure that it is a single peak with a symmetrical shape and single apex.
2. Extract the mass spectrum associated with the peak.
3. Select a background mass spectrum from a section of baseline (area lacking peaks) near the peak of interest. Subtract the background mass spectrum from the peak spectrum – commands vary between software packages.
4. Search the mass spectral libraries (NIST, WILEY, and other self-defined libraries) for a match (minimally 80%, 90% for structurally similar compounds such as terpenes) between the peak spectrum and the mass spectra of known library standards.
5. Closely compare the mass spectrum of the library standard against the mass spectrum of the unknown peak. This peak's spectrum should have all of the ion fragments present in the library standard plus additional ion fragments from other minor compounds. If the spectrum is missing ions present in the library standard within the m/z sampling range, there is not a match.
6. Compare the ion fragment patterns of the compound of interest and the library standard to see if the fragment ratios are similar.
7. Test the tentative identity of the unknown peak by running a synthetic chemical standard under identical chromatographic conditions as the sample of interest. To be a match, the synthetic standard should have an identical retention time and similar ion patterns as the peak of interest.
8. Further test the match by running the sample and the synthetic standard separately under different chromatographic conditions (usually a different column type) to determine if the similarities hold.
9. Confirm the match in retention time by co-injecting the sample with a known amount of the synthetic standard. The two compound peaks should overlap completely.
10. Determine the mass of the molecular ion fragment (M+1, the molecular mass plus one additional mass unit) of the unknown peak and the synthetic standard peak by running the sample and standards on CI GC-MS.

2.2.4.1.2. Quantification of volatile and non-volatile compounds with internal standards

The goal of quantification is to obtain an accurate estimate of volatile emission rates from an odour source. This is readily applicable to volatiles sampled using Super Q and Haysep Q adsorbents, and extracts (see sections 4 and 6 of this paper). One approach to quantification is to add a known amount of a synthetic chemical to a sample before processing the sample through extraction and separation (Heath and Manukian, 1994). Researchers usually select a chemical that is not present in their sample but has similar separation properties as the chemicals of interest. One internal standard used with honey bee volatiles is nonyl acetate (Carroll and Duehl, 2012). The internal standard automatically scales the peak areas to known amounts of material. One useful aspect of this internal standard is that quantitative errors in sample processing (i.e. fraction of sample injected on the GC, compound concentration or losses, pipetting errors) are automatically factored out, as both the internal standard and the sample compounds experience similar changes.

To make nonyl acetate internal standard:

1. Add 92.6 µl (80.0 mg) of nonyl acetate to 9.907 ml of the same solvent used for filter extraction (dichloromethane or hexane).
2. Vortex for a few seconds.
3. Add 100 µl of this stock solution to 9.900 ml solvent.
4. Vortex for a few seconds.

The concentration of this internal standard solution is 80 ng/µl.

5. Aliquot the internal standard solution into working amounts of 500 to 1,000 µl.

For long term storage, aliquot the internal standard solution into glass ampules and seal.

It is very important that solvent evaporation of internal standard solutions be kept to an absolute minimum to maintain an accurate concentration. Store the internal standard solutions in a -80°C freezer between uses.

The sample chromatogram will have a nonyl acetate peak that represents the 400 ng of nonyl acetate (5 µl of 80 ng/µl internal standard solution) that was added to the original sample during rinsing with solvent containing internal standard. Run a sample containing only the internal standard to determine the retention time and peak characteristics of the internal standard. Because the amount of internal standard added to the sample is known, conversion rates can be made between peak areas and compound amounts. Sample compound amounts are calculated from peak areas against the standard as:

$$\textit{Sample compound amount (ng)} = \frac{\textit{Area of compound peak (area counts)}}{\textit{Area of internal standard peak (area counts)}} \times 400\ \textit{ng}$$

2.3. Conclusion

To summarize, the most important features of a volatile collection and analysis design are to:

1. Isolate the sample of interest as much as possible without disturbing it.
2. Collect emitted volatiles with a technique that is as sensitive and efficient as possible while limiting the introduction of contaminants.

3. Collect background samples to help identify outside contaminants in the samples.
4. Use appropriate analytical techniques to detect and evaluate the biologically-important compounds of samples.
5. Confirm compound identities and quantities with appropriate chemical standards and techniques.
6. Test the biological activities of samples with bioassays on isolated native material. Then confirm the activities of suspected compounds with bioassays on synthetic chemical standards.

3. *Ex-situ* collection of honey bee odours and electrophysiology

3.1. Introduction

Honey bees produce airborne volatile organic compounds which serve as indicators of the 'status' of the colony. As such, the collection, analyses, bioactivity of identified honey bee odours is vital to understanding how social cohesion is maintained, regulated and influenced by various biotic factors such as foreign intruders (Torto *et al.*, 2005, 2007a, 2007b) and pathogens (Swanson *et al.*, 2009). Odours associated with honey bees have been used to improve colony vigour and to manage certain pest such as the small hive beetle *Aethina tumida*, (Teal *et al.*, 2006; Arbogast *et al.*, 2007; Torto *et al.*, 2007b).

A specialized method that provides information on honey bee detection and sensitivity to specific compounds is coupled Gas Chromatography-ElectroAntennographic Detection (GC-EAD) (Schneider, 1957; Baker *et al.*, 1985; Torto *et al.*, 2007a; Swanson *et al.,* 2009). This coupled system uses an insect antenna in tandem with a flame ionization detector (FID) to link the electrical activity stemming from neurons signalling receptor binding of chemicals to individual peaks. GC-EAD is an excellent analytical tool but works best for chemicals detected by many receptors thereby producing a strong electrical stimulation from the antenna. In this hybrid method, volatiles emerging from a GC column are split between a conventional GC detector (chemical sensor) and an antenna mounted between two electrodes (biological sensor). The interpretation of detected compounds requires an understanding of insect physiology, since antennal stimulation to chemicals represents activity which may indicate attraction or repellence. Some chemicals may be detected by a single receptor, transmitted by a single neuron and then amplified in the brain while others may stimulate several receptors and their associated receptor neurons. Honey bee chemosensory organs can be much more sensitive to bioactive compounds than analytical detectors, sometimes leading to strong electrophysiological responses to correspondingly weak chemically (FID) detected components. Although the honey bee antenna is most commonly used, other body parts having chemosensory activity can be used as biosensors. Antennae and body parts with weak electrophysiological activity can have their activity amplified by mounting multiple parallel sensory organs in tandem across a single electrode.

This section focuses on methods to collect honey bee odours outside of the colony environment (*ex-situ* volatile collection) and to carry out electrophysiological recordings using antenna or other chemosensory body parts of the honey bee.

3.2. Volatile collection

3.2.1. Setup and volatile sampling

Laboratory collection of headspace odours can be carried out using a static or dynamic sampling method. A detailed description of the merits and disadvantages of these two sampling techniques, adsorbents and their associated desorption techniques have been dealt with in the previous section (see section 2.2.2. on *In-situ* collection of volatiles). Briefly:

- Dynamic headspace sampling requires an air supply, an air purification system, flowmeters, quickfit glass containers to hold odour sources with air entry and exit ports, and a mesh screen metallic canister with a tight fitting lid to hold honey bees, copper/Teflon tubing connectors, adsorbents (Super Q, Tenax, and SPME fibres), humidifier and a vacuum supply.
- Static headspace sampling on the other hand simply requires glass containers with lids fitted with air ports to contain odour sources, SPME adsorbent fibres (suited for collecting both polar and non-polar chemicals) or gas tight glass syringes for sampling head space. Sample the headspace directly with a gas tight syringe by pulling 50 or 100 ml of odour and analyse on a GC or GC-EAD or GC-MS.

An example of a laboratory volatile collection setup for honey bees has been described by Torto *et al.* (2005). A similar setup is shown in Fig. 4, illustrated using 6 components. In this setup, medical air from a pressurised air tank (not shown in figure) is passed through a copper tubing (component 1) and then through activated charcoal (component 2) to purify it and into to a humidifier containing double distilled water (component 4). The humidified air is pushed through a y- or t-split (for treatment and control) or multiple ports (manifold) (component 5) to which odour sources enclosed in glass jars are connected to in parallel. The vacuum supply pulls air from the glass jar at a specified flow rate set on the flow meter (component 6) through the adsorbent filters.

Odour collections can be made from adult worker honey bees only; an entire honey bee comb bearing adult workers, bee brood, pollen and honey; honey comb containing bee larvae; just to name a few depending on the hive odour source of interest and the research

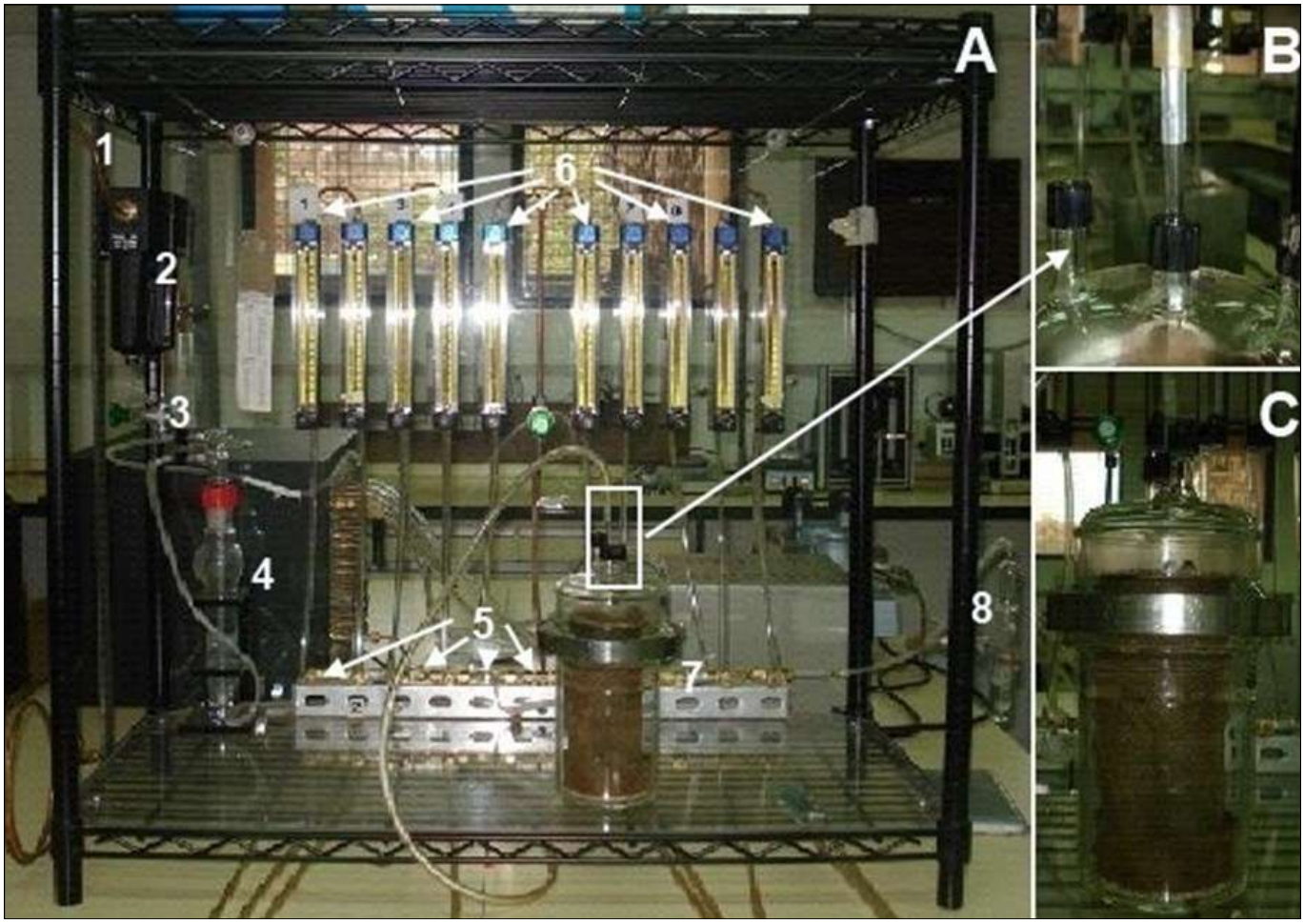

Fig. 4. **(A)** *Ex-situ* volatile collection setup, **(B)** Adsorbent filter fitted on lid and **(C)** glass jar with metallic canister containing worker honey bees. **(1)** copper tubes, **(2)** air filtering system (e.g. .activated charcoal), **(3)** air pressure regulator, **(4)** humidifier, **(5)** multiple air supply ports, **(6)** air flow meters, **(7)** glass jar containing odour source and 8-air exit monitor (water bubbler which give evidence of air flowing through the setup).

question being addressed. However, the experimenter should bear in mind that bee larvae usually become stressed in the absence of worker bees (see sections 2.2.2. and 2.2.3. on *in situ* volatile collection and analysis).

Once ready to collect odours in the laboratory and in possession of all the bits and pieces of equipment, following the steps below will ensure a successful process.

1. To collect odours from worker bees only, gently brush-off workers from a comb into a screen mesh metallic canister containing sugar source (a ball of cotton wool soaked in 50% sucrose solution or sucrose solution in a vial fitted with a dental cotton wick) and close it with its lid once the required population is obtained.
2. A setup consisting of a canister and sugar source without bees should also be prepared to serve as a control.
3. Return to the laboratory with the canisters and place them in an appropriate quickfit glass container (e.g. 2 or 5 l glass jar with a tight fitting lid).
4. To sample representative odours of the hive environment, gently push a honey bee comb or cut out a section of a comb with adult bees, brood, pollen and honey out of its frame.
5. Place in a clean quickfit glass jar with aeration ports and carry the confined comb and bees back to the laboratory for volatile collection.
6. Connect one aeration port attached to the glass jar to an air supply and the other to the a vacuum supply.
7. Pass clean air through the system for 15-20 min to purge out the alarm pheromone produced by the bees and any contaminants before collecting volatiles for a specified period.

3.2.2. Dynamic volatile collection

8. Briefly disconnect the vacuum line once the bees have become relatively calm.
9. Place a protective screen (Teflon sheath or screen mesh around the tip of an adsorbent (e.g. Porapak Q, Super Q or Tenax) filter to protect it from being blocked with wax by the bees.

10. Place the filter at the vacuum line's tip and reconnect it to the lid of the container.
    Avoid placing the vacuum supply source such as a pump and odour source on the same bench to prevent alarming the bees from the vibrations/noise from the pump.
11. Pull air out of the system at a desired flow rate and for a specific duration (see section 2.2.2. and 2.2.3. on *in situ* volatile collection and analysis). The sucrose solution serves a dual role; as a source of food to keep bees alive and to calm them.
12. Remove the filter and seal the ends with Teflon tape after volatile collection is complete.
13. Switch-off the vacuum.
14. Disassemble the odour container.
15. Return the bees to their colony of origin.
16. Elute the volatiles adsorbed on the filter using an appropriate volume of solvent (e.g. 200 µl) such as dichloromethane or redistilled ether in to a sample vial using a gentle flow of N_2 gas.
17. Label the sample bottle and store at -80°C prior to analysis.

3.2.3. Static volatile collection

Static odour sampling can be carried out using several sampling techniques. The use of SPME fibres is the most convenient in a closed head space environment (see section 2.2.2.2.1). For this procedure:

8. Gently disconnect the air supply and vacuum pumps from the odour containment glass jars after purging for 15-20 min.
9. Immediately close all ports with screw caps lined with flexible Teflon linings.
10. Insert a SPME fibre holder or gas tight syringe by pushing its needle through one of the flexible Teflon linings of the screw caps until the needle is fully within the glass jar.

11a. Expose the fibre (adsorbent) through the needle tip by pushing down the SPME holder plunger. Maintain this experimental setup for 0.5 - 12 h (or for a desired duration which should not be lengthy to avoid stressing the bees).

11b. For the gas tight syringe, maintain a closed experimental setup for 0.5 - 12 h and afterwards sample the head space odour by pulling a desired volume of air from the head space and analyse directly on a GC, coupled GC-EAD or GC-MS (see section 2.2.4.).

12. Retract the SPME fibre into the needle before withdrawing the needle from the Teflon lining.
13. Analyse sample as described in the previous section (see section 2.2.3. on *in situ* volatile collection and analysis)

3.3. Electrophysiology

3.3.1. Setup

Electrophysiological studies require expensive specialized equipment including software, which can be purchased from commercially available sources. A basic electrophysiological setup consists of four elements (Syntech, 2004).

- A biological sensor which is usually an antenna or any other chemosensory organ mounted across a pair of electrodes.
- Amplifier and signal processing electronics specially built to minimize noise and to control the baseline signal.
- Signal display and recording system which makes use of computer software for display, record and analysis of signals.
- Stimulus application system which ensures a continuous or discontinuous release of test stimuli over the antenna while its electrical activity is being measured.

Equipment similar to that described by Torto *et al.* (2005) can be used to measure bee antennal responses to various chemicals. A stimulus source consisting of an inert metallic delivery tube (Fig. 5a) with a hole at its basal end originating from the side of a gas chromatograph serves as the channel via which volatile stimuli are applied over the mounted antenna on a micro-manipulator or gel probe (Syntech, 2004). The micro-manipulator has two terminals; an indifferent and a 10x amplification recording terminal. Both terminals containing silver wires (0.1 mm in diameter) are sheathed with capillary tubes tapered at one end by drawing them out as heated tubes in pipette pullers, filled with insect saline solution such as Ephrussi solution (consisting of an aqueous mixture of Na, K, Mg, Ca and Cl) (Christensen, 2004), thereby converting them into conducting electrodes (Christensen, 2004). The manipulator base is connected to an earth cable to minimize internal electrical interference while its recording electrode is connected to an AC/DC-EAG amplifier. Signals from the recording electrodes are amplified and digitized by data acquisition electronics. This electronic equipment acquires signals from the amplifier and transforms them into digital wave-like signal depicting antennal responses as peaks recorded in millivolts (mV). Signal visualisation, recording and analysis is carried out using a specific software (e.g. GC-EAD, Syntech) installed on a personal computer (PC). All the EAD equipment except for the gas chromatograph, data acquisition electronics and PC are enclosed within a Faraday's cage to reduce external electrical interference. For more details of the setup, see the system's manual (e.g. Syntech, 2004).

3.3.2. Types of electrodes

Two types of electrodes are often used to record EAGs (electroantennograms); glass capillary and probe electrodes.

3.3.2.1. Glass capillary electrodes

A glass capillary electrode usually consists of a borosilicate glass capillary pulled out to produce a tapering end (~ 1 µm in diameter) in a pipette puller and filled afterwards with sensillum haemolymph mimics e.g. Ringer (see the *BEEBOOK* paper on cell cultures by Genersch *et al.*, 2013 for a recipe) or Ephrussi solution (Christensen, 2004). The saline-filled glass capillary is then pushed over a silver wire fixed into a micro-manipulator (Fig. 5a). This electrode type can be used to record EAGs from intact honey bees, excised honey bee heads and excised antennae.

3.3.2.2. Probe electrodes

A probe electrode is similar to a two-pronged fork with blunt flat tips (Fig. 5b). The probe is metallic and often made of silver or gold. An excised antenna or chemosensory organ is mounted across the prongs and held in place using conducting gel (Spectra 360 Electrode Gel). Modified versions of this electrode include the:

- Tetraprobe with a single base electrode and four (4) different recording electrodes which can be used to mount several antennae of the same insect species in tandem (Syntech).
- Quadroprobe similar in design to the Tetraprobe but with a detachable probe end which is useful in mounting several antennae from different insects.

3.3.3. Sensory organ preparation and mounting

No universal standard exists for preparing honey bee sensory organs for electroantennographic studies (Syntech, 2004). Various mounting techniques exist and the choice of technique to use depends on the objectives and experience of the experimenter. For recording bee antennal responses over a long period of time (over one hour), whole insect preparations should be the ideal choice (Syntech, 2004). For shorter durations lasting up to an hour, excised antennal preparations can be used. Both whole insect and excised antenna preparations can be carried out using saline-filled glass or probe electrodes (Torto *et al.*, 2005, 2007b). To prepare bee antenna for an electrophysiological recording:

1. Collect insects i.e. forager, guard or nurse bees at the hive entrance or comb using an aspirator (manual or automated) as required.
2. Immobilise the collected bees by placing them on ice for about 1-2 min.
3. Pick individual immobilised bees and insert each at the base of a pipette tip (100-1000 µL).
4. Gently blow the bee towards the tip whose apical portion has been cut-off to allow only the head of the bee to go through it in order to restrain it.
5. Plug the base of the pipette tip with cotton wool or paper towel to prevent the insect from crawling back inside the restraining tube.

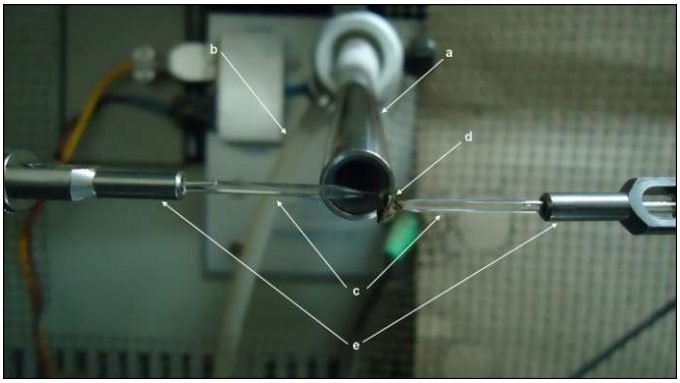

Fig. 5a. **(a)** The stimulus delivery line for the column effluent, **(b)** humidified air supply used to flush column effluent in the background (blurred), **(c)** Saline-filled glass electrodes **(d)** a mounted bee antenna showing both head and antenna **(e)** mounted electrode holders.

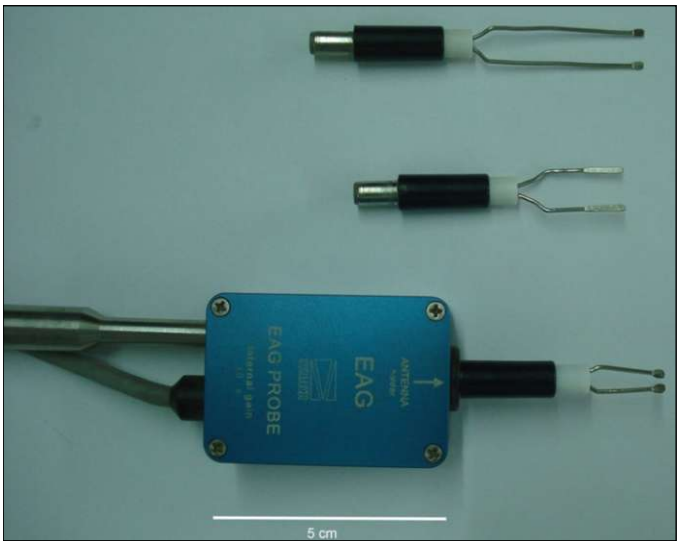

Fig. 5b. Gel electrodes and probe. Electrodes vary in their sizes fitting both large and small antennae or chemosensory organs.

6. Prepare the electrodes (saline-filled glass or gel electrodes) to be used for mounting. For saline electrodes, ensure that its drawn out tips have been cut to allow both antenna base and tip to fit through under a stereomicroscope. For gel electrodes, make sure that a sufficient amount of conducting gel is applied on the electrode tip.
7. Mount the excised antenna between glass micropipette electrodes on a micro-manipulator or probe electrodes covered with conducting gel (Syntech, 2004). For whole insect preparations, push the recording electrode into the distal end of the organ of interest (antennal tip) and the base or ground electrode into a body part close to the terminal end of the organ (which is often the eye for antennal preparations).

This should always be done under a stereomicroscope.

8. Ensure that saline electrodes are free of air bubbles as they can interfere with a smooth recording of EAG.
9. Make sure that the mounted antenna has its basal end connected to the ground/base/indifferent electrode while its apical portion is connected to the recording/different electrode (usually the one connected to the amplifier).

10. Move either the antennal preparation closer to the source of the stimulus or the stimulus dispenser close to the mounting.
11. When the amplifier light signal changes from red to green, it indicates that a complete circuit has been established and the mounting process is complete.

3.3.4. EAG recording

After the insect antenna or sensory organ has been successfully mounted, start recording the signal on the computer monitor. A stimulus should only be applied when a relatively stable baseline signal is obtained.

Depending on the nature of the application of the stimulus, EAG recordings can either be described as continuous or discontinuous. A continuous recording involves the continuous application of the stimulus over the antennal preparation throughout the recording session while a discontinuous recording refers to the application of stimulus at intervals. Coupled gas chromatography electroantennographic detection (GC-EAD) is the most widely used technique for continuous recording.

Coupled GC-EAD recordings can be used to record antennal responses to both natural and synthetic mixtures of chemicals. The GC chemically separates the mixture into individual components which are then detected by the antenna, followed by identification of the antennally-active components by coupled gas chromatography-mass spectrometry (GC-MS). However, a coupled GC-EAD system is expensive to setup and run, bulky and requires lengthy recording times. Compared to continuous GC-EAD recording, discontinuous EAG recording is a simple, less bulky and fast way of testing individual chemicals at different doses for antennal activity. Discontinuous recordings are mostly used in dose response studies to compare antennal responses to identified chemicals. This usually involves quantification of responses to the various chemicals, averaging these responses and comparing them using various conventional statistical tests (see the *BEEBOOK* paper on statistics (Pirk *et al.*, 2013)). The drawback of this method is that it is not suitable for testing mixtures of chemicals, especially natural unidentified mixtures, and it cannot be used to identify unknown compounds.

Both GC-EAD and EAG recording systems complement each other and it is advantageous to have both systems in place in any insect electrophysiology laboratory. A coupled GC-EAD system can easily be converted into stimulus puff EAG recording system by switching-off the GC and using the opening at the side of the metallic stimulus delivery tube to puff odours over mounted antenna.

3.3.5. Coupled gas chromatography-electroantennographic detection (GC-EAD) recording

In this continuous recording system, the stimulus (a natural extract or synthetic mixture of chemicals is applied by injection into a gas chromatograph where individual components are separated based on their physical and chemical properties while being carried through a column (see section 2.2.3.). One part of the column effluent is continuously flushed over the antenna while the other flows into the flame ionisation detector (FID).

1. Switch on the GC and programme it to run a specified separation method (see section 2.2.4. on *In situ* volatile collection) and wait for it to get ready.
2. Following successful antennal preparation, inject 1-5 µl of the extract depending upon its concentration into the GC through its injection port and run the method.
3. At the same time start the recording programme on the computer to synchronise the signal output of the GC's sensor (FID) with that of the EAD setup (antenna).
4. Allow the program method to run from start to finish. When the run is over, stop the recording and save the file on the PC. The file can then be retrieved later for analysis.
5. The recorded file contains two line tracings, the FID and EAD outputs (Fig. 6a).

Peaks on the FID trace represent the different chemical components separated on the column and detected by the FID, while those corresponding to EAD peaks indicate antennal detection to specific compounds.

3.3.6. Discontinuous EAG recording

Discontinuous EAG does not require a gas chromatograph, but rather a stimulus delivery system for delivery of odours over the antenna at intervals as puff (sometimes referred to as puff stimulation). Stimulus delivery systems are commercially available as stand-alone devices or as accessories which can be installed on to a GC (Fig. 5a). It also requires a preparation of the stimulus in a manner different from natural volatile extracts (Syntech, 2004) as follows:

1. Prepare a stock solution of 1 µg/µl (equivalent to 1 mg/ml) by dissolving 5 mg of a pure compound of interest to the experimenter in 5 ml of solvent (e.g. hexane, dichloromethane, acetone) and shake gently to dissolve the sample.
2. Serially dilute the stock solution to prepare a range of doses (e.g. 100 ng/µl, 10 ng/µl, 1 ng/µl, etc.) to be tested.
3. Place a cut piece of paper, preferably filter paper (3 cm long x 1 cm wide), in the wide end of a standard Pasteur pipette.
4. Apply a specified amount of the solution of the compound to be tested. For example apply 1 µl of a 1 µg/µl solution to test 1 µg of the compound, etc.
5. Allow the solvent to evaporate for 30 s to several min and gently push the filter paper completely into the Pasteur pipette. Seal both ends of the pipette using Teflon tape or parafilm.

6. Label the pipette by the side to indicate the stimulus type (code names for test compound and concentration).
7. Prepare three control stimuli consisting of:
   7a. a clean Pasteur pipette,
   7b. a pipette containing filter paper only,
   7c. a pipette with filter paper with only solvent applied.
   These three controls check for pipette, filter paper and solvent contamination respectively.
8. Place the smaller end of the pipette inside the hole on the side of the delivery tube (Fig. 5a) and connect its wider end to an air supply after removing the Teflon tape or parafilm.
9. Apply the stimulus over the antenna by puffing using the EAG puff pedal for about 0.5-3 s, in the order; control stimulus followed by the test stimuli and finally control stimuli again. You may randomize the application of the test and control stimuli on the antennae, and puff each test stimulus several times since only a fraction of the test compound is delivered in each puff.

Stimulus application should be done at intervals of 30-120 s to allow the antenna to recover from the previous stimulus.

10. Record antenna signals as described in section 3.3.5. and open files later for analysis (see Fig. 6b for an example of a stimulus-puff recording).

EAG recordings are limited in their sensitivity and specificity to certain chemicals because responses to these chemicals are a summation of the total depolarisations elicited by the chemicals across the mounted antenna at the level of the olfactory receptor neurons (ORNs) contained within each receptor cell (sensillum) (Christensen, 2004). As such, chemicals which elicit action potentials on very small number of ORNs are often not detected in EAG recordings. A newer technique, single-sensillum recording (SSR) partially overcomes EAG limitations by recording antennal electrical activity at the level of ORNs (Millar and Haynes, 1998; Christensen, 2004). Details on the equipment type, experimental protocols and analysis of results in SSR studies have been well described by Christensen (2004).

3.4. Chemical identification of electrophysiologically-active components

See section 2.2.4 on *In-situ* volatile collection for details of how volatile components can be identified. Briefly,

1. Collect volatiles on adsorbent and wash them-off the filters with an appropriate solvent (hexane, dichloromethane or redistilled ether).
2. Analyse aliquots of the volatile extracts using coupled GC-EAD and coupled GC-MS (linked gas chromatography mass spectrometry) using identical GC columns and oven conditions in both equipment. Add a specific amount of Internal Standard (highly recommended to facilitate matching and identification of peaks when comparing traces obtained from the GC-EAD and GC-MS) (see section 2.2.4. on *In-situ* volatile collection) for qualitative and quantitative comparisons.
3. Compare retention times of separated components from both GC-EAD and GC-MS traces and identify peaks representing EAD-active components in the mixture on the GC-MS trace.
4. Tentatively identify chemical structures of EAD-active components (based on their representative peaks) using their fragmentation patterns (mass spectral data) while comparing it with those already identified and stored in a mass spectra database (e.g. NIST, ADAMS) (see section 2.2.4. on *In situ* volatile analysis).
5. Individually prepare 50 – 100 ng/µl solutions of the tentatively identified components in an appropriate solvent from authentic compounds obtained from commercial sources or synthesized by a chemist and analyse via linked GC-EAD and GC-MS to confirm identities of EAD-active components. Repeat the same procedure using a mixture of the tentatively identified components from premix authentic compounds obtained from commercial sources or synthesized and constituted by a chemist. Using a mixture of compounds is advantageous in that it saves time and resources in cases where many EAG-active components (> 20) are present in the natural extract. You may also analyse each component separately.

3.5. Summary

In a nutshell, the following steps should serve as a guide for planning experiments designed for *ex-situ* volatile collections and analysis:

1. Electrophysiology is much of practical science, it takes practice to consistently set up organ preparations and adapting methods to different organisms takes some trial and error. Take the time initially to get a good feel for consistently setting up an organ to detect known chemicals before evaluating novel detections.
2. Design and carry out appropriate bioassays to demonstrate the involvement of honey bee emitted volatiles in bee behaviour (see the *BEEBOOK* paper on methods for behavioural research by Scheiner *et al.* (2013)).
3. Use appropriate analytical techniques to detect (coupled GC-EAD), identify (GC, linked GC-MS) and quantify (GC, linked GC -MS) biologically relevant chemicals present in the emitted odours (see section 3.3. and 3.4. of this paper).
4. Confirm biological activities of identified chemicals by using their authentic (pure synthetic versions) equivalents in bioassays.

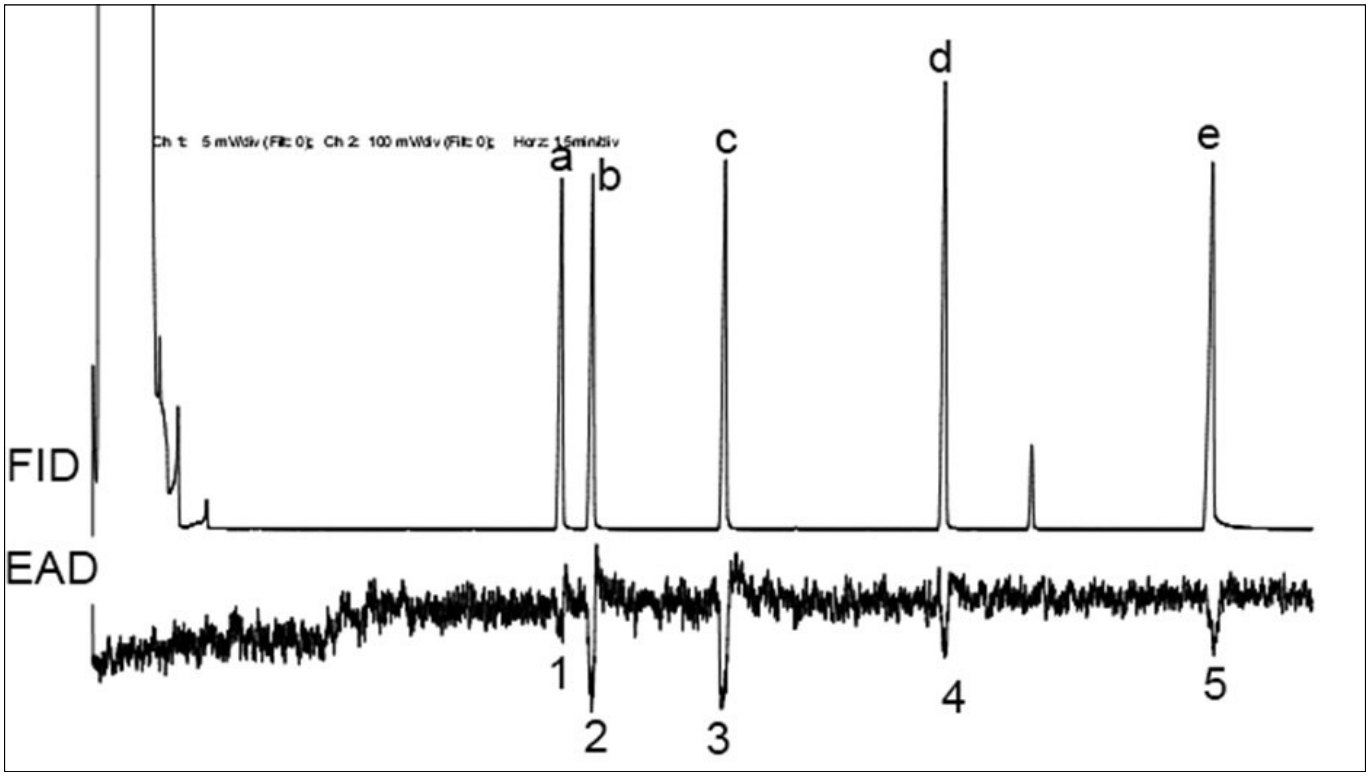

Fig. 6a. A continuous GC-EAD output showing antennal responses labelled 1-5 to a mixture of 6 chemicals. Notice that the peak representing a chemical (impurity) between peaks d and e is not detected by the antenna.

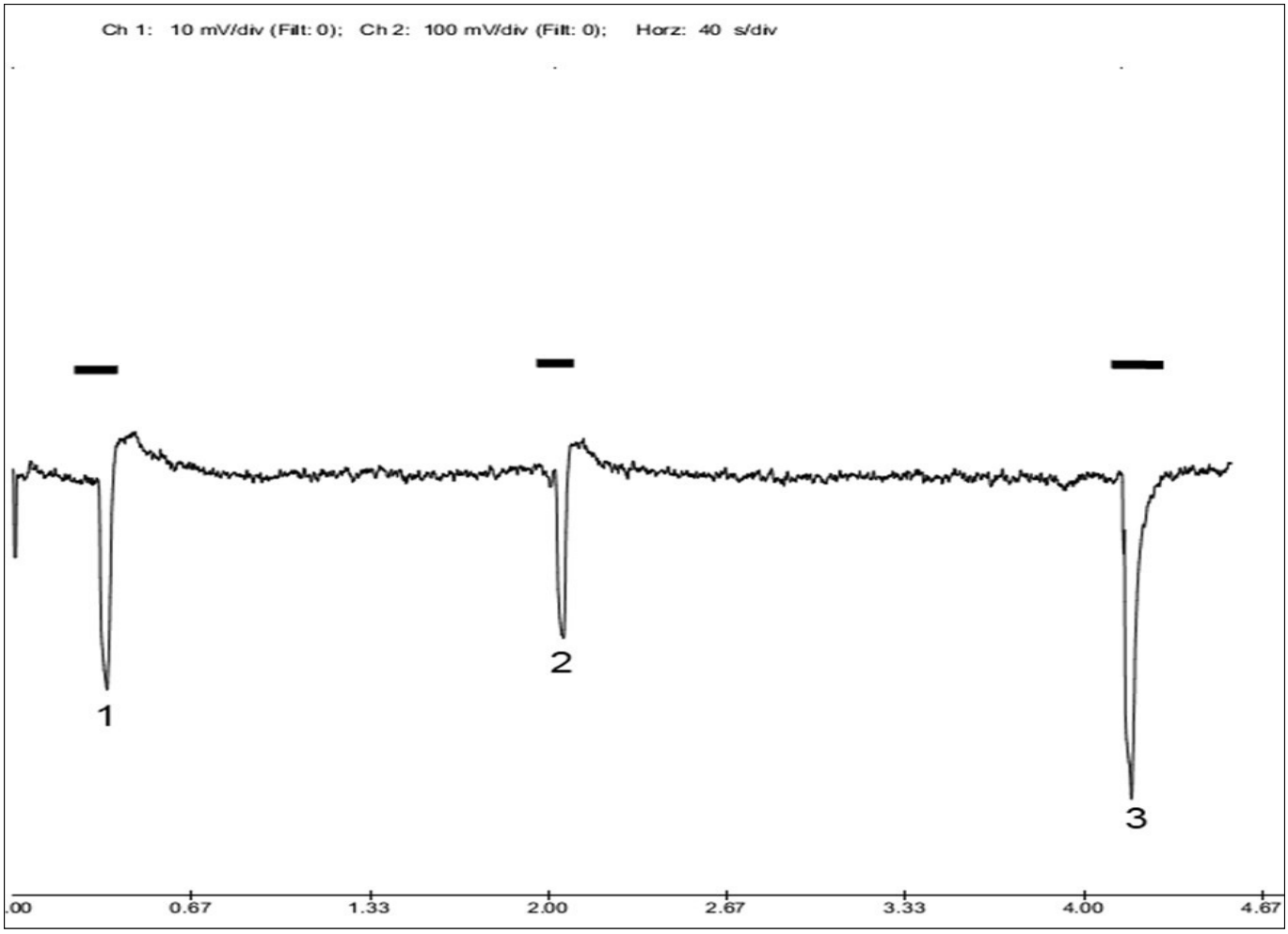

Fig. 6b. A discontinuous (puff stimulation) electro-antennal recording output showing antennal responses to three different doses (10 ng, 10 pg and 10 µg respectively) of a single chemical applied over the antenna at approximately 1 min intervals. Black horizontal bars above responses labeled 1 to 3 represent puff application.

4. Extraction and analysis of honey bee non-volatile cuticular hydrocarbons

4.1. Introduction

The honey bee cuticle is covered by a thin layer of non-volatile hydrocarbons that are used for several functions including prevention of dehydration and chemical communication. The study of honey bee cuticular hydrocarbons (CHC) has been carried out for chemotaxonomic characterization (Carlson and Bolton, 1984), nestmate recognition (Dani *et al.*, 2005) and elucidation of host-parasite relationships (Rickli *et al.*, 1994; Salvy *et al.*, 2001; Del Piccolo *et al.*, 2010).

Cuticular hydrocarbons of the honey bee include straight chain saturated and unsaturated hydrocarbons as well as branched saturated hydrocarbons. Chain length varies between 15 and 35, with odd numbered hydrocarbons being the most abundant; unsaturation is common at the 9 position in monoenes, but other positions are possible in longer chain hydrocarbons (Blomquist *et al.*, 1980; Francis *et al.*, 1985; Francis *et al.*, 1989; Carlson *et al.*, 1989). Some excellent reviews about insect CHC analysis have been published that can be used as a reference (e.g. Blomquist, 2010).

4.2. Techniques for analysing honey bee CHC

In general, the study of honey bee cuticular HC involves the following steps: extraction, sample preparation, identification and data-analysis, as detailed in the following sections.

4.2.1. Extraction

Modern analytical equipment easily allows for the analysis of CHC from single specimens.

The most used method for extracting CHC is, by far, solvent extraction. This can be done using the following protocol.

1. Transfer a single bee that has been anesthetised by chilling (see the section on Standard methods for immobilising, terminating, and storing adult *Apis mellifera* in the *BEEBOOK* miscellaneous paper (Human *et al.*, 2013), into a small glass container with a convenient amount of an apolar solvent (e.g. 1 ml of hexane HPLC grade).

Use clean forceps to avoid any possible contamination. Glass containers that are used need to be carefully washed, rinsed with hexane and kept overnight at high temperature before use; other materials should not be used to avoid contamination.

2. Leave the specimens for extraction at room temperature. The duration of the extraction can vary and preliminary tests are advisable to determine the best duration according to the amount of compounds obtained. In general, 2-10 min can be regarded as a good compromise between extraction efficiency and the need to keep the working time reasonably short.
3. Transfer the extract into a vial and store at -20°C until use.
4. In case an absolute quantification of CHC has to be carried out, add a convenient internal standard to the sample before extraction (see section 2.2.4.2 above for more details on this subject).

Such extracts are normally suitable for analysis without further processing apart from solvent evaporation under a stream of nitrogen to concentrate the sample for the analysis.

Other extraction methods can be used to sample CHC; in particular Nazzi *et al.* (2002 and 2004) used solid phase microextraction (SPME) to study short chain hydrocarbons released by honey bee pupae infested by the parasitic mite *Varroa destructor*; Nazzi *et al.* (2002) rinsed the gelatin capsules used for rearing the bees from larval stage to emergence, to sample the hydrocarbons released during pupation. The use of SPME for extracting CHC from living specimens is increasingly being used in other insects and details of this method are available (Bland *et al.*, 2001). For more details on other extraction methods see section 2.2.2 above.

4.2.2. Sample preparation

Crude hexane honey bee extracts are normally already suitable for subsequent analysis without further purification. In some cases CHC are separated for the purpose of behavioural bioassays; in particular, HC can be separated from oxygenated compounds using column chromatography on silica gel.

To do so the following method can be used:

1. Prepare a column, this is done by packing a Pasteur pipette (clogged at its tip with glass wool to prevent the gel running down the pipette) with 100-500 mg of silica gel (200–400 mesh, 60 Å).
2. Condition the column by passing hexane through it 2-3 times.
3. Add the sample to the column using a small volume of solvent.
4. Elute the column with 1-5 ml of hexane to collect the HC.

Several elutions can be done to ensure collection of all HCs.

5. Elute the column with 1-5 ml of ether or acetone if interested in more polar compounds.

To further fractionate CHCs, saturated and unsaturated components in the apolar fraction can be separated.

6. Prepare a column packed with 100-500 mg of 10% silver nitrate on silica gel (200–400 mesh, 60 Å).
7. Elute the column with 1-5 ml of hexane to collect saturated hydrocarbons.
8. Elute the column with 1-5 ml of ether to collect unsaturated HC.

In order to remove silver ions,

9. Reduce the ether fraction under nitrogen.
10. Pass the eluate through an identical silica column.
11. Elute with hexane.

4.2.3. Identification

4.2.3.1. Analysis

Nowadays, the most used method for HC identification is mass spectrometry coupled to gas-chromatography (see section 2.2.4 for more details on GC-MS analysis), although satisfactory results can be obtained also using stand-alone gas-chromatography using the retention index method. In this case, peaks in the chromatogram are identified according to their retention index that is calculated from the retention time of the peak and that of adjacent reference alkanes. For the analysis, 0.1 insect equivalent (i.e. 1/10 of the material that can be extracted from a single insect) in 1 µl of hexane can be injected in the chromatograph (injector temperature can be set to 300°C) in splitless mode. A capillary column is normally used; many authors found DB-5 or DB-1 columns particularly suited for the purpose. The temperature of the GC is ramped from 40-50°C to 300-320°C and maintained at that temperature for 20 minutes or more.

The compounds in the extracts are identified by comparison of retention times and spectra (if GC-MS is used) with those of authentic standards. Quantification is based on the peak area with reference of that of the internal standard; a standard solution of hydrocarbons should also be analysed to calculate calibration factors. For more details on quantitative analysis see section 2.2.4.2.

4.2.3.2. Double bond position in unsaturated hydrocarbons

The double-bond position of unsaturated hydrocarbons detected in crude extracts can be assessed by GC-MS after derivatisation with dimethyl disulfide (DMDS) (Carlson *et al.*, 1989). Given its importance, the method will be described in detail.

To derivatise a few µg of HC in 200 µl of hexane, use the following protocol:

1. Add 200 µl of DMDS and 100 µl of iodine solution (60 mg in 1 ml of ether) to catalyse the reaction.
2. Keep at 40°C for 4 hours.
3. Dilute with hexane with 5% sodium thiosulfate to neutralize the iodine.
4. Collect the organic phase which contains the DMDS adducts of the unsaturated hydrocarbons.
5. Remove moisture from the solution by adding a small amount of anhydrous Na_2SO_4 to it.
6. Reduce the solvent under a stream of nitrogen to the desired concentration.

Derivatised alkenes give a single chromatographic peak with a characteristic spectrum composed of two prominent fragments. These are related to the cleavage of the bond between the carbon atoms carrying the methyl sulphide substituent, originally the location of the double bond. The m/z values of these fragments are identified by the series 61+n14; for example, the alkene 9-heptadecene ($CH_3(CH_2)_7$=CH $(CH_2)_6CH_3$) will give a peak with two prominent fragments at 159 ($CH_3(CH_2)_6CHSCH_3$) and 173 m/z ($CH_3(CH_2)_7CHSCH_3$); the molecular ion is normally seen (in the case of 9-heptadecene, this has m/z 332, that is the sum of the two fragments described above).

4.2.3.3. Stereochemistry of alkenes (determining the identities of geometric isomers of unsaturated hydrocarbons)

Carbon-carbon double bonds of alkenes can exist in two alternative forms, known as isomers, using the named (*E*)-(*Z*) notation. To determine the stereochemistry of the alkenes that are present in a sample, they can be co-chromatographed with authentic standards on silver nitrate impregnated thin-layer chromatography plates using hexane as a solvent. This allows separation of the stereoisomers so that the sections of the plate corresponding to the (*Z*) and (*E*) alkenes can then be extracted with ether and analysed by GC-MS (Nazzi *et al.*, 2002).

4.2.3.4. Branching position

The point where the chain of carbon atoms is branched in non-linear hydrocarbons can be determined from the mass spectrum taking into account both the mass spectrum and the Kovats index (Carlson *et al.*, 1998).

4.2.3.5. Synthesis for the purpose of identification

Although the study of retention time and mass spectrum normally provides reliable data for the identification, injection and coinjection represent the definitive proof of the identity. If standard compounds are not readily available they have to be synthesized using organic chemistry techniques that are outside the purpose of this review. Some of these techniques have been used extensively; in particular the Wittig reaction for the synthesis of unsaturated HC. In a few cases suitable alternatives (e.g. the partial hydrogenation of the corresponding alkyne obtained by alkylation of a convenient terminal alkyne with 1-bromoalkanes of suitable chain length according to Sonnet, 1984) have been used giving excellent results (Nazzi *et al.*, 2002).

4.2.4. Data analysis

For the analysis of differences between cuticular compounds or profiles, data can be arranged in a matrix with as many rows as the number of the studied hydrocarbons and one column for each analysed honey bee. In many cases the percentage composition (e.g. the proportion of a compound relative to the whole set of hydrocarbons is used for the analysis, in other cases the absolute amount of each hydrocarbon is used; sometimes this is expressed as the absolute quantity of a compound relative to the weight of the insect. If the percentage composition is used, data transformation according to Reyment (1989) is common using the following formula:

$$Z_{i,j}=\log[X_{i,j}/g(X_j)]$$

where:

$Z_{i,j}$ the transformed area of peak i for specimen j;

$X_{i,j}$ represents the area of peak i for specimen j;

$g(X_j)$ the geometric mean of the areas of all peaks for specimen j.

Different methods for data analysis are applied according to the purpose of the study. Given the distribution of data, possible differences between experimental groups can be tested using parametric methods such as ANOVA if experimental groups are three or more, or Student's t test if only two groups are considered. In this case, the number of tests to be carried out corresponds to the number of CHC considered, that can be rather high causing possible errors related to multiple comparisons; therefore probabilities from the test should be adjusted to allow for possible false positives using convenient formulas (e.g. Bonferroni correction that is very common and conservative, see the *BEEBOOK* paper on statistics by Pirk *et al.* (2013)).

In many cases the whole set of CHC is considered using multivariate techniques such as principal components or discriminant analysis. This can be carried out with most commercial statistical packages and this allows for a plot of the specimens on the plane formed by the derived functions accounting for most of the variability. Possible differences between groups are denoted by isolated clouds of points grouped around the centroids, whose distance from other centroids can be tested for its significance with standard methods.

Discriminant analysis is carried out when samples belong to predefined groups; in this case, to account for multicollinearity, a preliminary principal component analysis is carried out and the discriminant analysis is applied on the extracted factors.

5. Bee attraction bioassay

5.1. Introduction

Chemical communication in the honey bee is very complex with 15 exocrine glands known to produce various pheromonal chemicals. Among these pheromones, the queen retinue pheromone (QRP) is considered the most important as it is essential to maintaining social cohesion in the honey bee colony. This pheromone is composed of 9 components derived from various glands in the queen and elicits 'aggregation', antennating, licking and grooming of the queen by worker bees (Slessor *et al.*, 1988; Wossler and Crewe, 1999; Keeling *et al.*, 2003; Katzav-Gozansky *et al.,* 2001; Slessor *et al.*, 2005). The attractiveness of the queen and her pheromonal cues to worker bees is not stable but varies with the age and reproductive status of the queen, worker sensitivity, seasonal change and genetics (De Hazan *et al.*, 1989; Pankiw *et al.*, 1994, 2000; Kocher *et al.*, 2009; Wossler *et al.*, 2006).

Over the years, several compounds have been tested for their role as queen attractants using various bioassay setups. This section focuses on solvent extraction of pheromones from glands and the evaluation of both crude extract and its components in bioassays. Although we present these methods based on queen pheromones, worker and drone secretions can be analysed in the same manner.

5.2. Stimuli preparation

The prior knowledge regarding the chemical cues (source, nature and etc.) determines the procedure for stimuli preparation. If the glandular origin of the secretion is known, then a chemical extract of the gland/s can be used following these steps:

1. Dissect the glands of interest under double distilled water.
2. Clean unwanted tissues attached to the gland.
3. Wash once or twice with double distilled water.
4. Transfer the gland into a glass vial
5. Extract with solvent.

As described by Millar and Sims (1998) the glands can often be extracted by soaking for a few minutes to hours. Furthermore, the extraction is solvent dependent with solvents such as alcohols penetrating membranes more effectively and yielding greater extraction of cellular content than less polar solvents (e.g. hexane). Although this approach provides relatively large quantities of compounds in the extracts, it might not correctly represent the composition of the emitted odour from the gland. The latter can be better assessed by *in situ* or *ex situ* head space collections (Millar and Sims, 1998) (see sections 2 and 3). The crude extracts can be tested directly or after fractionation using a hand-made column (Katzav-Gozansky *et al.,* 2001) (see section 4.2.2, steps 1-5) or commercially available columns.

Analysis and quantification of the extracted components can be achieved by GC-MS with an internal standard added during the preparation of the extract as previously described in section 4.2.3.1. The amount of the glandular secretion is calculated based on the average amount of several analysed queens, all components included. This amount is considered as queen equivalent (Qeq). Where the chemical nature of the suspected mixture is known, a synthetic blend of it prepared in the natural ratio of the individual components can be used. For example in the case of Dufour's gland, esters can be synthesized from commercially available alcohols and acid chlorides following standard procedures as described by Francke *et al.*, 2000 and Katzav-Gozansky *et al.* (2001), detailed below. The blend of the esters is prepared based on the relative proportions of the esters present in the queens' total glandular extracts. The doses used are calculated as queen equivalents (Qeq).

5.2.1. Preparation of synthetic esters

Esters can be synthesized according to the following standard procedure (Francke *et al.*, 2000):

1. Dissolve 10 mmol of the alcohol in 10 ml absolute pyridine.
2. Add a catalytic amount (ca. 10 mg) of 4-dimethylamino pyridine to facilitate the planned reaction.
3. Cool the solution in an ice bath.

4. Stir the solution and add 1.1 equivalent of the acid chloride, dissolved in 10 ml hexane.
5. Continue to stir the resulting mixture for one hour at room temperature.
6. Quench the reaction by slowly adding 100 ml of aqueous sodium hydrogen carbonate while stirring the mixture.
7. Extract the aqueous phase with two subsequent 20 ml portions of hexane.
8. Wash the combined hexane solution with 30 ml portions of each diluted hydrochloric acid, aqueous sodium hydrogen carbonate, and brine.
9. Dry the solution with anhydrous magnesium sulfate and remove the white material by filtration.
10. Concentrate the clear solution under reduced pressure of Nitrogen.
11. Chromatograph the resulting crude product on silica gel (Merck 60–200 mesh) using hexane/ethyl acetate 30:1 as the eluent.
12. Check the purity of the final product and provide its structural proof, by GC/MS (see section 2.2.4.1.1) and other spectroscopic methods (e.g. nuclear magnetic resonance (NMR)) in consultation with a chemist.

5.3. Stimuli presentation techniques

5.3.1. Use of surrogates

To study the impact of queen pheromone "bouquet" and its components on workers attraction, a surrogate queen is used. The surrogate material needs to be as chemically neutral as possible. Glass slides/ micropipettes are commonly used as surrogates. Apply the cues to be tested, for example glandular extracts, and its suspected chemical component(s) or corresponding solvent (control) on the surrogate and allow the solvent to evaporate for about 5 min. The other possibility is to use workers as a substrate to test a chemical. Presenting a secretion on a bee vs. an inanimate object such as a glass slide provides a more natural situation. When using this procedure, apply the treatment slowly on the worker thorax and allow the solvent to evaporate before the bioassay. The disadvantage of this procedure is that worker behaviour and chemical signals may interfere with the assay.

5.3.2. Stimuli preparation

To prepare a stimulus for testing in assays, potential solvent effects on the living honey bee used as a surrogate queen can be a challenge. Different solvents such as methanol (Kaminsky *et al.*, 1990), ethanol (Katzav-Gozansky *et al.*, 2003), dichloromethane (Wossler and Crewe, 1999) and isopropanol (Kocher *et al.*, 2009) can be used for stimuli presentations on the glass, but for living workers, ethanol is recommended (Katzav-Gozansky *et al.*, 2003). Irrespective of the solvent used, best results can be obtained by preparing the lure just prior to the bioassay.

5.4. Bioassays:

Using bioassays to assess the effect of a stimulus can pose various challenges. To identify behaviour modifying cues, a simple and repeatable bioassay, in which all parameters are well controlled, with the experimental conditions kept as close as possible to the natural ones is recommended. The bioassay should also be a discriminative and quantitative test that measures the induction of the tested behaviour. In studies of workers attraction to the queen, commonly used bioassays can be carried in or out of the colony. Each has its own advantages and shortcomings detailed below.

5.4.1. In colony assays

These can be performed in observation hives of various sizes, established for at least 2 weeks prior to experimentation (see the *BEEBOOK* paper on behavioural studies (Scheiner *et al.*, 2013)). The surrogates can be introduced and removed through portals in the walls situated on either side of the frame.

Pros: This setup almost completely mimics the natural situation. The presence of a large variety of worker bees creates a good chance to record a good response. It is suitable for evaluating changes in queen attractiveness.

Cons: The system is complex, includes a large number of uncontrollable cues changing with time. Moreover, the complexity of such bioassays and its operational cost is high, not only due to the price of the hive itself but all the necessary preparations. The colonies need to be established days in advance, thus making replication difficult and problematic for a routine screening of compounds in question. Behaviour of the bees can be affected by colony and weather conditions.

5.4.2. Choice assays on groups in semi natural conditions (micro-hives)

In this assay, caged bees can be used (see the *BEEBOOK* paper on maintaining adult *Apis mellifera* in cages under *in vitro* laboratory conditions (Williams *et al.*, 2013)). The cage must be fitted with transparent walls allowing for observation, with a small comb glued in the middle and two side openings for lure introduction. Briefly,

1. Place freshly emerged (about 1 day old) bees into the cage (Dor *et al.*, 2005; Malka *et al.*, 2007)
2. Feed them with sugar solution and pollen cakes ad libitum.
3. Keep the cages in a temperature controlled chamber simulating hive conditions (of 30-33°C and 50-70% relative humidity).
4. After a few (1-3) days, of acclimatization in darkness, present workers with the choice of lures.

5. Count the number of workers on the lure licking and antennating at specific time intervals determined in preliminary studies.

Pros: This type of assay offers greater control over environmental conditions; remotely resembles the natural situation of bees as they are kept on a comb; allows for maintenance of bees for about three weeks and enables the comparing of responses of the same group of bees to a series of doses or different compounds. It also enables the conduction of age-specific tests.

Cons: Such bioassays demand construction of special cages and possession of a temperature controlled chamber.

5.4.3. Arena tests

A disposable plastic Petri dish (15 x 2 cm) or 9 cm in diameter lined with a filter paper, can be used as a bioassay arena (Kaminsky *et al.*, 1990; Wossler and Crewe, 1999). The system is especially suitable for a two choice bioasay. For stimulus introduction, side slits for insertion of the tested material or openings for introduction of bees on both sides of the cover can be easily prepared (Katzav-Gozansky *et al.*, 2003). Individual behaviour of 10-20 worker bees can be observed at a time in such an arena. The best approach is to use workers aged 1-12 days which normally attend to the queen (Seeley, 1982). If the exact age of the bees is unknown, workers can be collected from the open brood area of a queenright colony and used for the screening bioassay. Freshly collected bees are best for this study. However, if it is intended to test the responsiveness of workers from a special age and task, these need to be prepared in advance by marking bees at emergence (see the section on obtaining brood and workers of known age in the *BEEBOOK* paper on miscellaneous methods (Human *et al.*, 2013)).

1. Introduce the glass slides (treatment and control) or marked workers (Fig. 7) simultaneously to the arena through the side openings.
2. Place the arena in a temperature controlled room (25-27°C). Either red or day light will do for this assay.
3. Record behaviour towards each one at pre-determined time intervals.

Since the volatility of the tested component is expected to affect the active space of the chemical and thus the detection time, the length of the observation need to be determined in preliminary studies. This set up enables simultaneous evaluation of multiple arenas.

Pros: It is fast and highly reproducible. It is also a sensitive bioassay. Especially well suited for evaluation of chemicals of low volatility. It requires minimal equipment investment. Positive response can be obtained with low amount of material i.e. 10^{-7} Qeq of synthetic 4 component queen mandibular pheromone (Kaminski *et al.*, 1990) and 1/3 Qeq of Dufours gland extract (Katzav-Gozansky *et al.*, 2003). This bioassay enables the testing of worker responses based on their specific ages and tasks.

Cons: This type of assay examines honey bee behaviour out of the natural context; the assay conducted in closed atmosphere with mostly still air can be problematic for evaluation of highly volatile compounds as the arenas could become saturated with volatiles rather quickly, thus eliminating the gradient to which the test organism could respond (Hare, 1998).

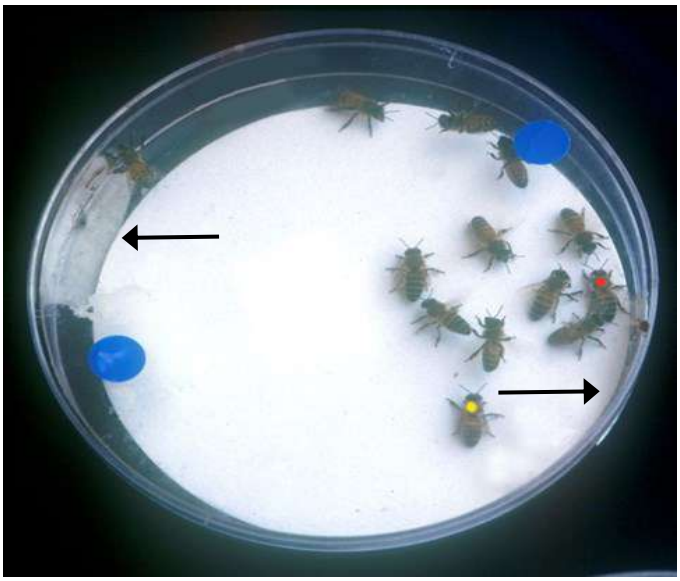

Fig. 7. The arena bioassay in the Petri dish. The red marked bee is pheromone treated while the yellow one is solvent treated control. The blue marks indicate openings for bees' introduction. Arrows indicate slots, for the glass slide introduction, when the latter are used as the surrogates.

5.4.3.1 An example of arena choice bioassay using live workers

Evaluation of the attractiveness of queen Dufour's gland pheromone extract on workers (Katzav-Gozansky *et al.*, 2003).

1. Apply the test material and also the solvent on two individually marked nurse bees in each assay.
2. The amount of secretion applied onto a glass or live bee should be calculated relative to 1 queen equivalent.

To illustrate, let's assume that 1 queen equivalent (Qeq) of Dufour's gland secretion is equal to 20 µg (all constituents included). If the total ester content amounts to 14 µg, then for 1 Qeq of a blend of the esters, prepare a synthetic mixture of the gland esters in ethanol based on the relative proportion of each ester present in the total glandular extract (see Table 2) (Katzav-Gozansky *et al.*, 1997).

3. Apply the gland extract (2 µl), its synthetic constituents (main esters) dissolved in ethanol using a micropipette.

4. Allow the solvent to evaporate for about 5 min.
5. Colour-mark the treated bees on the thorax after applying the extracts on the abdomen and allow the paint to dry for another 5-10 mins.
6. Introduce simultaneously extract and solvent treated bees into the arena.
7. Observe the activity of the bees for 5 min and record the number of workers attracted to the test material (contacting the worker) at 30-sec time intervals (total of 10 times).

For each replicate the sum of contacts for treatment vs. control over 5 min is used as a measure of attraction. The level of preference of the treatment vs. control can be presented as the percentage of time intervals in which there is preference towards the treated bee. Statistical analyses can be performed by any commonly used program that performs non-parametric statistical tests such as Wilcoxon Signed Rank test, Kruskal-Wallis ANOVA or repeated measures ANOVA after appropriate transformation (see also the *BEEBOOK* paper on statistics by Pirk *et al.* (2013)).

Table 2. Ester composition of Dufour's gland secretion of *Apis mellifera* queens and the amount used for preparation of ester synthetic blend (1 Qeq = 14 µg/gland). *- main compounds.

Esters	**Amount of synthetic ester compound (µg)**
Tetradecyl dodecanoate	0.14
Tetradecyl (Z)-9-tetradecenoate	Trace
Tetradecyl tetradecanoate*	2.88
Tetradecyl (Z)-9-hexadecenoate Tetradecyl (Z)-11-hexadecenoate*	Trace 3.46
Tetradecyl hexadecanoate Hexadecyl tetradecanoate*	1 2.78
Hexadecenyl hexadecenoate	0.88
Tetradecyl (Z)-9-octadecenoate*	1
Hexadecyl (Z)-9-hexadecenoate	0.94
Hexadecyl hexadecanoate	1.02
Octadecyl hexadecanoate	0.28

5.5. Summary

We have described both field and laboratory bioassays with their cons and pros for evaluating queen chemical cues inducing and sustaining the retinue of workers around the queen. This behaviour seems to be mediated by signals that are derived from a number of sources. The discussed bioassay systems offer a means for evaluation of the short range queen attractive compounds from various sources. These bioassays are useful for testing any suspected attractive source as well as for evaluating changes in worker responses to queen-retinue-inducing-signals and changes in the queen's abilities to produce such cues. Still, retinue behaviour does not necessarily mirror workers' response to the whole queen's chemical bouquet. Isolation of cues indicating queen quality, long range queen attractiveness etc., need to be guided by separate and specific bioassays. Moreover, in order to clearly distinguish the roles of volatile versus contact cues, the responding workers should be separated from the queen by double mesh screen to prevent any contact (Katzav-Gozansky *et al.*, 2004).

6. *In vitro* bioassay for studying mechanisms regulating pheromonal gland activity in honey bees

6.1. Introduction

The pheromonal system of the honey bee, *Apis mellifera* L. is complex and composed of several caste specific chemical signals mediating various colonial activities in an integrative manner. These caste specific signals are known to be produced by over 15 exocrine glands in workers and queens (Free, 1987; Blum, 1992). Pheromone production by these glands shows age, task and caste-based variations (Blum, 1992; Winston, 1987; Pankiw *et al.*, 1998; Robinson and Huang, 1998), which suggest plasticity in pheromone biosynthesis. Such biosynthetic plasticity has been demonstrated for the mandibular glands (Plettner *et al.*, 1996), Dufour's glands (Katzav-Gozansky *et al.*, 1997; Martin and Jones, 2004) and may also be true for some of the other exocrine glands.

Regulation of the biosynthesis of these pheromones within glands is mediated by hormones. For instance juvenile hormone (JH) is known to regulate the activity of the mandibular and Koshevnikov glands (Robinson, 1985) while pheromone production in the Dufour glands is regulated by unidentified brain factors (Katzav-Gozansky *et al.*, 2007). It is probable that pheromone biosynthesis in other exocrine glands are similarly regulated by various hormones.

To study the mechanisms behind the control of pheromone production, it is important to isolate the gland of interest and evaluate its performance *in vitro* via manipulation with potential regulatory factors. This is especially crucial when studying organs of a social organism whose function is affected at any moment by multiple external cues followed by activation of internal cues. The *in vitro* isolation of the gland enables the separation of the target tissue from the impact of suspected factors on its performance both at the organismal and molecular levels. At the organismal level we consider pheromone production, whilst at the molecular level we refer to gene expression evaluation via genomic and proteomic tools. So far, proteomic tools (see the *BEEBOOK* paper on physiology and biochemistry methods (Hartfelder *et al.*, 2013)) have been used in studies of mandibular

glandular performance *in vivo* (Hasegawa *et al.*, 2009; Malka *et al.*, 2009). The effect of each factor, their interaction and regulation mechanisms can be delineated using classical pharmacological methods but also with innovative molecular tools (e.g. RNAi-RNA interference, see the *BEEBOOK* paper on molecular methods by Evans *et al.* (2013)) without the interference of any social factors.

The idea of isolating pheromone producing glands was first successfully explored studying the control mechanism of sex-pheromone production in female moths (Soroker and Rafaeli, 1987), and thereafter to study ant postpharyngeal gland secretion (Soroker and Hefetz, 2000) and to discover the source of the locust pheromone phenylacetonitrile (Seidelmann *et al.*, 2003). This approach has been used largely to study moth sex-pheromone production and control (Rafaeli, 2009). In the case of honey bee exocrine glands it has only been briefly explored to study pheromone production by Dufour's and Mandibular glands (Katzav-Gozansky *et al.*, 2000 and Soroker and Katzav-Gozansky, unpublished data).

6.2. Methods

While developing a specific method for target tissue studies, several issues should be taken into consideration, in particular related to tissue isolation (section 6.2.1.), incubation conditions (section 6.2.6), appropriate precursors (section 6.2.4) and extraction procedures (section 6.2.8):

6.2.1. Isolation of organs/tissues

Incomplete isolation of the gland from other organs and tissues may negatively affect the glandular performance. For example, residues of fat body or other destroyed tissues can contribute degradation factors such as proteases, RNAases, etc. These can severely reduce glandular activity and thus obscure the studied effects. For example, Dufour's glands should be completely separated and cleared from the sting and the poison gland whilst mandibular glands need to be separated and cleared from labial, hypopharyngial glands and muscles. While dissecting the labial glands, the head glands need to be carefully separated from the hypopharyngeal gland and the thoracic glands for precise separation from the thoracic muscles.

6.2.2. Maintaining the normal performance of the gland in an artificial medium

In the body, the gland is surrounded by haemolymph characterized by specific composition (pH, inorganic salts, sugars, amino acids). Bee medium (Kaatz *et al.*, 1985, modified, as described in section 6.2.8.2.) has successfully been used in the *in vitro* incubation of honey bee Dufour's glands (Katzav -Gozansky *et al.*, 2000) to support glandular function.

6.2.3. Media contamination by microorganisms

Media contamination by microorganisms may severely affect the results directly interfering with glandular performance or by producing substances that may bias the results. Thus, only sterilised media should be used. Although the bees are themselves not sterile, the incubation should be maintained with care to reduce contamination especially when long term incubation is required. In some cases, surface sterilization of the bee by brief dipping in 70% ethanol prior to tissue dissection is recommended.

6.2.4. Selection and labelling of an appropriate precursor

Pheromonal signals, for example those produced by the mandibular gland often consist of multiple components (Blum, 1992). In addition, not all pheromonal components are necessarily derived from the same biosynthetic pathway. Thus precursors need to be selected according to the suspected biosynthetic pathway. For example, acetate is an appropriate precursor for evaluation of *de novo* biosynthesis in the case of pheromones that are derived from the fatty acid biosynthesis pathway such as Dufour's gland esters, hydrocarbons, 11- eicosanol and the acids of mandibular gland pheromone. However, for evaluation of other stages of synthesis, fatty acids or other compounds of appropriate length, saturated and unsaturated, should be used following the corresponding biosynthetic pathway (Blomquist *et al.*, 1987; Martin and Jones, 2004; Plettner *et al.*, 1995, 1996; Stanley-Samuelson, *et al.*, 1988).

Precursors can be labelled with stable or radioactive isotopes. Selection of a type of labelling will determine the methods for analysis of the biosynthetic product and which may in turn affect the detection sensitivity. Using radioactive labelled precursors containing $^{14}C/^{3}H$ has advantages by providing fast evaluation and high sensitivity, but these demand authorization from appropriate agencies as they may pose environmental risks. Labelling precursors with stable isotopes (^{13}C) is less hazardous but demands GC-MS analysis (see section 2.2.4). Precursors such as acetates/propionates/fatty acids are commercially available.

6.2.5. Incubation conditions

Incubation conditions (temperature and humidity) should be defined based on preliminary studies reflecting normal physiological and environmental conditions. Humidity should be maintained to prevent water evaporation and media concentration during the incubation process. Temperature can be maintained by incubating the samples in temperature controlled conditions (water bath or temperature controlled dry blocks). To prevent evaporation, the incubation vials must be kept sealed with protective cover or Parafilm. The number of glands (1 or 2 glands) per incubation may vary and depend mainly on the sensitivity of the detection method and on their secretion rate.

6.2.6. Determining optimum incubation time

Optimum incubation time should be adjusted by preliminary time course experiments, adjusted to the rate of biosynthesis and sensitivity of detection and depending on incubation temperature. However, care should be taken not to extend it over the period of 24 hours due to potential problems of tissue viability and bacterial contamination (unless sterile conditions prevail) and tissue deterioration.

6.2.7. Extraction of pheromone

Extraction of biosynthesized pheromonal components is the final step of an *in vitro* incubation. It involves extraction of the product from the incubation medium by an appropriate solvent and this depends on the chemical properties of the product. Recommended solvents for extracting pheromonal components are pentane/hexane/heptane or dichloromethane. The first group of solvents is especially suited for non-polar compounds such as hydrocarbons while dicholoromethane, a moderately polar solvent, efficiently extracts components such as aldehydes and ketones.

6.2.8. Product analysis

The various newly synthesized components can be separated and analysed using methods as determined by the nature of the precursor label. When radioactive labelling is involved, the major compounds or classes of compounds can be separated by a number of techniques depending on the nature of products, for example HPLC separation with radioactive detection. Other options include thin-layer chromatography (TLC) separation followed by phosphor imager detection (Katzav-Gozansky *et al.*, 2000) as described in section 6.2.8.3. For stable isotopes, GC-MS or LC-MS using single ion monitoring can be used (Tsfadia *et al.*, 2008). Below, we take the example of Dufour's and Mandibular glands for the description of the analysis of exocrine glands products (after Katzav-Gozansky *et al.*, 1997).

6.2.8.1. Dissection and sample preparation

1. Chill freshly collected bees on ice prior to dissection.
2. Dissect the exocrine glands under a stereo microscope (X20) using the medium specified below (see section 6.2.8.2.).
3. Wash the glands twice in fresh medium.
4. Transfer 1 or 2 glands to 80 - 100 µl medium supplemented with a labelled precursor (1 µCi [1-^{14}C] sodium acetate. An aliquot of ethanolic solution of sodium acetate , 1 µCi/gland, is dispensed to a vial, ethanol is evaporated to dryness under N_2 prior to addition of the incubation medium (56 mCi/mmol, Perkin Elmer).

For best results, worker glands should be incubated in pairs whilst the queen gland can be individually incubated (see section 6.2.8.2. for incubation medium preparation).

5. Incubate glands at 39°C, for 4-20 hours.

6.2.8.2 Bee incubation medium preparation based on Kaatz (1985)

Amino Acids (AA) (50 ml)

1. Dissolve L-Cysteine (5 mg) in 2 ml 1M HCl,
2. Add L-Tyrosine (10 mg) to 40 ml of double distilled water.
3. Dissolve the remainder Amino Acids one after the other: 60 mg L-alanine, 51 mg L-arginine, 20 mg L-aspargine, 10 mg L-aspartic acid, 25 mg L-glutamic acid, 100 mg L-glutamine, 20 mg L-glycine, 20 mg L-histidine, 10 mg L-isoleucine, 55 mg L-lysine, 5 mg L-methionine, 20 mg L-phenylalanine, 330 mg L-proline, 20 mg DL-serine, 15 mg L-threonine, 5 mg L-tryptophan, 15 mg L-valine.
4. Add double-distilled water to make up the volume to 50 ml.

Inorganic Salts (IS) (20 ml):

Dissolve 179 mg KCl, 40.6 mg $MgCl_2*6H_20$, 49.3 mg $MgSO_4*7H_2O$, 69 mg $NaH_2PO_4*H_2O$, 33.6 mg $NaHCO_3$, in 20 ml DDW.

Sugars (15ml):

Dissolve 400 mg glucose, 250 mg fructose and 6700 mg sucrose in 15ml DDW.

Mixture of all components

1. Mix the three groups of components (AA, IS and sugars) with piperazine-N,N'-bis(2-ethanesulfonic acid buffer (PIPES) (756 mg).
2. Titrate with NaOH until PIPES is completely dissolved.
3. When all components are dissolved, add NaCl (100 mg).
4. Adjust the pH with NaOH to 6.7.
5. Add 2ml of $CaCl_2*2H_2O$ (56.45 mg/ml in DDW) to the medium.
6. Bring the medium to a final volume of 100 ml.
7. Sterilize the medium by filtration via Millipore 0.22 µm.

6.2.8.3. Isolation and identification of Dufour's biosynthesis products.

The glands and incubation media are extracted in 350 µl dichloromethane for 24 h after incubation and then subjected to TLC. To isolate Dufour's gland biosynthesis products, carry out TLC of the extract using silica gel coated plates (polygram Sil G). The radioactivity of the various TLC fractions can be determined by a phosphor imager (IP Autoradiography System). Quantification of the radioactive fractions (Rfs) is achieved by comparison of its radiation to a standard radiation curve generated using different doses of radioactive acetate. TLC separation is performed in two successive steps:

1. Run the TLC plate using hexane to separate the components.
2. Air-dry the plate in a fume hood.
3. Subject the dried TLC plate to a second separation in a mixture of hexane: diethyl ether: acetic acid (70:30:1) (v/v), as the running solvent.
4. Identify the various lipid classes by comparing their Rf values with those of co-chromatographed standards (unsaturated).
5. Visualize by iodine vapour (in pre-prepared saturated tank).

In case of Dufours gland, suitable standards are: cis-9-tricosane (hydrocarbons); oleic acid (fatty acids); 11-eicosenol (alcohol); palmitic acid myristhyl ester and palmitoleic acid stearyl ester (esters); trinervonin and triolen (triglycerides). This procedure can be validated by using unlabelled products followed by GC/MS analysis as described below.

1. Incubate Dufour's glands as described in section 6.2.8.2. using cold acetate as a precursor (0.3 mg/ml).
2. Apply the glandular extracts on TLC along with commercial TLC standards (as above).
3. Perform TLC separation as previously described and allow the plate to dry.
4. Cut off the part of plates containing the commercial standards and expose it to iodine vapour to prevent modifications to compounds in the sample lanes designated for GC-MS analysis.
5. Mark the position of the standards.
6. In the sample lanes scrape the silica gel of the areas corresponding to standards on the TLC, into a glass vial and extract with chloroform, evaporate to dryness, reconstitute in dichloromethane.
7. Concentrate using N_2 and subject the product to GC-MS to identify relevant peaks as described in section 2.2.4.

Pros: The obvious advantage of the above described system is in its ability to study the activity of the exocrine gland detached from its original controlled environment. By manipulating the environment of the gland, factors and mechanisms regulating glandular activity can be isolated. This technique enables the researcher to separate between the effects of social regulation and possible physiological constraints of any studied gland.

Cons: However, the isolated gland function may not necessarily represent the full range of glandular functions as some of these may be dependent on unknown precursors received from elsewhere in the body of the bee. Thus, it is advisable to compare in vitro function of the gland with in vivo labelling studies, followed by glandular extraction and determination of de novo synthesized products.

6.3. Summary

In this section we have provided a step-by-step approach method for conducting an *in vitro* bioassay. We have also provided the pros and cons associated with using certain solvents, reagents and conditions for isolating gland biosynthetic products. Further reading using the references provide is recommended.

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