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REVIEW ARTICLE

Standard methods for varroa research

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Summary

Very rapidly after *Varroa destructor* invaded apiaries of *Apis mellifera*, the devastating effect of this mite prompted an active research effort to understand and control this parasite. Over a few decades, varroa has spread to most countries exploiting *A. mellifera*. As a consequence, a large number of teams have worked with this organism, developing a diversity of research methods. Often different approaches have been followed to achieve the same goal. The diversity of methods made the results difficult to compare, thus hindering our understanding of this parasite. In this paper, we provide easy to use protocols for the collection, identification, diagnosis, rearing, breeding, marking and measurement of infestation rates and fertility of *V. destructor*. We also describe experimental protocols to study orientation and feeding of the mite, to infest colonies or cells and measure the mite's susceptibility to acaricides. Where relevant, we describe which mite should be used for bioassays since their behaviour is influenced by their physiological state. We also give a method to determine the damage threshold above which varroa damages colonies. This tool is fundamental to be able to implement integrated control concepts. We have described pros and cons for all methods for the user to know which method to use under which circumstances. These methods could be embraced as standards by the community when designing and performing research on *V. destructor*.

Métodos estándar de la investigación en varroa étodos

Resumen

Poco tiempo después de que el ácaro *Varroa destructor* invadiera las colmenas de *Apis mellifera*, su efecto devastador produjo un efectivo esfuerzo investigador para comprender y controlar este parásito. En unas pocas décadas, la varroasis se ha extendido a la mayoría de los países que explotan a *A. mellifera*. Como consecuencia, un gran número de equipos han trabajado con este organismo desarrollando diversos métodos de investigación. A menudo, se han utilizado diferentes enfoques para lograr el mismo objetivo. La diversidad de métodos hizo que los resultados fueran difíciles de comparar, lo que dificulta la comprensión de este parásito. En este artículo se proporcionan protocolos fáciles de usar para la recolección, identificación, diagnóstico, cría, cruzamiento, marcaje y medición de los índices de infestación y la fertilidad de *V. destructor*. También se describen los protocolos experimentales para el estudio de la orientación y la alimentación de los ácaros, la infestación de colonias o células y para medir la susceptibilidad del ácaro a los acaricidas. Cuando es pertinente, se describe qué ácaro se debe utilizar para los bioensayos puesto que su comportamiento está influido por su estado fisiológico. También proporcionamos un método para

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determinar el umbral de daño más allá del cual varroa causa daños a las colonias. Esta herramienta es fundamental para poder poner en práctica el concepto de control integrado. Hemos descrito los pros y los contras de todos los métodos para que el usuario sepa qué método utilizar según las circunstancias. Estos métodos podrían ser adoptados como estándares por la comunidad para el diseño y la realización de investigaciones sobre *V. destructor*.

大蜂螨研究的标准方法

自狄斯瓦螨侵袭西方蜜蜂蜂场以来，其带来的毁灭性危害促进了该领域的研究工作。在过去的几十年里，大蜂螨已分布到大多数饲养西方蜜蜂的国家。由此许多研究团队开展了蜂螨的研究工作，并形成了多种研究方法。但往往是运用不同的方法解决了同一问题，同时也造成了实验结果难以比较，妨碍了我们对大蜂螨的认知。本文我们提出了一些简单实用的实验方案，可用于开展大蜂螨感染率和生殖力方面的研究，包含了收集、鉴定、诊断、饲养、育种、标记和检测技术。还提供了研究蜂螨定位和饲养蜂螨，蜂螨侵染蜂群、侵染巢房以及蜂螨对杀螨剂的耐药性的相关实验方案。在相关内容中还描述了如何选择蜂螨开展生物学实验，因为蜂螨的行为在其不同的生理阶段是不同的。还给出了测定蜂螨对蜂群危害的临界值的方法，这是实施蜂螨综合治理的基本工具。对所有的方法我们都描述了其优、缺点，以帮助研究者选择合适的方法开展工作。这些方法也可作为标准方法介绍给广大从事大蜂螨研究或治理的工作者。

Keywords: COLOSS, *BEEBOOK*, *Varroa destructor*, *Apis mellifera*, research method, protocol, orientation, feeding, marking, taxonomy, bioassay, damage threshold, acaricide, artificial infestation, breeding, honey bee

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1. Introduction

Most honey bee researchers consider the ectoparasitic mite *Varroa destructor* to be the most damaging enemy of the honey bee. It has been recently identified as one of the major factor responsible for colony losses worldwide (e.g. Brodschneider *et al.*, 2010; Chauzat *et al.*, 2010; Dahle, 2010; Genersch *et al.*, 2010; Guzman-Novoa *et al.*, 2010; Schäfer *et al.*, 2010; Topolska *et al.*, 2010; vanEngelsdorp *et al.*, 2011; Martin *et al.*, 2012; Nazzi *et al.*, 2012). Both the development of new and innovative control methods against the mite and further studies on the complex interaction with the honey bee should be a priority in bee health research (Dietemann *et al.*, 2012). The use of standardised methods by those studying the mite will greatly increase the impact of such work. When reviewing the literature, researchers should take note that publications prior to 2000 mention *V. jacobsoni* instead of *V. destructor*. The species name was changed after Anderson and Trueman (2000) demonstrated with molecular tools that the invasive population was not the species from Indonesia described by Oudemans in 1904.

2. Taxonomy and systematics

2.1. Taxonomy

Varroa mites were first discovered more than 100 years ago on the Asian honey bee (*Apis cerana*) in Java, Indonesia and named *Varroa jacobsoni* (Oudemans, 1904). They were assigned to a new genus, *Varroa*, and eventually to a new family, Varroidae (Delfinado-Baker and Baker, 1974). At present the genus contains four species. Since the initial discovery, it has become clear that varroa mites are native brood parasites of a group of cavity nesting Asian honey bees that are closely related to *A. cerana*. These include, *A. cerana* itself (which is distributed throughout most of Asia), *A. koschevnikovi* (Borneo and surrounding regions), *A. nigrocincta* (Sulawesi) and *A. nuluensis* (Borneo). These bees are still undergoing taxonomic revision as seen by the recent proposal to elevate the plains honey bee of south India to a new species, *A. indica*, and separate it from *A. cerana* (Lo *et al.*, 2010). At present, varroa mites are only known to infest *A. cerana*, *A. koschevnikovi* and *A. nigrocincta*, although very few surveys for mites have been reported for *A. nigrocincta*, *A. nuluensis* or *A. indica* and those mites that have been found on *A. nigrocincta* in Sulawesi were most likely not native to that bee, but rather to sympatric *A. cerana* (Anderson and Trueman, 2000).

It is not exactly certain when the European honey bee (*A. mellifera*) first came in contact with varroa but it certainly occurred after that bee was introduced into Asia by man (De Jong *et al.*, 1982a). There are specimens of varroa in the Acarological Collection at Oregon State University, USA, that were collected from *A. mellifera* in China during the middle of the last century (Akratanakul and Burgett, 1975). The varroa mites that have since utilized *A. mellifera* as a host are all members of *V. destructor*, the most recently described species of the genus, and are native to *A. cerana* in northeast Asia (Anderson and Trueman, 2000). Hence, the current four recognized species of varroa came about through a long process of speciation on Asian honey bee hosts and, given the rather uncertain taxonomic status of those bees, it is possible that new varroa species await discovery. Prolonged co-evolution of *V. destructor* and *A. mellifera* may yet see these mites also becoming genetically diverse (Oldroyd, 1999), particularly as they gradually adapt to exist on isolated populations of *A. mellifera*. However, the movement of bee stocks around the world by man and the beekeeping practice of re-queening large numbers of *A. mellifera* colonies on a regular basis with queens from a common source will, to some extent, counter natural evolutionary processes that may eventually lead to varroa speciation on *A. mellifera*.

Various methods have been used over the years to determine variation within varroa, all of which have contributed to the current level of taxonomic understanding. The most common and simple methods of identifying species have been those that provide measurements of mite physical characteristics (morphology). These methods are discussed below. The initial discoveries of *V. jacobsoni* on *A. cerana*, *V. underwoodi* on *A. cerana* and *V. rindereri* on *A. koschevnikovi* all resulted from morphological studies.

More recently, molecular methods have helped clarify varroa taxonomy and have proven particularly useful for identifying genetic variation within species and even identifying cryptic species. These methods, also described below, played a crucial role in the discovery of a new species, *V. destructor*, and in showing that it was that species, not *V. jacobsoni* as previously thought, that had colonized *A. mellifera* after its introduction into Asia (Anderson and Trueman, 2000).

The current taxonomy of varroa on Asian honey bees can be summarized as follows (after Lindquist *et al.*, 2009):

Kingdom: Animalia
Phylum: Arthropoda
Class: Arachnida
Subclass: Acari
Superorder: Parasitiformes
Order: Mesostigmata
Family: Varroidae
Genus: *Varroa*
Species:
- *V. jacobsoni* (Oudemans, 1904)
- *V. underwoodi* (Delfinado-Baker and Aggarwal, 1987)
- *V. rindereri* (De Guzman and Delfinado-Baker, 1996)
- *V. destructor* (Anderson and Trueman, 2000).

The taxonomic status of three genetically distinct varroa types that infest *A. cerana* in the Philippines remains unresolved at this time (Anderson, 2000; Anderson and Trueman, 2000).

Mites of just two 'haplogroups' of *V. destructor* (see section 2.4.5. 'Haplogroup and haplotype identification') have colonized *A. mellifera* globally. Of the two, those belonging to a Korea haplogroup are the most common and widespread on *A. mellifera*, being present in Europe, the Middle East, Africa, Asia, the Americas and New Zealand. Mites of a Japan haplogroup are less common on *A. mellifera*, and are only found in Thailand, Japan and the Americas (Anderson and Trueman, 2000; Warrit *et al*., 2006). At the present time Australia remains the only large landmass on earth on which the resident *A. mellifera* are free of varroa.

2.2. Collection of mites for identification

The best varroa specimens for laboratory analyses are those that have been collected live and preserved immediately. A benefit of sampling live mites is that they can be submerged in hot water prior to their preservation. This relaxes internal body tissues and exposes hard-to see organs, such as the chelicerae, which usually remain hidden from sight in mites collected directly into alcohol.

2.2.1. Mite appearance

Adult females are large (about 1.5 mm in width) and reddish-brown in colour, whereas males and female nymph stages are smaller and cream or white in colour. All stages are easily seen by the naked eye (Fig. 1). Each of the different life stages may be carefully removed from cells with the aid of a fine pair of forceps (such as #55 biologie forceps, Cat. No. 11255, from FST Fine Science Tools Inc.; Canada; Fig. 2) or soft paintbrush and dunked immediately into preserving fluid in a collection vial. Mites dunked into a vial of alcohol will immediately die and sink to the bottom, whereas those dunked into a vial of RNAlater will float on the surface and crawl around the inside of the vial before eventually dying some time later.

2.2.2. Where to find mites

Live adult mites, nymphs and eggs are most easily found in capped brood cells of bee colonies in which adult female mites are reproducing. In *A. cerana* colonies this is restricted to drone cells, but in *A. mellifera* colonies it may be either drone or worker cells. After removing the wax cappings and bee brood, the presence of white faecal deposits on cell walls (Fig. 3) is a sure indicator of the presence of reproducing females. Collecting mites from brood cells with offspring also provides evidence that these mites indeed reproduce on the bee species they have been collected from, as mites sometimes drift to and from colonies of foreign species on which they are unable to reproduce (Anderson and Trueman, 2000; Koeniger *et al*., 2002), which might confuse the host-specificity attributed to them. Only live adult female varroa can be collected from broodless bee colonies. These are generally found on the bodies or in body cavities of worker bees.

Fig. 1. A mite family with mother mite (reddish brown) and different stages of offspring at the bottom of a cell from which the honey bee pupa was removed. Photo: Denis Anderson.

Fig. 2. Tool kit to collect *Varroa* spp. mites. Photo: Denis Anderson.

Fig. 3. In this section of a cell (the bottom is on the right side), the pearly white faeces deposit is visible on the upper and back walls. Mature and immature varroa mites are also visible.

Photo: Swiss Bee Research Institute

2.2.3. Sampling techniques

Varroa spp. mites can be sampled from brood or adult workers. Sampling techniques are described below in section 3.1. 'Collecting mites'.

2.2.4. Storage of mite samples

2.2.4.1. Storage medium and conditions

Mites collected in the field should be preserved immediately in 70-95% ethyl alcohol or RNAlater. This ensures the specimens are not damaged and, even if they are kept this way at room temperature, are good for morphological analyses for at least a few months, but often much longer. However, if specimens are to be used in DNA analysis, they should be stored in a cool environment, such as a fridge at 4°C or freezer at -20°C, within a few days of collection to slow the degradation of DNA in body tissues. Specimens frozen at -20°C remain viable for several years, but to remain viable longer, they should be stored at -70°C (see the section on 'Storing dead adults' in the *BEEBOOK* paper on miscellaneous methods (Human *et al*., 2013)).

2.2.4.2. Storage and collection container

Ideal containers for collecting mites are small and made from tough plastic, such as the small plastic 1.5 ml cryogenic vial supplied by Nalgene®, shown in Fig. 2. This vial may hold hundreds of mite specimens and has a large white-coloured area on its outside for a label. Importantly, its lid is secured on a thread that runs down the outside of the vial. This ensures that no preserving fluid is forced from the vial as it is being closed, which could result in smudging or complete removal of the label. The label should contain essential information, such as the date of collection, name of host bee, location and name of collector, using a fine point permanent marker pen. To overcome external labels becoming removed from the collection vial, a small piece of paper on which the collection data have been written with a pencil (alcohol resistant) may be inserted in the vial, with the sample.

2.2.5. Sample shipping

Specimens should be transported to their destination as soon as possible after collection. Some airlines prohibit the carriage of biological specimens preserved in alcohol on aircraft, whilst others are less stringent. It pays to check airline policy in this regard before attempting to send or carry specimens preserved in alcohol. A convenient way to avoid this problem is to pour the alcohol off the specimens shortly before transportation. In this way the specimens will still remain covered with a very small amount of alcohol and thus remain saturated in alcohol and preserved during transport. However, upon arrival the specimens should be again well-covered in fresh alcohol before storage. Some transportation courier services have arrangements in place with airlines to transport biological specimens preserved in alcohol on aircraft.

Some countries (e.g. Australia and the USA) require an official quarantine import permit to accompany imported varroa mite specimens. Other countries (e.g. Brazil) may prohibit the exportation of specimen due to specific laws on biopiracy. Therefore, before sending or transporting specimens to a particular country, that country's policy on importing biological specimens should be checked and followed.

2.3. Morphological methods for identifying varroa

The four recognized species of varroa are readily identified morphologically and are shown for comparison in Fig. 4.

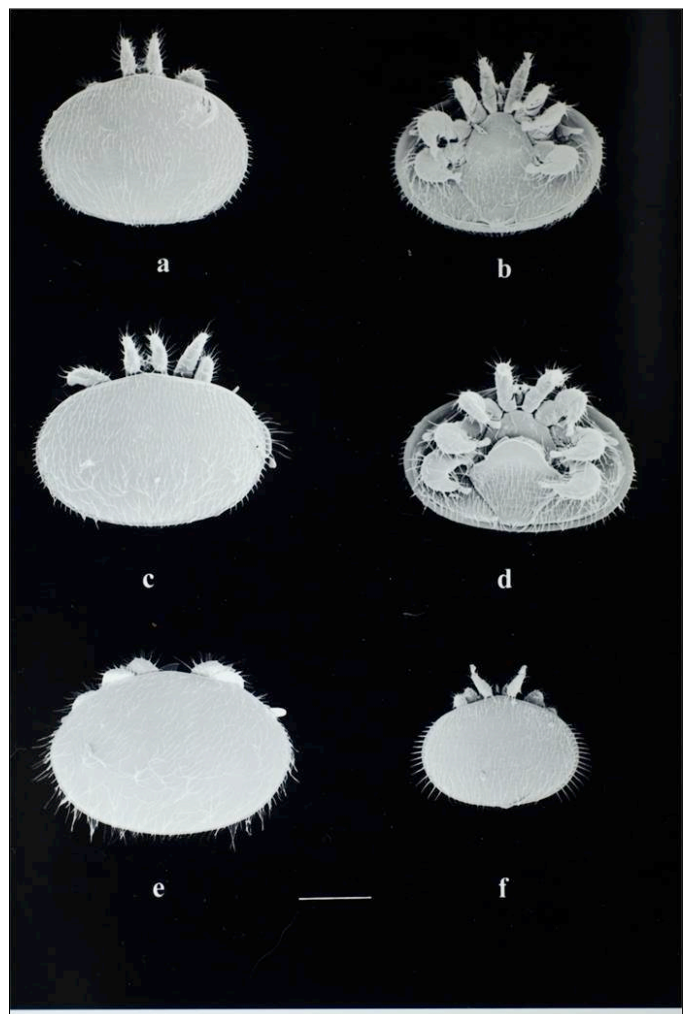

Fig. 4. The four species of *Varroa*: ***a.*** *V. jacobsoni* dorsal view; ***b.*** *V. jacobsoni* ventral view; ***c.*** *V. destructor* dorsal view; ***d.*** *V. destructor* ventral view; ***e.*** *V. rindereri*; ***f.*** *V. underwoodi.*

Photo: Denis Anderson.

2.3.1. Sample preparation

Morphological analyses are best carried out on mite specimens that have been mounted on glass microscope slides. For this, a specimen must first be cleared of its soft tissues before being mounted on a slide.

This is achieved as follows.

1. Remove specimen from preserving medium.
2. Immerse specimen in Nesbitt's Solution (see recipe below) in the depression of a concave slide.
3. Wait until the specimen becomes saturated with Nesbitt's Solution, and then push it under the surface of the solution to make it sink to the bottom, using a fine needle.
4. Place a cover slip over the depression of the slide.
5. Warm the slide in an oven for 1 hour at 45°C.
   The specimen should become free of body tissue and appear transparent, but older specimens may require further clearing in the oven for several hours or overnight.

This procedure can be speeded-up by warming the slide over a flame or hotplate for a few seconds, instead of placing it in an oven. However, extreme care should be taken to avoid boiling the Nesbitt's Solution, which will destroy the specimen. Laboratory gloves and coat should be worn when clearing specimens.

The cleared specimen is then mounted as follows:

6. Remove specimen from the Nesbitt's Solution and transfer it to a drop of Hoyer's Mounting Medium (see recipe below) on a glass microscope slide.
   Note: the drop should be just large enough to form a thin layer when a cover slip is placed on top, without overflowing around the edges of the cover slip.
7. Push the specimen down through the Hoyer's so that it rests on the slide, using a fine needle.
8. Gently lower a cover slip (thickness No. 1, diameter 16 mm) over the drop of Hoyer's, starting from the edge of the drop and letting it slowly settle over the drop under its own weight, spreading the Hoyer's as it goes.
9. Place the slide horizontally to cure in an oven at 45°C for at least 2 weeks.
10. Label and store slide.

Hoyer's medium does not completely harden and remains water-soluble, so that the slide can be reheated and specimen floated off the slide for dissection or re-mounting. For long-term storage or for transporting, the edges of the cover slip should be sealed with some water-resistant material, such as clear fingernail varnish. Laboratory gloves and coat should be worn when mounting specimens.

2.3.1.1. Recipe for Nesbitt's Solution:

- 60 g of chloral hydrate.
- 10 ml of concentrated (35.4%) hydrochloric acid.
- All dissolved in 100 ml of distilled water.

Note: care should be taken in preparing this solution, as it is highly corrosive to skin and microscope.

2.3.1.2. Recipe for Hoyer's medium:

- 30 g of gum Arabic.
- 200 g of chloral hydrate.
- 20 ml of glycerol.
- All dissolved in 50 ml of distilled water.

Note: the mixture needs to be stirred and warmed gently to allow the gum Arabic to dissolve, then filtered through muslin and stored in an airtight container, but not a container with a screw cap, as the cap will become permanently stuck.

2.3.2. Sample identification

Mounted mite specimens are best examined with dissecting or compound light microscopes that have been fitted with ocular micrometers. The following measurements should be considered.

- Body size (length and width).
- Structure and setation (i.e. stiff hair, bristle) of dorsal shield.
- Structure and chaetotaxy of the sternal, epigynal, anal and metapodal shields, peritreme, tritosternum and hypostome (see Fernandez and Coineau, 2007 for a description of varroa morphology).
- Number, arrangement and morphology of setae on the legs and palps.

The two species *V. destructor* and *V. jacobsoni* are morphologically similar, except in body size and shape. *V. jacobsoni* is much smaller and more circular in shape than *V. destructor* (Fig. 4). Nevertheless, some *V. jacobsoni* (e.g. those found on *A. cerana* in Laos, mainland Asia) are much larger than other *V. jacobsoni*. Hence it is always best to confirm a diagnosis of either of these species with additional molecular information.

In case varroa work is conducted in Asia where several species cohabit, we provide a determination key adapted from Oldroyd and Wongsiri (2006) and Warrit and Lekprayoon (2011) to differentiate those mites. Varroa mites have body as wide or wider as it is long. This characteristic distinguishes it from other Asian parasitic mite genera *Tropilaelaps* (with a body longer than it is wide) and *Euvarroa* (triangular shaped body).

2.4. Molecular methods and systematics

Molecular technology was first used in varroa research during the 1990s to look for variation within and among mite populations (Kraus and Hunt, 1995; De Guzman *et al.*, 1997, 1998, 1999; Anderson and Fuchs, 1998). Initially it was expensive and was only used by specialised laboratories. Currently, the landscape has changed and a number of quick and easy commercial kits can be purchased for extracting DNA from tissue and any number of laboratories will sequence DNA for a reasonable fee within hours of its extraction.

Sequence data from small DNA fragments (< 1,000 base pairs) has been particularly useful in providing 'snap-shots' of genetic variation across the entire varroa genome and for use in phylogenetic

Key to identification of varroa species * 'gutters' protruding from the spiracle on the ventral side, towards the edge of the body at the level of the third pair of legs (see Fernandez and Coineau, 2007).

1. a. Peritremes* are long and looping up from the ventral side, extending beyond the lateral margin of the dorsal shield and thus sometimes visible on a dorsal view.	*Varroa rindereri* (Fig. 4e) primarily found parasitizing *A. koschevnikovi*
b. Peritremes not extending beyond the lateral margin of the dorsal shield.	2.
2. a. Setae of the lateral margin long and slender	*Varroa underwoodi* (Fig. 4f) primarily found parasitizing *A. dorsata, A. laboriosa and A. breviligula*
b. Setae shorter and stout.	3.
3. a. Body size ratio (width to length) 1.2-1.3:1	*Varroa jacobsoni* (Fig. 4a, b) parasitize *A. cerana* on Sundaland, including *A. nigrocincta* on Sulawesi
b. Body size ratio ≥ 1.4	*Varroa destructor* (Fig. 4c, d) parasitize *A. cerana* on Mainland Asia, and *A. mellifera* worldwide

analyses or as molecular markers (Avise, 2004). Sequencing involves three basic steps described below in sections 2.4.1., 2.4.2. and 2.4.3.:

1. Extraction of total DNA from mite tissue.
2. Amplifying (making copies of) fragments of that DNA using Polymerase Chain Reaction (PCR).
3. Sequencing the amplified fragments.

2.4.1. DNA extraction

DNA is sourced from the tissue of *Varroa* spp. mites that have been collected and preserved in 70% ethyl alcohol or RNAlater (as described in section 2.2.4.1. 'Storage medium and conditions'). Any tissues can be used, but if the tissue is dissected from a single appendage (such as a leg), the rest of the mite can be used for other purposes. See the section on 'CTAB genomic DNA extraction from adult bees' of the *BEEBOOK* paper on molecular methods (Evans *et al.*, 2013)) for extraction methods.

2.4.2. DNA amplification

DNA amplification requires a PCR machine (such as an Eppendorf Mastercycler®) and a set of specific forward and reverse primers. The machine is initially programmed to carry out a number of cycles to amplify the DNA (see the section 'DNA methods' of the *BEEBOOK* paper on molecular methods (Evans *et al.*, 2013).

A commonly used method for amplifying *Varroa* spp. DNA consists of:

1. PCR thermo-cycles of 5 min pre-denaturation at 94°C.
2. 35 cycles of denaturation at 94°C for 1 min.
3. 1 min annealing at 52°C.
4. 2 min extension at 72°C.
5. Final extension at 72°C for 5 min.

2.4.3. DNA sequencing

Amplified DNA can be then sent to a laboratory for sequencing and the sequence can then be compared with sequences in GenBank using BLAST (see the section 'Obtaining and formatting sequences of interest for phylogenetics' of the *BEEBOOK* paper on molecular methods (Evans *et al.*, 2013)). The critical part of DNA sequencing is to decide which gene to sequence for a particular outcome, and only trial and error will determine this. Fortunately, studies have already shown that sequences obtained from specific regions of the mitochondrial DNA (mtDNA) of varroa are useful for examining inter and intra-species variation (Anderson and Fuchs, 1998; Anderson and Trueman, 2000; Navajas *et al.*, 2010).

2.4.4. Species identification

A 458 DNA base-pair fragment of varroa mtDNA *cox1* gene has proved useful in identifying mites to a particular species (Anderson and Trueman, 2000). To do this, a sequence of the fragment is obtained from a mite and compared to other sequences of the same region deposited in the GenBank database. If this sequence shows 2% or less difference from the one in the database, then it is considered to be a member of this particular species. Fragments from each of the 4 recognized species differ from each other by about 6%

(Anderson and Trueman, 2000). Sequences of the *cox1* gene fragment have been obtained from all *Varroa* spp. mites that have been identified to date by molecular methods. Hence, sequences of this fragment should be incorporated into all new molecular studies on varroa mites, as it places this new work in context with what has gone before.

2.4.5. Haplogroup and haplotype identification

The varroa *cox1* gene marker is also useful for identifying mites of large discreet populations within a species (such as island populations) (Anderson and Trueman, 2000). As smaller populations within these larger populations can be identified by concatenated (joined) sequence data obtained from the mtDNA *cox3*, *atp6* and *cytb* genes, the larger discreet populations have been referred to as 'haplogroups' and the smaller populations within them 'haplotypes' (Navajas *et al.*, 2010). The primer sequences for amplifying all these fragments, together with the size of fragments amplified, are shown in Table 1.

As the varroa *cox1* gene marker has proved useful for resolving mites from haplogroups to a particular species it has also been useful in phylogenetic studies. A phylogenetic tree of the all the currently known and published haplogroups with species is shown in Fig. 5 (see the section 'Phylogenetic analysis of sequence data' of the *BEEBOOK* paper on molecular methods (Evans *et al.*, 2013) for methods to perform phylogenetic analyses).

2.4.6. Kinship determination with microsatellites

Microsatellites are useful markers for measuring kinship or paternity relationships within varroa populations. These consist of repeating sequence of base-pairs DNA (such as CACACA) at a single locus (see the section 'Microsatellites' in the *BEEBOOK* paper on molecular methods (Evans *et al.*, 2013)). Loci with long repeats have more alleles than loci with short repeats and therefore often allow for a progenitor of a particular allele to be identified. Microsatellite loci in varroa have been published by Evans (2000) and Solignac *et al.* (2005).

2.5. Perspectives on the taxonomy of *Varroa* spp.

More research is needed to clarify the taxonomy of varroa mites from *A. cerana* in the Philippines. This will require examinations of nuclear DNA sequences obtained from these mites, as their mtDNA sequences do not provide the resolution needed to determine their identity (Fig. 5). From published research, there are three distinct mite types in the Philippines, two from the northern island of Luzon and another from the southern island of Mindanao (Anderson and Trueman, 2000). These could well be distinct species. New varroa species may also yet be found on other Asian honey bees, particularly on *A. nigrocincta* in Sulawesi, *A. nuluensis* in Borneo and *A. indica* from southern India.

An interesting feature of varroa mites on Asia honey bees is that most of them lack the ability to reproduce on *A. mellifera*. This is not from lack of trying though, for when *A. mellifera* colonies are introduced to different regions of Asia female mites that are indigenous to the local Asian honey bee readily invade the introduced colonies and enter brood cells that are about to be capped, in preparation for reproduction. However, they do not go on to lay eggs or produce offspring. Since at least the middle of the last century, only a few mite types have been able to reproduce on *A. mellifera*, the most successful of which is the Korea type of *V. destructor*. This suggests that female varroa mites must recognize specific signals on the host bee in order to successfully reproduce. Even though these signals may be fundamentally the same between different honey bee types and species, they may vary between honey bee populations. Identifying these signals and the genes that control them, could lead to the genes being targeted for particular purposes, such as control. This kind of research will require a good understanding of both the parasite and host genomes. Even though our understanding of the honey bee genome has improved in recent years, studies have only recently commenced on sequencing the varroa genome (Cornman *et al.*, 2010). As our understanding of the varroa genome improves, too will our understanding of varroa taxonomy and ways by which the mite can be controlled on European honey bees.

Table 1. Primer sequences (and their names) used in varroa research to amplify fragments of a particular size (base pairs) of mtDNA genes. From Anderson and Fuchs (1998); Navajas *et al.* (2010).

mtDNA Gene	Fragment Size (bp)	Primer Sequence	Primer Name
Cox1	458	GG(A/G) GG(A/T) GA(C/T) CC(A/T) AAT (C/T)T(A/T) TAT CAAC	COXF
		CCT GT(A/T) A(A/T)A ATA GCA AAT AC	COXRa
Cox1	929	CTT GTA ATC ATA AGG ATA TTG GAAC	10KbCOIF1
		AAT ACC AGT GGG AAC CGC	6,5KbCOIR
Atp6-cox3	818	GAC ATA TAT CAG TAA CAA TGAG	16KbATP6F
		GAC TCC AAG TAA TAG TAA AACC	16KbCOIIIR
Cytb	985	GCA GCT TTA GTG GAT TTA CCT AC	10KbCytbF-1
		CTA CAG GAC ACG ATC CCA AG	10KbCytbPRIM

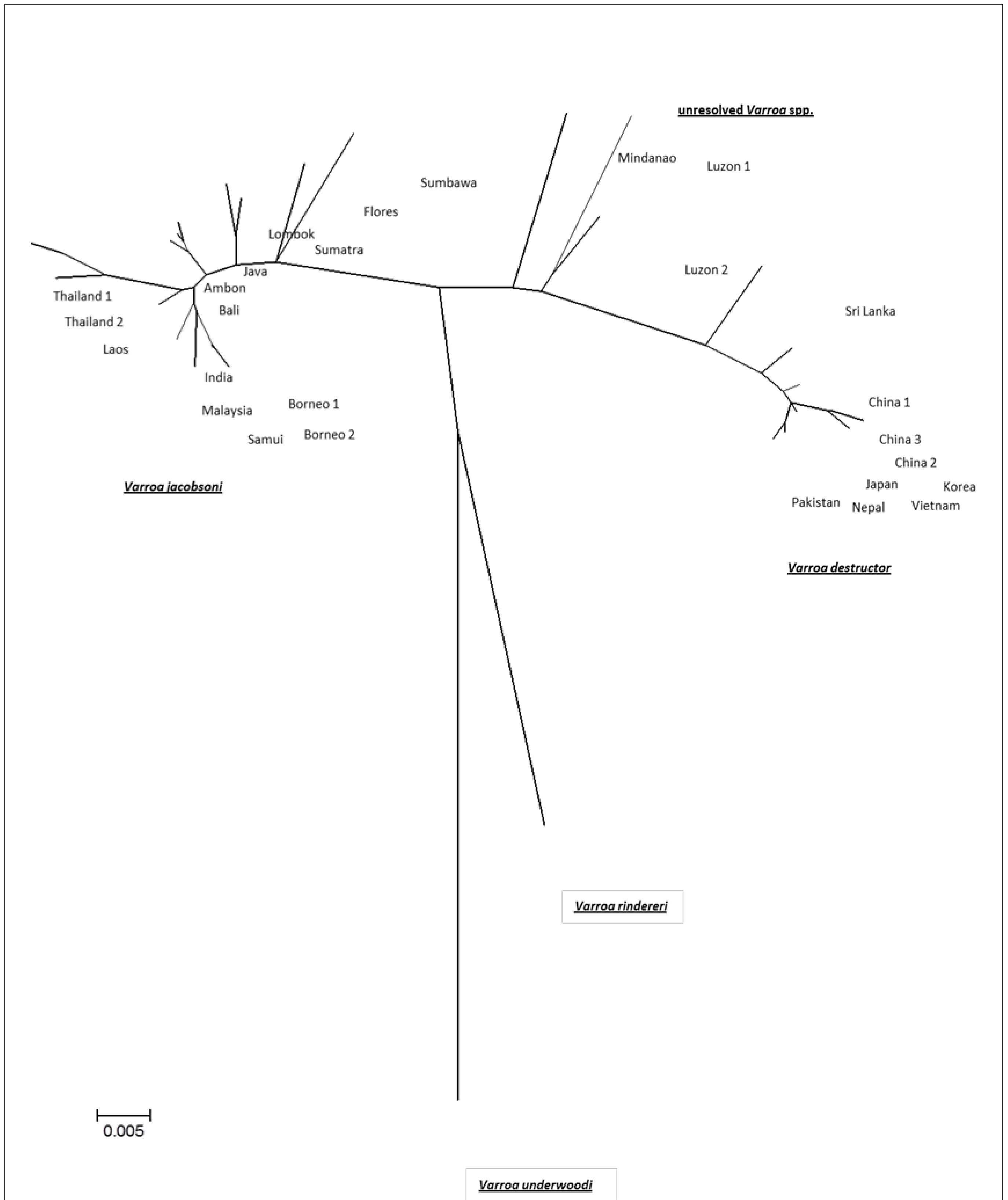

Fig. 5. A phylogenetic tree of all the currently known and published haplogroups.

3. Laboratory techniques

3.1. Collecting mites

There are several ways to collect varroa mites for experiments. Some methods described below provide mites of unknown age, which have reproduced an unknown number of times. Other methods provide mites in which oogenesis has been triggered. Which method is adopted depends on the physiological state of mites needed for the experiment (see section 3.6. 'Bioassays').

3.1.1. Manual collection

Phoretic mites can be picked up by hand from their host with a fine bristle brush or a small mouth aspirator.

1. Collect honey bees from a colony. Manual collection is easier when the colony is highly infested (see section 4.6. 'Breeding mites in colonies' for a method to obtain regular supply of highly infested colonies), but to collect 'healthy' mites it is recommended that the host colony does not have symptoms of extreme infestation such as crippled bees.
2. Catch honey bees one by one and examine them for the presence of mites.
   Mites may run freely over the bee's body or be hidden between two sternites. Finding and collecting them sometimes necessitates grasping the bee by the thorax and sting apparatus with forceps to stretch the abdomen, thus making the mites visible and reachable.
3. Honey bees can be treated with CO_2 or cooled down to facilitate the physical collection. CO_2 affects the bees' physiology (Czekońska, 2009), but recent results indicate that a short treatment with CO_2 does not affect fertility and fecundity of varroa female artificially introduced into brood cells (Rosenkranz *et al.*, unpublished data). The effect of cooling on mites is not known and might affect mite survival. An alternative to CO_2 and cooling treatment is: (i) to let the bees crawl out of their container one by one so they can be caught easily or (ii) to cut off the head of the bees; mites tend to leave dead bees within a short time.
4. Place the mites collected in a mite-tight container with a source of humidity (a wet cotton plug or ball of paper) to prevent the mites desiccating.

Pros: allows for the collection of mites that have not been stressed by a treatment with water or powdered sugar (see sections 3.1.2. 'Icing sugar' and 3.1.3. 'Washing with water'). This is advantage if mites are used in long lasting experiments.

Cons: tedious, few mites can be sampled in a short time.

3.1.2. Icing sugar

Icing sugar can be used to detach mites from their host collected in a jar (Macedo *et al.*, 2002) or still in the colony.

Material needed: a wide mouthed jar with a lid of which the centre part is replaced by a 2mm hardware cloth or mesh (Fig. 6a).

1. Place 300 bees in the jar and close the lid.
2. Pour 1 heaping table spoon (at least 7g) of powdered sugar through the mesh or cloth (Fig. 6a).
3. Roll the jar to cover all the bees with sugar (Fig. 6b).
4. Let stand for 1 min.
5. Turn jar upside down over a white surface (Fig. 6c).
6. Shake for 1 min.
7. Place the fallen mites and sugar (Fig. 6d) in a sieve and rinse with 1X phosphate-buffered saline (or other similar saline solution) to rid them of icing sugar particles (Fig. 6e).
8. Place mites on absorbent paper to help them dry up (Fig. 6f).
9. Place the mites collected in a mite-tight container to prevent them escaping.
   Place a source of humidity in the container to prevent the mites desiccating until they are used for experiments.

This can also be done using the entire colony fitted with a mesh floor:

1. Remove each frame containing adult bees.
2. Sprinkle with icing sugar so that the frames are all covered.
3. Place back into the colony.
4. Remove the excess icing sugar with the mites from the floor at 10-20 min intervals.
5. Pour over a sieve to remove the sugar and collect the mites.
6. Rinse with 1X phosphate-buffered saline (or other similar saline solution) to rid them of icing sugar particles.
7. Place mites on absorbent paper to help them dry up (Fig. 6f).
8. Place the mites collected in a mite-tight container to prevent them escaping.
   Place a source of humidity in the container to prevent the mites desiccating.

Pros: fast and allows for several hundreds of mites to be collected in short time. The treatment is bee-friendly since few individuals die during the process. Workers collected in the jars can be placed back in their colonies where they will be cleaned by their nestmates.

Cons: decreases lifespan of mites (Macedo *et al.*, 2002). This can be a problem if they need to be used for long lasting experiments (> 3 days).

3.1.3. Washing with water

1. Collect bees from a colony in a bee tight container.
2. Fill the container with 1X phosphate-buffered saline (or other similar saline solution) to prevent the bees flying away and shake.

Fig. 6. Collecting mites with icing sugar: ***a.*** a heaped table spoon of powdered sugar is poured on 300 honey bees kept in a jar through the lid equipped with a mesh. Photo by V. Dietemann

Fig. 6. b. rolling the jar on its side ensures that bees are covered with the sugar. Photo by V. Dietemann

3. Pour the content of the container over a first sieve (aperture: 2000 µm) to collect all the bees.
4. Place a second sieve (aperture < 0.5 mm) underneath to collect the mites.
5. Place mites on absorbent paper immediately after washing them off to help them dry up (Fig. 6f).

Fig. 6. c. the jar is turned upside down and shaken to dislodge the mites. Photo by V. Dietemann

Fig. 6. d. mites (2 darker points) and sugar fallen through the mesh on the paper. Photo by V. Dietemann

6. Place the mites collected in a mite-tight container with a humidity source to prevent the mites desiccating.

Pros: fast and allows for several hundreds of mites to be collected in a short time.

Cons: effect on lifespan of mites unknown; this can be a problem if they need to be used for long lasting experiments. The treatment it is not bee-friendly since many can die during the process.

3.1.4. Collecting mites from brood

3.1.4.1. Collecting mites from L5 larvae

Mites at a similar physiological stage can be collected from recently capped brood cells (after Chiesa *et al.*, 1989)

1. Remove a brood comb with L5 larvae ready to be capped in the evening of the day preceding the experiment.
2. Mark the capped cells with a convenient marker (e.g. correcting fluid, queen marker, felt pen).
3. Replace the comb in its colony of origin.
   Bees will continue capping mature cells.
4. The following morning, transfer the comb to the laboratory and unseal the unmarked cells that have been capped overnight.

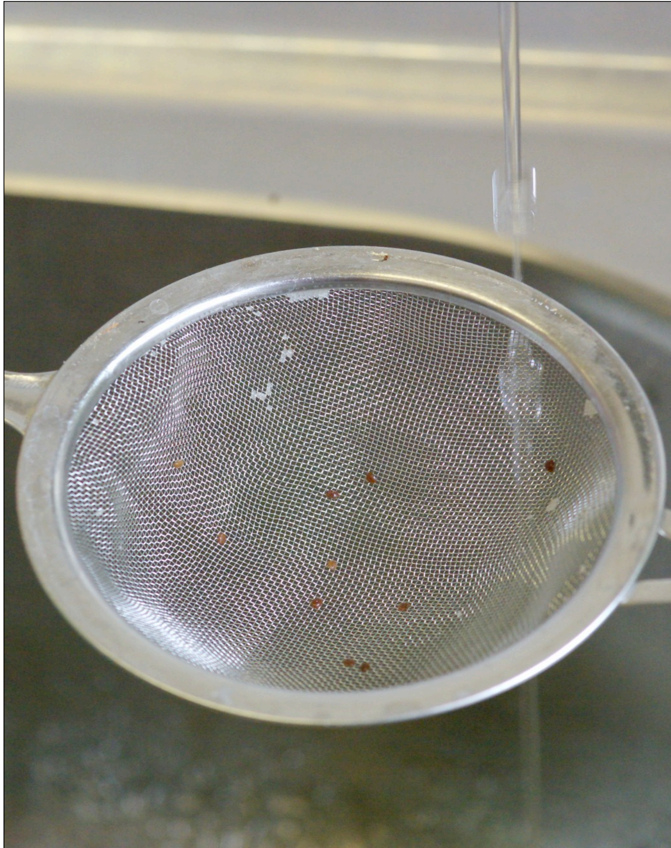

Fig. 6. e. the mites and sugar collected are placed in a sieve over which saline solution is poured to rid the mites from sugar particles. Photo by V. Dietemann

Fig. 6. f. the mites are placed on an absorbing paper to accelerate their drying. Photo by V. Dietemann

5. Place the comb in an incubator at 34.5°C, 60-70% RH.
6. Infested and non-infested larvae deprived of the capping spontaneously emerge from brood cells in a short time.
7. Collect mites that have fallen from their cells with their host.
8. Place the mites collected in a mite-tight container to prevent them escaping.
9. Place a source of humidity in the container to prevent the mites desiccating.

Pros: easy collection, all mites are at the same physiological stage.

Cons: there is no knowledge of the mite's age and of how many reproductive cycles she already performed.

3.1.4.2. Collecting mites from capped cells

3.1.4.2.1. Opening each cell

Brood mites can be picked up by hand from their host with a fine bristle brush or a small mouth aspirator after opening the cells they infest and removing the pupa. To obtain mites at a given time during the reproductive cycle, the collection can be made from brood of known age (see the section 'Obtaining brood and adults of known age' in the *BEEBOOK* paper on miscellaneous methods (Human *et al.*, 2013)). For this the queen is caged on an empty frame at the necessary date (see also section 4.6. 'Breeding mites in colonies').

1. Uncap the cell with fine forceps or scalpel.
2. Push away the cell walls to free the developing larva or pupa.
3. With soft forceps pull the larva or pupa out.
4. Carefully look on the larva or pupa and on the cell walls for mites.
5. Place the mites collected in a mite-tight container.
6. Place a source of humidity in the container to prevent the mites desiccating.

Pros: This is the less damaging collection method for the mite.

Cons: It is the most time consuming collection method.

3.1.4.2.2. Opening large number of cells and washing the brood

A quicker method for collecting large numbers of live mites from capped brood cells it to uncap large quantities of brood and force the mites out by knocking them out of the cells or by washing them off the brood. For this:

1. Uncap a large number of cells.
2. Remove all developing bee brood.
   These two steps can be done at once using an uncapping fork used for honey extraction.
3. Turn the comb upside-down over a sheet of white paper.
4. Tap on its upper surface to dislodge mites from the cells.
5. Collect the dislodged mites, sometimes in the hundreds, from the paper.

An alternative method to increase the number of mites sampled is to:

1. Uncap the brood cells.
2. Flush the comb with lukewarm water that will dislodge the brood and mites.
3. Collect dislodged brood and mites in a first sieve (5 mm mesh) that will retain the bees.

4. Place a second sieve (0.5 mm mesh) underneath the first to retain mites.
5. Flush with more water.
6. Dry the mites by placing them on absorbent paper (Fig. 6f).

Pros: easy collection of high mite numbers (depending on colony infestation rate).

Cons: mites of unknown physiological stage; possible shortening of life expectancy after washing from brood; a large number of mites remain in the comb with the knocking method.

3.2. Rearing mites in the laboratory

It is relatively easy to maintain varroa mites during their phoretic phase (on honey bees or pupae) in the laboratory. Their maintenance in cages kept in incubator is necessary for screening of varroacides, or while marking before transfer to colonies for example. In contrast, few laboratory breeding methods for varroa are available. The conditions reigning in their breeding environment, i.e. in the cells in which honey bees develop, are so particular that it is very difficult to replicate them artificially. Obtaining a full life cycle in the laboratory therefore remains a challenge that few overcame (Donzé and Guérin, 1994; Nazzi and Milani, 1994). This section describes methods to keep or breed varroa in the laboratory. These methods do not yet allow their breeding in large quantities as would be desired for experimentation, but allow observation of mite behaviour and testing of products that may affect the mite's life cycle.

3.2.1. Maintaining mites in the laboratory

3.2.1.1. Maintaining mites on adult honey bees

Mites can be maintained on bees in hoarding cages (see section 'Cages to keep bees *in vitro* in the laboratory' in the *BEEBOOK* paper on maintaining adult bees *in vitro* (Williams *et al.*, 2013)). A temperature of around 33ºC and RH of 60-70% is also adapted for mites. Mortality can be high in case phoretic mites are used. Their age is unknown at the time of collection and the variability in their life expectancy is therefore high. However, mites will commonly survive for 1 week or longer in bee cages established in this way. It is recommended using mite-tight cages or keeping each cage in a dish to avoid any escaped mites from entering another cage of a different treatment group. This procedure can be used in acaricide toxicity *in vitro* assays, in assays where the investigator is attempting to trace the movement of a compound (such as dsRNA) from a bee food (sugar water, pollen patties, etc.), through bees, and into mites, or in other similar assays.

A common method for establishing *in vitro* studies with varroa-infested adult honey bees involves individually selecting worker bees carrying mites from combs/frames in colonies and placing them in cups or cages. This method is relatively time-consuming and can be particularly difficult for researchers with limited bee experience. An alternative method includes the separate collection of bees and mites followed by the infestation of the bees with the collected mites. Mites can be transferred onto the caged bees using a brush or, if mites were collected in a container, they can be introduced in a cage with bees. The mites readily spread across the bees within minutes. A primary benefit of the latter method is that it is feasible for a single, inexperienced experimenter to accomplish quickly. Additionally, this method obviates the need for maintaining colonies with high varroa populations (which are preferred when infested bees are chosen individually from frames, see section 4.6. 'Breeding mites in colonies').

Collecting bees and mites:

1. Collect worker bees from frames and place in cages as described in section 'Cages to keep bees *in vitro* in the laboratory' of the *BEEBOOK* paper on maintaining adult bees *in vitro* (Williams *et al.*, 2013)).
   The number of bees placed in each cage can vary depending on experimental requirements.
2. Collect mites using the methods described in section 3.1. 'Collecting mites'.

In vitro infestation of bees with varroa:

3. Add 5-10 mites to a small (5 cm) Petri dish containing a filter paper circle wetted with 1:1 sugar syrup (by volume) or water.
4. Place the Petri dish with mites on the bottom of the cage containing worker bees.
5. Tap the cage lightly against the laboratory bench or table to cause the bees to drop from the top of the cage to the bottom and contact the mites.
   This artificial infestation procedure relies on the questing behaviour of mites, which readily attach to bees that contact them. The sugar syrup impregnated in the filter paper will keep the bees for an increased duration at the bottom of the cage to give more time for mites to find a host.
6. Remove the Petri dish after all of the mites have attached to bees.
7. Repeat steps 5-6 until the desired number of mites has been added to the cage. Partitioning the mite additions, rather than trying to add them all to the cup at one time, results in a more even distribution of mites on bees. Be aware that some bees will inevitably carry multiple mites and some bees will have no mites.
   More mites should be added to each cage than are needed for the experiment. For example, add 30 mites to a cage when the experiment calls for the live recovery of 25 mites later in the study. This is necessary because some mites will fail to attach to any bee. Regardless, the number of varroa mites per cage or per bee can vary based on the predetermined experimental criteria.

8. Add a sugar syrup feeder and water supply to the cage (see section 'Cages to keep bees *in vitro* in the laboratory' in the *BEEBOOK* paper on maintaining adult bees *in vitro* (Williams *et al.*, 2013)) after the mites have been added.
   Bees must stay hydrated and fed to ensure mite survival.

If keeping mites on brood is possible in Eppendorf tubes (see section 3.2.1.2. 'Maintaining mites on honey bee brood'), keeping mites on adult bees works better in hoarding cages. Ventilation within the Eppendorf tubes is poor and the health of the bees kept in them decreases rapidly.

3.2.1.2. Maintaining mites on honey bee brood

Adult mites can be kept for many days on bee larvae or pupae in the laboratory, under standardized thermo- and hygrometric conditions necessary for the brood (34.5°C and 60-70% RH). Brood must be replaced regularly with younger individuals before they reach the adult stage (Beetsma and Zonneveld, 1992). Small Petri dishes with a few immature honey bees can be used for this purpose. In order to improve mite feeding and survival, a close contact between the mite and the brood item is desirable. Some authors confine mites individually on a larva or pupa in smaller containers (1ml Eppendorf tube). To increase humidity in the Eppendorf tube, a wet piece of cotton wool is pushed into the bottom of the tube. Excess water accumulating on top of the cotton wool needs to be removed by shaking the tube to prevent mites from adhering to droplets. Piercing the lid with 1-2 holes (diameter < 1 mm) will improve ventilation and respiration. Up to 8 mites can be kept in one tube. Mites can be transferred into the Eppendorf tube using a brush. Once all mites are transferred, the pupa/larva can then be placed in the tube.

Pros: easy for keeping mites alive.
Cons: does not result in oviposition by the mites.

3.2.1.3. Artificial diet

To facilitate the keeping and rearing of the mite in the laboratory, attempts were made to develop rearing methods based on artificial diets that mites could suck through a synthetic membrane (Bruce *et al.*, 1988; Bruce *et al.*, 1991). Unfortunately, despite both membrane and diet seeming suitable for the purpose, satisfactory survival and reproduction were not achieved.

3.2.2. Breeding mites in the laboratory

In order to obtain the whole life cycle of varroa within the cells under laboratory conditions for observations and experimentation, rearing methods within artificial cells made of different materials were designed. Tested materials for cells include wax, glass, plastic, gelatin (Nazzi and Milani, 1994 and citations therein). In general, reproduction is very difficult to obtain, due to the seemingly high number of cues necessary for the mite to reproduce successfully. However, in some cases reproduction rates close to the natural ones were obtained. In particular, Donzé and Guérin (1994, 1997) obtained complete reproductive cycles in artificial cells. The cells used where first kept in the hive until sealing, then brought to the laboratory and placed in an incubator. Despite the low acceptance and infestation rate of these cells, it allows using cells of natural size and naturally infested. Nazzi and Milani (1994) developed a method that allow normal mite reproduction under laboratory conditions in cells in which larvae were introduced and which were infested artificially to allow more control on the process.

3.2.2.1. Natural infestation

Material needed: cylindrical transparent polystyrol cells (internal dimensions: 5.1 mm diameter x 14 mm length for workers and 6.7 mm diameter x 16 mm long for drones)

1. Incorporate the cells at an inclination of 5-10 degrees in groups of 60-70 in wax combs.
2. Coat with honey to increase acceptance by the workers and stimulate cleaning behaviour.
3. In a heavily varroa infested colony, confine the queen on the artificial cells for 12 h.
4. Release the queen after this period.
5. Record the time of cell capping at 1-2 h interval some 8.5 days after oviposition by the queen.
6. Remove the cells from the colony and the comb after they have been capped.
7. Place in an incubator at 34.5°C and 60-70% RH.

Pros: transparent cells allowing observation of behaviour, natural infestation, natural cell size.
Cons: tedious, low acceptance and infestation rates.

3.2.2.2. Artificial infestation

1. Collect mites and L5 bee larvae from natural brood combs as described in section 3.1.4. 'Collecting mites from brood'.
2. Place the larva in a gelatin cell by holding its dorsum between thumb and first finger to get it stretched.
3. Insert the mite with a fine paint brush.
   Gelatin cells of different diameters were tested; as a general rule, the narrower the cell diameter the higher the reproduction, but the higher the chance of injuring the larva while inserting into the cell; the best compromise is achieved with gelatin cells of 6.5 mm diameter.
4. Place in an incubator at 34.5°C, 75% RH.
5. Place the cells so that pupae are laying on their back.
   Geotaxis is an important cue for varroa behaviour (Donzé and Guerin, 1994).
6. Fix the cell to a substrate to avoid rolling and manipulate only occasionally for observations.

Pros: high percentage of fertile mites and offspring number close to natural infestations in colonies can be obtained; transparent cells allow observation of behaviour; *in vitro* procedure allows complete control over the infestation state of the bee since workers do not have the opportunity to remove infested brood.

Cons: tedious; non-natural infestation; non-natural cell size.

Perspectives: With the aim of developing a complete rearing method for the mite, Nazzi and Milani (unpublished data) and Dietemann, Zheng and Su (unpublished data) carried out preliminary trials aiming at obtaining several reproduction cycles in the laboratory, i.e. artificially breeding mites that were born under laboratory conditions. Attempts were discouraging and continuing efforts are needed.

3.3. Assessing reproduction in the laboratory

3.3.1. Assessing fertility

Assessing fertility of mites reproducing in artificial (see section 3.2. 'Rearing mites in the laboratory') or natural cells follows the same principle than described for field methods (see section 4.3.1. 'Assessing reproductive success'). A difference is that if transparent cells are used (Donzé and Guérin, 1994; Nazzi and Milani, 1994), one does not need to open the cell to count offspring. Offspring production and survival can thus be monitored over time.

3.3.2. Assessing oogenesis

Assessing oogenesis of laboratory reared mites requires dissection of female mites and tissue dyeing (see the section on marking techniques 3.4.1. 'Oogenesis').

3.4. Marking techniques

3.4.1. Oogenesis

Activation of the oocyte (i.e. oogenesis) is followed by the incorporation of euplasmatic material and/or yolk proteins. Marking a whole mount of the mites' ovaries with toluidine blue is a rapid method to confirm such incorporation and therefore initiation of oogenesis (Garrido *et al.*, 2000).

1. Remove ventral shield of the mites with thin dissecting forceps under a binocular microscope.
2. Excise ovary together with spermatheca and lyrate organ.
3. Place in PBS buffer (phosphate buffered saline, pH 7.2–7.4).
4. Fix in formalin (4%) for 30 min.
5. Wash three times with PBS buffer.
6. Incubate in toluidine blue (0.005%) for 30 min.
   The duration of incubation might need optimization, which can be tested on the coloration of activated oocytes approximately 12 h after cell sealing.
7. Rinse with PBS buffer for 15 min.
8. Repeat rinsing twice more.
9. Verify the colouring of the oocytes under a microscope at 400x magnification.

Pros: easier and faster than the alternative histological method. Detects initiation of oogenesis with high resolution.

Cons: somewhat tedious; subjective grading of oocyte colour.

3.4.2. Feeding site

Varroa mothers pierce the cuticle of honey bee larvae and pupae on which they (Fig. 7a) and their offspring feed (Fig. 7b). In late pupae, the wound can be seen under the binocular thanks to the scarring process of the cuticle (Fig. 7c). It can also be located by observation of the feeding mites, events that are relatively rare and need an artificial in vitro system to be observed (see section 3.2.2. 'Breeding mites in the laboratory'). In most cases, no wound can be seen on larvae or pupae and a staining method is necessary to find it (Fig. 7d). The ability to locate the wound might be necessary for behavioural studies of feeding behaviour or reproduction or for secondary disease transmission studies (Kanbar and Engels, 2003). By extension, this method can also be used for all cases in which a perforation of the cuticle of immature honey bees has to be made visible (e.g. injection of pathogens or hormones).

Kanbar and Engels (2004) designed a vital staining method that allows the visualisation of feeding sites. They used Trypan blue, a dye that enters damaged cells (Roche, 1999), i.e. cells around the hole pierced by mother mites in the late 5th instar larvae, prepupae or pupae cuticule. Feeding sites could thus be stained durably on live individuals and observed over time (Herrmann *et al.*, 2005). Staining can be detected until the stage when the cuticule darkens to the point of hiding the dyed blue cells. At this point the dyeing is not any longer necessary.

Staining method:

1. Sample larva, pre-pupae or pupae to be stained from varroa infested cells (see section 3.1. 'Collecting mites').
2. Immerse them for 30 min in a volume of Ringer-based staining medium sufficient to cover the major part of the body surface.
   The larvae and pupae survive this treatment (Kanbar and Engels, 2003).
3. Rinse in pure Ringer solution for 3 min.
   Ringer solution: see Table 1 of the *BEEBOOK* paper on cell cultures (Genersch *et al.*, 2013) for a recipe.
   Vital staining medium: 100ml Ringer solution, 0.01 g Trypan blue adjusted to pH 6.8 with KOH (0.1M).

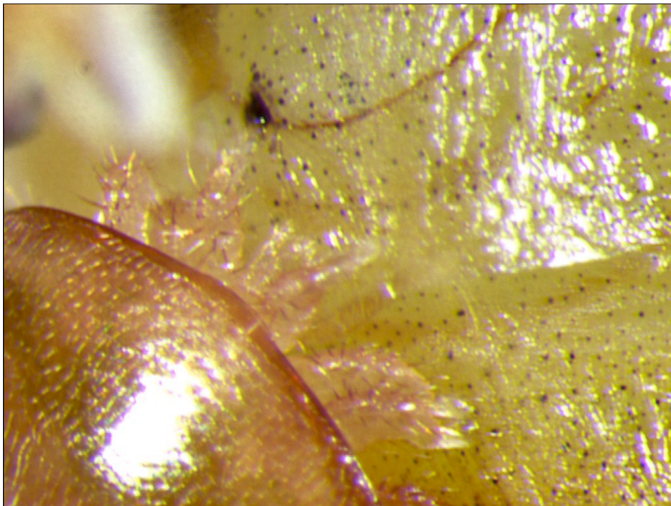

Fig. 7. a. Adult varroa mite sucking haemolymph of a pupa at the feeding site (black dot on top, left from centre).

Fig. 7. b. varroa nymph sucking haemolymph of a pupa atthe feeding site (between the nymph's legs).

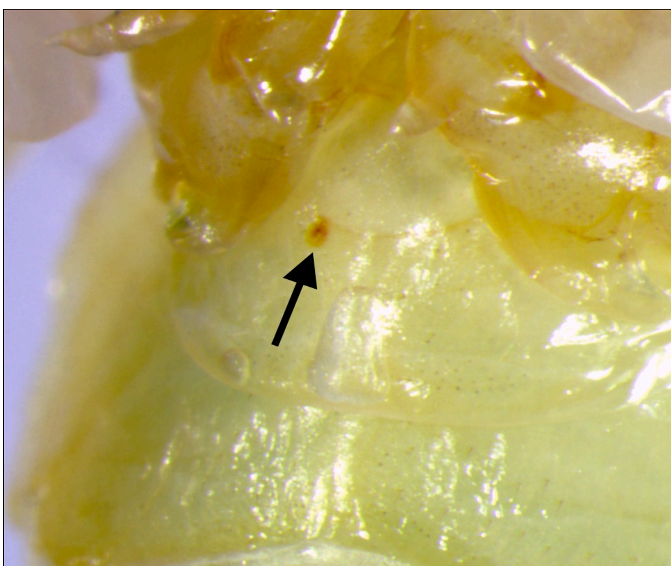

Fig. 7. c. feeding site with melanisation (arrow). It is visible without staining. Such instances are more frequent in older pupae.

Fig. 7. d. a feeding site (blue dot) on a white pupa after staining with Trypan blue. Photos: Swiss Bee Research Institute.

3.4.3. Marking mites

Varroa mites can be marked with paint markers, enamel paint for models, correction fluid polyester glitter or fluorescent pigment (Schultz, 1984; Harris, 2001; Kirrane *et al.*, 2012). Methods with paint are faster to use and have been shown safer for the mites. Toxicity is mainly due to the solvents incorporated in the products.

For paint application, soft tools are preferable to toothpick or other hard tools to avoid injuring the mites. A droplet of paint can be placed on a microscope slide and little quantities collected with a very thin paintbrush or a fishing line (Kirrane *et al.*, 2012) for application on the mite. The hair of a paintbrush can be cut off leaving enough hair to obtain the desired size. The right size of the application tool is obtained when the paint dot is visible, but does not impair the behaviour of the mite.

If the mites are used for behavioural observations during the phoretic or reproductive stage, care should be taken to produce a flat paint mark that enables the mites to push herself between the bees' sternites and feed, or to allow the mite's free movement within the restricted space between pupae and cell walls.

Before use in large scale experiment, a toxicity test should be performed to ensure that the paint chosen is not toxic to the mite: marked and sham-treated mites should be kept in similar conditions and their longevity compared. Marked mites should live as long as unmarked mites. Refer to section 3.2. 'Rearing mites in the laboratory' for rearing methods. To minimise the risk of the paint dot coming off the cuticle and thus prevent recognition of the marked mite at the end of the experiment, preliminary tests with different brands should be made to select a long lasting paint.

3.5. Infecting varroa with secondary diseases

3.5.1. Microinjection

Microinjection techniques specific to varroa have not yet been developed. For basic microinjection techniques please refer to the *BEEBOOK* paper on miscellaneous methods (Human *et al.*, 2013). Campbell *et al.* (2010) mention various problems when testing such methods. The manipulation is laborious, requires specialised equipment and resulted in the death of most mites injected.

3.5.2. Dipping

Dipping mites in a solution containing dsRNA resulted in gene knockdown (Campbell *et al.*, 2010), demonstrating the potential of this procedure as a way to infect or contaminate mites with micro-organisms or genetic material. This study showed that when proper osmolality of the dipping solution is established, a high number of varroa mites (80%) can survive long immersion periods (14 h at 4°C) that might be necessary for infection/contamination. To achieve the right osmolality, 0.9% NaCl was used. Dipping methods have rarely been used for varroa, and optimisation work should be done to improve the survival of the mite after immersion.

3.6. Bioassays

The influence of both physical and chemical factors on *V. destructor* has been studied by means of different bioassays. So far, bioassays have been essential in two fields of varroa research: the study of the semiochemicals involved in the interactions between the mite and honey bee and the study of mite resistance towards acaricides.

3.6.1. Experimental conditions

The literature on the subject reveals some critical aspects that must be considered when conducting bioassays with varroa, including the environment, the chemicals tested and the origin of mites.

3.6.1.1. Environment

The mite spends its life in the hive, under strictly controlled environmental conditions; in order to get a realistic representation of the reactions of the mite towards a given chemical, this should be tested under the same conditions, that is a temperature around 32-35°C and a relative humidity around 70%.

3.6.1.2. Dosage of chemicals

In order to avoid any misjudgement about their real effect, semiochemicals should be tested at doses that are close to their biological range. For example, most chemicals, when tested at too high a dose, become repellent. Unfortunately, this aspect has often been overlooked in the study of varroa chemical ecology (for some examples see Milani, 2002). Dose-response studies, where the biological activity of a given compound is tested at different doses in a logarithmic scale, are mandatory in current research.

3.6.1.3. Mites to be used in the tests

In chemical ecology studies, the mites that are used in the bioassays should be those involved in the process under study (e.g. if the study is about cell invasion, the mites that invade the brood cells are used). In efficacy study of acaricides, the life stage of mites tested must correspond to that that will be exposed to the product under scrutiny.

In contrast to many other arthropods whose ecology is studied by means of bioassays, no artificial rearing method is currently available for the varroa mite (see section 3.2. 'Rearing mites in the laboratory'); thus standardisation is a difficult task when it comes to collecting mites that are homogenous for age, physiological condition, mating status etc. A solution to this problem is to use mites that are at the same stage of their life cycle (see section 3.1. 'Collecting mites').

3.6.2. Bioassays in varroa chemical ecology

Bioassays are a fundamental resource in the study of behaviour modifying chemicals, all the way from the demonstration of their existence, through all steps of isolation, until the final confirmation of their identity. Some of the bioassays used so far in varroa chemical ecology were simple adaptations of those already used for the study of arthropod semiochemicals (Baker and Cardé, 1984). In particular, the response of the varroa mite towards different odour sources and pure compounds was tested using several general purpose setups, including four-arms olfactometers (Le Conte *et al.*, 1989), servospheres (Rickli *et al.*, 1992), Y-mazes (Kraus, 1993), wind-tunnels (Kuenen and Calderone, 2002) and observation arenas (Rickli *et al.*, 1994), in other cases, bioassays were specifically designed for the varroa mite. In this section, we will concentrate on the latter. The chemical stimuli that influence the behaviour of the mite during the following stages of the mite's biological cycle have been studied by means of bioassays: cell invasion, mating, oviposition, phoretic phase.

3.6.2.1. Cell invasion

In this case, the attention is on the cues influencing the entrance of the mite into the brood cell containing an L5 bee larvae (see the section 'Obtaining brood and adults of known age' in the *BEEBOOK* paper on miscellaneous methods (Human *et al.*, 2013) for a description of larval stages), in the 20-60 hours preceding capping (Rosenkranz *et al.*, 2010). After the first studies with the star olfactometer (see Le Conte *et al.*, 1989), bioassays better adapted to the varroa mite were used; here a bioassay that appeared to be suitable to test both attractants linked to worker cell invasion (Rosenkranz, 1993; Nazzi *et al.*, 2001; Nazzi *et al.*, 2004; Aumeier *et al.*, 2002) and repellents involved in the avoidance of queen cells (Nazzi *et al.*, 2009) is presented.

3.6.2.1.1. Mites to be used

What determines the end of the phoretic phase of mites is not known. Using phoretic mites for this assay is therefore not ideal since the randomly sampled mites might not be 'motivated' to enter cells and

respond to the stimulus provided. It is therefore possible to use mites which have recently expressed this behaviour and will repeat it again given the right circumstances. However, several experiments confirm that during the season most of the phoretic mites are willing to reproduce (e.g. Rosenkranz and Bartalszky, 1996; Martin and Cook, 1996; Garrido and Rosenkranz, 2003; Frey *et al.*, in preparation) and might also be used for these kinds of experiments (see section 3.1. 'Collecting mites' for a description of how to obtain them).

3.6.2.1.2. Experimental setup

This bioassay was described by Nazzi *et al.* (2001) and represents a modification of the device used by Rosenkranz (1993).

1. Use an arena consisting of a glass plate with four wells (7 mm diameter; 8 mm deep) equidistant (1 cm) from the centre.
2. Mount a glass lid on a circular metal ring (5.6 cm diameter) to confine the mites in the arena.
3. Apply the treatment to two opposite wells while the other two wells are used as controls.
4. Place one bee larva or dummy into each well.
5. Place one adult female mite in the centre of the arena between the four wells with a fine paint brush (Fig. 8).
6. Keep the arenas in a chamber at 34.5°C and 60-70% RH for the duration of the bioassay.
7. Note the position of the mites every 5 min for 30 min.

In order to obtain sufficient sample size, twenty arenas are used at a time and tests are replicated on different days for several times (typically a minimum of three) using a total of at least 60 mites.

3.6.2.1.3. Data analysis

A sampled randomization test is used because the distribution of the variables to be compared is unknown. Such test is preferred over a conventional parametric statistics since they often lead to an overestimate of the significance of differences.

For each arena, the number of times the mite is observed in the treated and control wells, respectively, over the 30 min period are used as scores for the statistical analysis. This is done regardless of whether the mite had changed wells between observations or just stayed in the same well. Then, a matrix is constructed with as many rows as the number of mites used in the bioassay, and two columns containing the scores for treated and control wells for each of the tested mites. The treated and control scores in a given set of data are compared by a sampled randomization test (Manly, 1997; Sokal and Rohlf, 1995). The randomization distribution should be resampled a sufficient number of times (e.g. 10^6 times).

3.6.2.2. Oogenesis

About 70 h after cell sealing, the mite begin egg-laying, which continues at 30 h intervals until 5-7 offspring are produced (Rosenkranz *et al.*, 2010). The cues influencing oviposition were studied using artificial brood cells made of different materials (e.g. wax, glass, gelatine) (Milani and Chiesa, 1990; Trouiller and Milani, 1999).

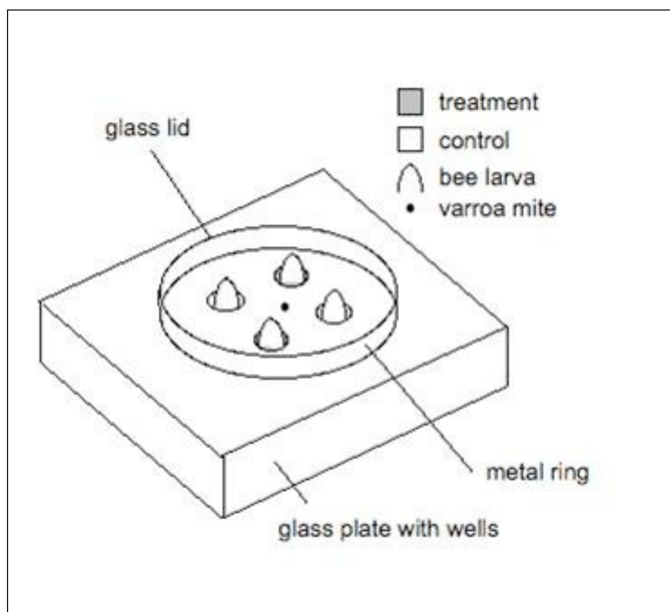

Fig. 8. Arena used for the bioassays on cell invasion behaviour.

3.6.2.2.1. Mites used in the bioassay

To study the factors that affect oogenesis, mites that have not yet been stimulated to start oogenesis must be used. Indeed, if a mite has been in contact with a L5 larva for 0-6 h, oogenesis is initiated (Garrido *et al.*, 2000). Phoretic mites must therefore be used. However, not all phoretic mites will initiate reproduction and oogenesis since they are of various age and physiological status. To study factors triggering oviposition once mite oogenesis has been activated and completed, mites must be collected at least 24 h after capping, but no later than 132 h (after which they stop reproducing, Nazzi unpublished) and introduced in cells containing a pupa young enough to allow the normal reproductive cycle. See section 3.1.4.2.1. 'Opening each cell' for methods to obtain mites from their cells. For these studies, the different phases of the mite´s ontogenetic development and the time-dependent course of the gonocycle must be considered (Steiner *et al.*, 1994).

3.6.2.2.2. Experimental setup to test the activation of oogenesis

Bioassay to assess the effect of volatile or non-volatile compounds for their effect on oogenesis activation.

3.6.2.2.2.1. In the field

1. Treat brood cells in colonies with the stimulus under testing or treat mites. Beware of solvent toxicity for bees and mites.
2. Open cells a minimum of 70 h after mite introduction.

3. Investigate the presence of eggs in the cells.
   Opening the cells later (e.g. between 7 and 10 days) will facilitate detection of eggs and offspring, but increase the chances of removal by hygienic workers.

Pros: less time consuming compared to laboratory bioassay (see section 3.6.2.2.2.2.). Many brood cells can be treated under natural conditions.

Cons: stimuli cannot be tested independently from other factors (larvae, nurse bee activity).

3.6.2.2.2.2. In the laboratory

This protocol follows Garrido and Rosenkranz (2004) to test the effect of volatiles on activation of oogenesis:

1. Offer volatile compounds on a piece of filter paper placed in a 0.2 ml PCR tube.
2. Add a female mite in the tube.
3. Prevent the mite reaching the filter paper with a plastic gauze.
4. Remove the mites eight hours after the exposure to the putative triggering factor.
5. Dissect the reproductive tract of mites.
6. Dye the reproductive tract to determine the development stage of the terminal oocytes (see marking techniques section 3.4.1. 'Oogenesis').

Pros: test of single stimulus possible.

Cons: time consuming. Due to the lack of nutrition of the mite the test can only be performed for about 8 h.

3.6.2.2.3. Experimental setup to test oviposition

1. Treat the cell or the mite with the compound under testing. Beware of solvent toxicity for bees and mites.
2. Transfer the mites into cells containing a host at an early enough developmental stage, which allows reproduction by the mite to proceed normally.
3. Monitor reproduction after a given interval of time.
   When fecundity measurement is needed, the cell should be opened one day before emergence. This period can be shorter if only fertility (i.e. answering the reproduction yes or no) is of interest, but it must be passed 70 h after capping (Table 2).

3.6.2.3. Orientation inside the sealed cell

Careful observations of the behaviour of the mite inside the brood cell carried out by Donzé and Guérin (1994) revealed a well-structured spatial and time allocation of its activity. The chemicals involved in this crucial stage of the mite's life cycle were studied mostly using bioassays based on observation arenas (Donzé *et al.*, 1998; Calderone and Lin, 2001).

3.6.2.3.1. Mites to be used

Mites should be sampled from naturally infested cells at the stage at which the behaviour under scrutiny is expressed (see section 3.1. 'Collecting mites').

3.6.2.3.2. Experimental setup

Donzé *et al.* (1998) used a modification of the bioassay described by Rickli *et al.* (1994) to study the chemicals inducing arrestment of the mite.

1. Use a glass plate cleaned with acetone and pentane.
2. Draw three concentric circles with 12 and 24 and 36 mm diameter on the underside of the glass (Fig. 9).
3. Apply the compound tested on top of the glass plate on the ring delimited by the 12 and 24 mm circles.
4. Place a mite in the centre of the circles (mites not reaching the treated area within 300 s are not considered).
5. Observe the mite's walking activity.
6. Note the time spent in the treated area.
   This time is used as a measure of the arrestment activity of the stimulus under testing.
7. Stop the assay when the mite crosses the outer 36 mm circle, or after 300 s.

3.6.2.3.3. Data analysis

In order to check for significant differences between treatments and controls, the times spent inside the area treated with different stimuli are compared using the non-parametric tests of Mann–Whitney and Friedman (for simple and repeated experiments, respectively). Due to some unbalanced replications, a generalization of the Friedman test is used (Del Fabbro and Nazzi, 2008).

3.6.2.4. Phoretic phase

After emerging from the brood cell the varroa mite enters the phoretic phase, that is spent on adult bees and lasts until a new brood cell is invaded for reproducing. Bioassays have been used by several authors to work on the chemical cues affecting the mite on the adult bees.

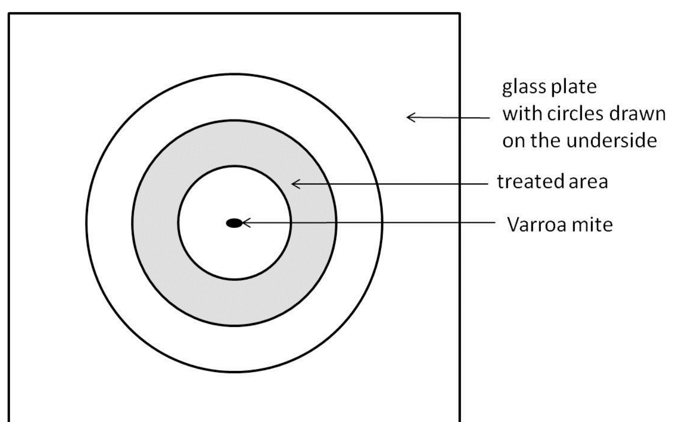

Fig. 9. Test arena for arrestment bioassays.

Table 2. Minimal time after capping for which various mite reproduction parameters can be accurately measured. Adapted from Martin, 1994 and 1995a. *see Fig. 16 for a physogastric mite. In comparison, the segments of a non-physogastric mite appear connected and not separated by white borders (intersegmental membrane).

Mite reproduction classification	**Time from cell capping (hours)**	**Comments**
abnormal	> 60	after first egg should have been laid
abnormal with only single male	> 140	after second egg should have hatched
non-reproducing	30-50 > 70	if mother mite non-physogastric* absence of eggs
mite dead trapped in cell wall	> 30	after cocoon spinning by larva is complete
mite dead in cell	> 0	at any time

Kraus (1994) used a simple two-choice bioassay to test several chemicals for their effect on the mite as a screening procedure to identify possible substances to be used in biological control methods. He and others used laboratory bioassays to investigate the stimuli affecting the host choice by the mite (Hoppe and Ritter, 1988; Kraus, 1990, 1994; Del Piccolo *et al*., 2010). These bioassays are all based on the same kind of setup. Here the bioassay described by Del Piccolo *et al*. (2010), that was used to study the preference of the varroa mite for pollen and nurse bees, is presented.

3.6.2.4.1. Mites to be used

Mites are sampled with the host that carries them. Mites are separated from their host bee by means of a mouth aspirator or a paintbrush. Collection of mites with sugar powder method is not recommended given the possible effects of the sugar on mite vitality (see section 3.1. 'Collecting mites').

3.6.2.4.2. Experimental setup

1. Clean a small glass Petri dish (60 mm diameter) with acetone and hexane or pentane.
2. Place 2 dead adult bees at 2 diametrically opposite sides of the Petri dish, close to the walls (Fig. 10).
3. Treat one bee with the substance tested, treat the other (control) bee with the solvent used to transfer the tested substance on the first bee.
   Use a volume of solvent as small as possible to avoid perturbing the layer of cuticular hydrocarbons. In case of a removal / restoration bioassay the bees' cuticle need be washed with a solvent to remove the hydrocarbons before the tested profile is applied.
4. Place the Petri dishes in a thermostatic cabinet, in darkness, at 34.5°C and 60-70% RH.
5. Place one adult female mite in the centre of the Petri dish.
6. Note mite position every 10 min for 60 min.
   Three positions are considered: mite on the treated bee, mite on the control bee, mite not on bees.
7. Test 10 mites in different Petri dishes simultaneously and replicate 6 times.
   Alternate side of treated and control bees for each replicate to control for the influence of external factors on mite locomotion.

3.6.2.4.3. Data analysis

For each Petri dish, a score is calculated summing the number of mites that were found on the bees during the six observations. This figure can vary between 0 and 6 and is representative of the time the varroa mite spends on the bees. The score can thus be considered as a measure of the preference of the mite for the stimulus under testing. Data from all the replicates are organized in a matrix with as many rows as the number of mites used in the bioassay, and 2 columns containing the scores of the 2 stimuli to be compared. As the variables under study have an unknown distribution, the scores of different stimuli in a data set are compared by a sample randomization test (Sokal and Rohlf, 1995; Manly, 1997). The randomization distribution should be re-sampled a sufficient number of time (e.g. 10^6 times).

Active chemicals identified by means of laboratory bioassays can be tested in the field. For methodologies see section 4.5.4. 'Testing varroacides in the field'. This guideline describes the testing of acaricidal effects; however, it can also be used when using substances that do not kill mites, but disturb their orientation and reproduction.

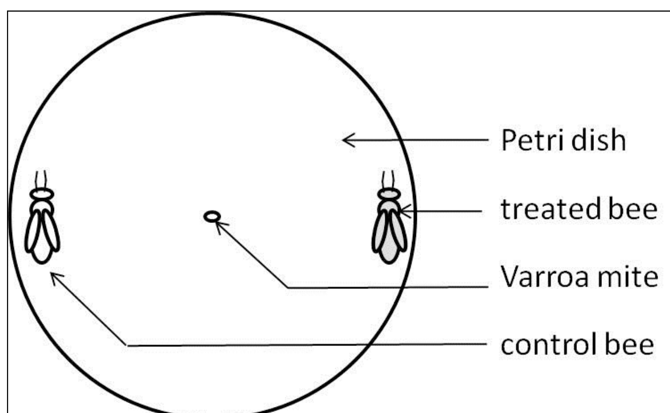

Fig. 10. Test arena for phoretic mite attraction cues.

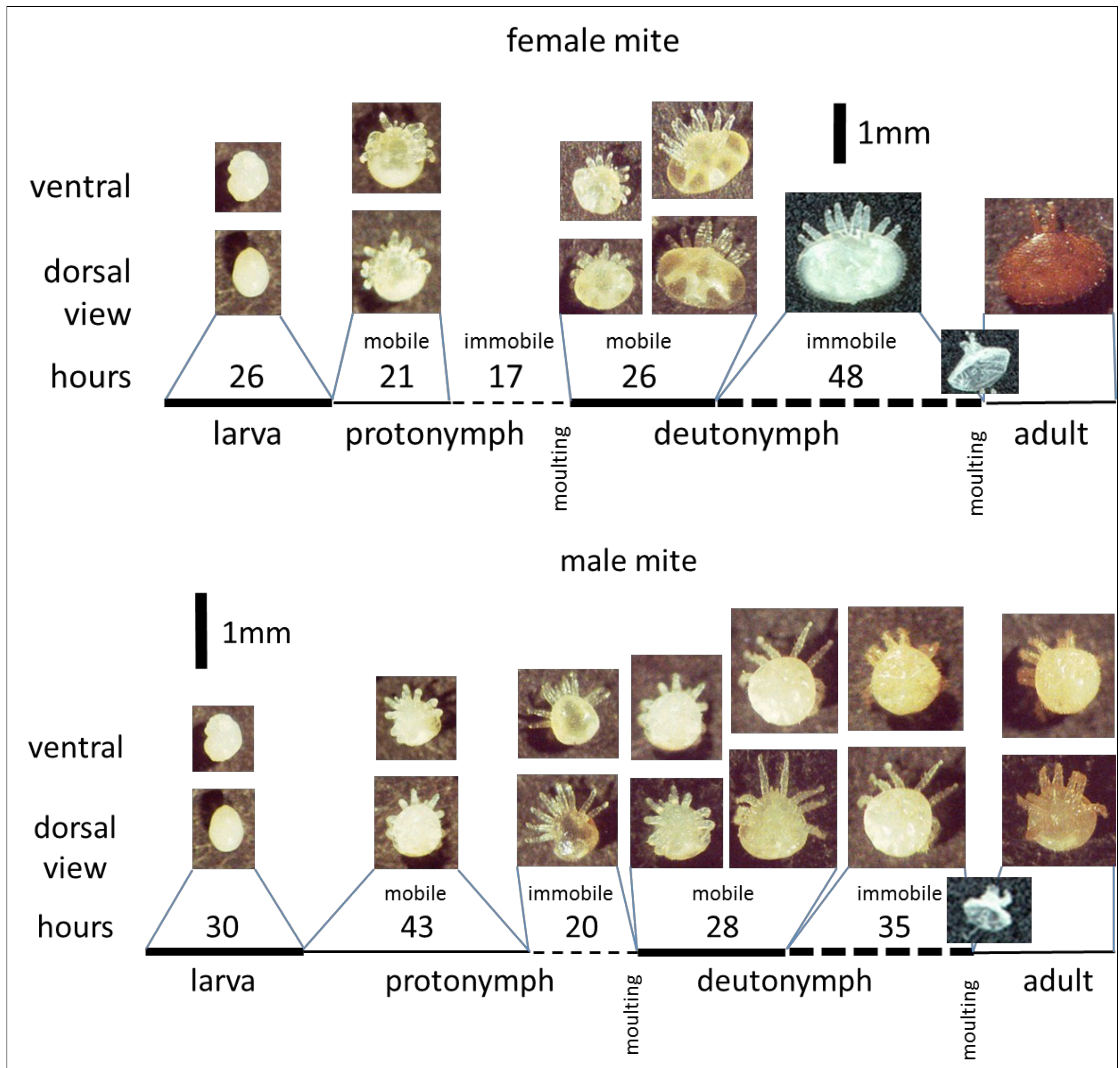

Fig. 11. Ventral and dorsal views of developmental stages of *Varroa destructor* females (above) and males (below) *on A. mellifera* brood. Approximate developmental time is given above the horizontal lines of different thicknesses which delimit the stages. Solid lines denote mobile phases, dashed lines immobile phases prior moulting (after Donzé *et al.*, 1994). Immobile and mobile phases can only be distinguished in live material, not in frozen samples. Photos: R Nannelli and S J Martin

The effect expected is not mite death, but a reduction in mite population size in the colony which can also be detected with this method.

3.6.2.5. Mating bioassays

This bioassay allows the observation and analysis of the mating behaviour of mites under laboratory conditions and is also suitable for testing substances which might stimulate or disturb the mating behaviour.

3.6.2.5.1. Mites used in the bioassay

Adult males and all other relevant mite stages for the mating bioassay can be found in worker brood cells 8-9 days after cell capping. See section 3.1.4.2.1. 'Opening each cell' for the description of how to collect mites from cells. Females shortly after the adult moult should be used for the general observation of the mating behaviour and disturbance experiments. Deutonymphs (Fig. 11) are not attractive for males and can be used as "dummies" when stimulating cues are tested.

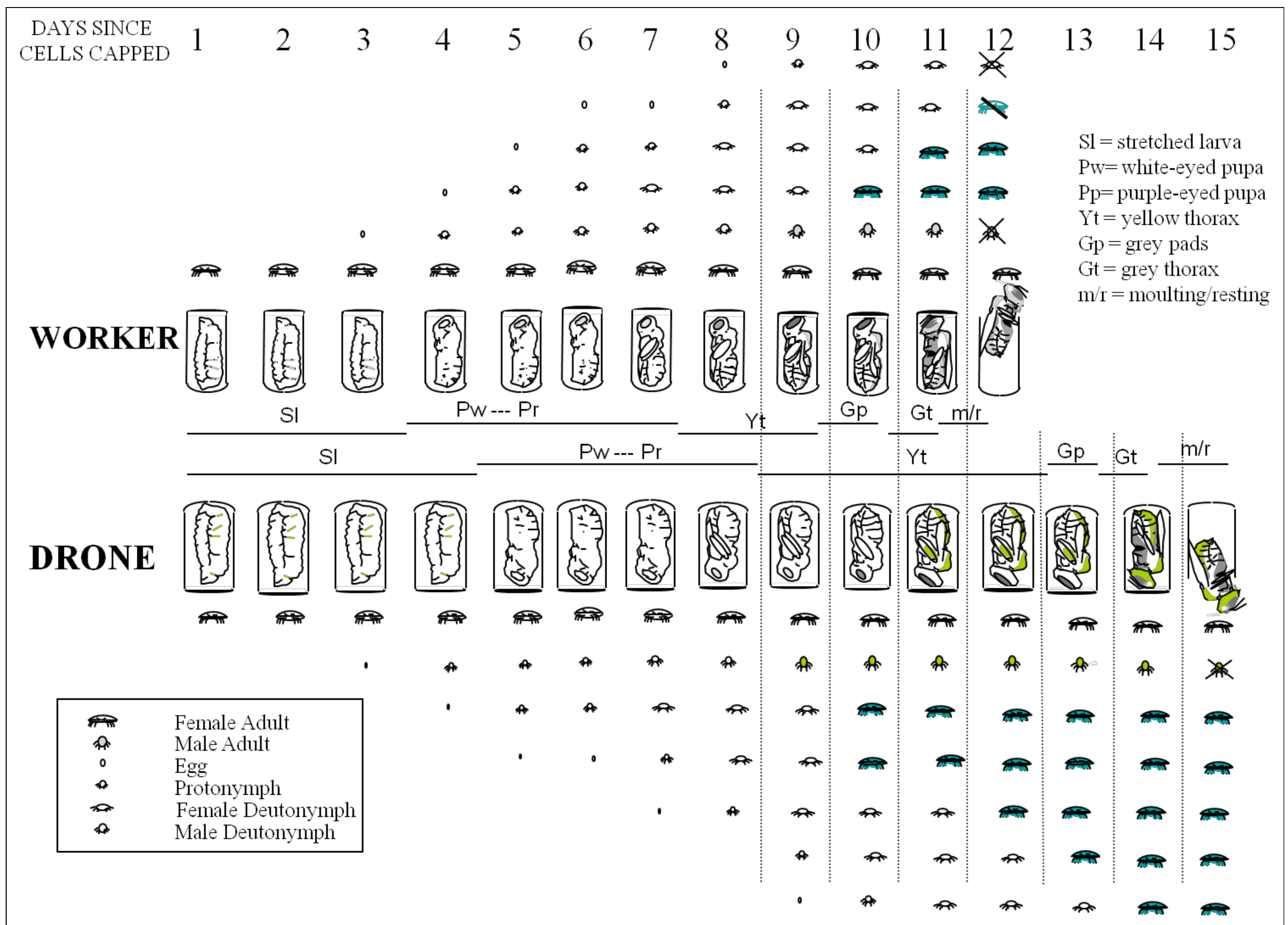

Fig. 12. Development chart of varroa mites and their honey bee host, *A. mellifera*.

The mites should be separated according to sex (see section 4.3.3. 'How to measure reproductive success' and Figs 11 and 12) and kept in groups of maximum 5 individuals at 28-30°C in order to avoid unwanted copulations and a decrease of fitness.

3.6.2.5.2. Experimental setup

This bioassay is described by Ziegelmann *et al.* (2012).

1. Queen cell cups (e.g. Nicot system®) can be used as test arena.
   It is recommended to embed the cell cups in a glass Petri dish with wax.
2. Ensure a temperature of 28-30°C in the cell cup by placing the setup on a hotplate.
3. Transfer the relevant mite stages into the cell cup.
   When extracts or single substances are tested, follow steps 4 and 5, if only behaviour is observed, go to 6.
4. Apply volatile test substances to a piece of filter paper (size: 1.5 mm x 15 mm).
5. Place volatile substances in the vicinity of the female; apply non-volatile substances directly to the female mite.
   For the application of test substance, chose a solvent which does not harm or repel the female.
6. Cover with a glass plate to prevent mites from escaping.
7. Record the male responses with e.g. the Observer software (Noldus Information Technology) for 5 or 10 minutes.
8. Categorise male responses as follows: 1. movement around female; 2. mounting the female's dorsum; and 3. copulation attempt on the female's venter.

3.6.3. Bioassays to quantify the susceptibility of the varroa mite to acaricides

Acaricide resistance represents a dramatic problem for apiculture and has been related to widespread losses of bee colonies. To reduce the impact of such losses, a prompt detection of resistant varroa population is vital and reliable methods for testing the susceptibility of the varroa mite to different acaricides are a fundamental resource, notwithstanding the possible use in basic research on the mode of action of pesticides (Milani, 1999). There is also a need to discover new varroacidal substances. For both purposes a simple and fast bioassay is necessary. A convenient bioassay was devised by Milani (1995) for the study of acaricides that are active by contact (i.e. the active ingredient contaminates the cuticle of the bees and is taken up by the mite by indirect contact). This is the case of most acaricides used currently (e.g. pyrethroids and some organic acids). The bioassay

described has been used to test the activity of several acaricides including tau-fluvalinate, flumethrin (Milani, 1995), perizin and Cekafix (Milani and Della Vedova, 1996), oxalic and citric acids (Milani, 2001), as well as for the study of reversion of resistance (Milani and Della Vedova, 2002). Other acaricides that are widely used for the control of the varroa mite are air-borne and the bioassay above is not suitable. For these cases a new bioassay developed by the honey bee laboratory of the University of Udine is presented in section 3.6.3.3.

3.6.3.1. Mites used in susceptibility bioassays

For this assay, mites that would be exposed to the product in colonies in the apiaries should be used. Some products affect only phoretic mites, others also affect mites in brood cells. Adult mites at the reproductive stage might have different susceptibility compared to phoretic mites or to their offspring because their cuticle is not hardened or because of physiological differences. Milani (1995) and Milani and Della Vedova (1996) tested compounds only affecting phoretic mites, but showed that mortality was more homogeneous in mites collected from the brood. This might be due to a more homogeneous physiological status compared to phoretic mites. It is therefore recommended to test all life stages to obtain a complete picture of mite susceptibility.

When brood mites are tested, they are collected from combs (or pieces of comb) of infested colonies after opening and inspection of capped cells. Mites parasitizing brood of different developmental stage have different susceptibilities to acaricides (Milani and Della Vedova, 1996). For the tests, they are therefore grouped according to the age of their brood host and assayed separately. The age of larvae or pupae inhabiting these cells can be pre-determined by marking at the capping stage and opening it at a given time. Alternatively, the approximate age of the brood can be inferred on the basis of the morphology and pigmentation of the larva or the pupa (see section 'Obtaining brood and adults of known age' in the *BEEBOOK* paper on miscellaneous methods (Human *et al*., 2013)). Varroa mites from different brood stages can be pooled if previous results indicate no differences among development stages (Milani, 1995). Differences between mite developmental stages might also influence their susceptibility to active ingredients, independently of host development, but this has not been shown yet.

Mites are kept on their host larva or pupa in glass Petri dishes until a sufficient number is collected. This ensures they can feed if hungry and the availability of their own host ensures that their physiological status is not changed. Since mites might stray from their host larva or pupa and climb onto another, only hosts at the same development stage should be kept in any given dish.

3.6.3.2. Bioassays for contact substances

1. A stainless steel ring (56 mm inner diameter, 2–3 mm height) and 2 glass circles (62 mm diameter; Na-Ca glass) are cleaned with acetone and hexane or pentane to form the testing arena.
2. Apply the product to be tested and the control solution on the arena pieces.
   Various concentrations of the products are tested. See the *BEEBOOK* paper on methods for toxicological studies (Medrzycki *et al*., 2013) to define these concentrations.
   The application of the active ingredient on the arena pieces varies according to its physico-chemical properties.

2.1. For water soluble active ingredients (polar compound):
   - 2.1.1. Mix the active ingredient with a convenient solvent.
   - 2.1.2. Spray the glass disks and ring with a solution of the compound as evenly as possible.

   This can be done by means of a "Potter precision spray tower" (e.g. Burkard Manufacturing Co; UK) (Milani, 2001). To do so, the reservoir is loaded with 1 ml of solution; the distance of the sprayed surface from the bottom end of the tube is set at 11 mm and a nozzle 0.0275 inches is used. The pressure is adjusted (usually in the range 350–500 hPa) until the amount of solution deposited is 1 ± 0.05 mg/cm^2. Alternatively, if such piece of equipment is not available a glass Petri dish can be used as arena. The solution (active ingredient in a solvent of low boiling point) is poured in the dish so as to cover the whole bottom of the dish and left to evaporate under a fume hood. Depending on the surface tension of the active ingredient, this will result in an uniform layer of substance at the bottom of the dish. Varroa mites can thus be exposed to the substance in the Petri dish. This method cannot be used when the surface tension of the ingredient is too high and droplets are formed on the Petri dish.

2.2. For lipid soluble (apolar compound) active ingredients:
   - 2.2.1. Melt 10 g of paraffin wax (e.g. Merck 7151, melting point 46-48°C) in a glass container kept in a water bath at 60°C.
   - 2.2.2. Dissolve the required amount of the active ingredient in a convenient solvent (e.g. hexane or acetone).
   - 2.2.3. Add this solution to the melted wax. The solvent alone is added to the control wax.
   - 2.2.4. Weigh glass disks and iron rings before coating it with the wax.
   - 2.2.5. Stir the mixture for 30 min.
   - 2.2.6. Immerse the steel rings in the molten paraffin wax, one side of the glass disks is coated by lowering the disks onto the molten paraffin.
   - 2.2.7. Weigh glass disks and iron rings after coating. Discard the arenas (ring + glass circles) with a total amount of coating outside the range 1.6-2.0 g.
   - 2.2.8. Keep the arena pieces for at least 24 h at room temperature to allow for the solvent to evaporate.
   - 2.2.9. Store at 32.5°C until they are used.

3. Place the ring between the glass circles so as to build a cage. The cages are used within 60 h of preparation, for not more than three assays.
4. Introduce 10 to 15 varroa mites in this cage and bind the pieces together with droplets of melted wax.
   Mites collected from spinning larvae, stretched larvae, white eyed pupae and dark eyed with white and pale body are used. See the section 'Obtaining brood and adults of known age' estimating pupa age of the *BEEBOOK* paper on miscellaneous methods (Human *et al*., 2013) for determining the age of pupae.
5. After 4 h transfer mites into a clean glass Petri dish (60 mm diameter) with two or three worker larvae taken from cells 0–24 h after capping (obtained as described in section 3.1.4. 'Collecting mites from the brood') or with two or three white eye pupae (4-5 days after capping).
6. Observe the mites under a dissecting microscope, 4 (i.e. at the time of transfer into the Petri dish), 24 and 48 h after the beginning of the treatment and classify as:
   6.1. Mobile: they walk around when on their legs, non-stimulated or after stimulation.
   6.2. Paralysed: they move one or more appendages, non-stimulated or after stimulation, but they cannot move around.
   6.3. Dead: immobile and do not react to 3 subsequent stimulations.

   A clean tooth pick or needle can be used to stimulate the mites by touching their legs. New tooth picks or cleaned needles should be used for stimulating control groups to avoid their contamination with residues of active ingredients from treated mites.

The assays are carried out at 32.5°C and 60-70% RH. If the mortality in the controls exceeds 30%, the replicate is excluded. Each experiment is replicated with a sufficient number of series of cages. To determine the sample size, refer to the *BEEBOOK* paper on methods for toxicological studies (Medrzycki *et al*., 2013) and the *BEEBOOK* paper on statistics (Pirk *et al*., 2013). If mites are scarce, more replications are carried out and more mites are assayed at doses around the median lethal density, to increase statistical resolution in this region.

3.6.3.3. Bioassays for volatile substances

1. Dissolve the active ingredient (e.g. thymol) in a suitable solvent (e.g. diethyl ether) at the concentration 0.5 g/ml.
2. Treat a circular area (diameter = 6 cm) of the inner side of the lid of a glass Petri dish (diameter = 14 cm) with 250 µl of the solution.
3. Let the solvent evaporate.
4. Place 10 to 15 varroa mites on the bottom of the Petri dish and keep different groups inside the closed container for 0, 15, 30, 45, 90, 135 min at room temperature.
   Mites of the same origin as for the bioassay for susceptibility to contact substances are used (see section 3.6.3.2. 'Bioassays for contact substances').
5. After each interval, transfer the group into small Petri dishes (diameter = 6 cm) with one bee larva for every five mites.
6. Place in an incubator at 34.5°C and 60-70% RH.
7. Monitor mite survival at 48 hours.

3.6.3.4. Data analysis

The data are analysed using the probit transformation. The natural mortality rate is taken into account using the iterative approach, according to Finney (1949). The concentrations which kill a given proportion of mites and their fiducial limits are computed according to Finney (1971). Refer to the *BEEBOOK* paper on toxicological methods (Medrzycki *et al*., 2013) for these calculations.

4. Field methods

4.1. Diagnostic techniques

The OIE manual describes three methods to diagnose the presence of varroa mites in colonies (OIE *Terrestrial Manual* 2008). Debris, adult and brood examination are reported here.

4.1.1. Debris examination

Hives must be equipped with a bottom board on which debris are collected. The board must be protected by a mesh to prevent bees from discarding the dead mites. The mesh size should allow the mites to fall through. To increase probability to detect mites, colonies can be treated with a varroacide. After a few days, dead mites can be observed on the boards. In case a large quantity debris prevents easy detection of mites, debris can be cleaned from the varroa board and examined using a flotation procedure:

1. Dry debris for 24 h.
2. Flood with industrial grade alcohol.
3. Stir continuously for 1 minute or up to 10-20 min if debris contain wax or propolis particles.
4. Investigate the surface of the alcohol for the presence of mites.

4.1.2. Brood examination

Since varroa mites prefer drone brood, the probability to detect them on male pupae is higher than on worker brood. However, in absence of drone brood worker brood is used. When a large number of samples are examined, a rough determination of the degree of infection can be obtained.

1. Remove the cappings of the brood cells with a knife or fork.

2. Flush the pupae out of the combs with a stream of warm water over a sieve (mesh width 2–3 mm).
3. Collect the mites in a second sieve (mesh width 1 mm) placed below the first.
4. Examine the contents of the second sieve on a bright plate, where the mites can be easily identified and counted.

When a smaller number of samples are examined,

1. Open individual cells.
2. Remove larva, pre-pupa or pupa.
3. Examine cell walls using an appropriate source of light.
4. Identify infected cells by the presence of small white spots – the faeces of the mite (Fig. 3).
5. Confirm the presence of the mites themselves in the cell or on the brood.

4.1.3. Bee examination

1. Collect 200–250 bees from both sides of at least 3 unsealed brood combs.
2. Kill the bees in a container filled with alcohol.
3. Stir the container for 10 min.
4. Separate the bees from the mites by pouring the alcohol over a sieve with a mesh size of approximately 2–3 mm.

4.2. Measuring colony infestation rate

Three methods to estimate colony infestation have been designed (Ritter, 1981; De Jong *et al.*, 1982b). Acaricides can be used to kill all mites in a colony. Mites will fall to the bottom of the hive and can be counted (Branco *et al.*, 2006). Without the use of acaricides, the natural mortality can be quantified from the bottom of the hive to determine the population size of the live mites. Alternatively, the infestation rates of adults and brood can be estimated from adult and brood samples. When the first two methods are used, ants must be prevented access to bottom boards. Their scavenging habit will result in the disappearance of dead mites before they can be counted and will thus bias the results (Dainat *et al.*, 2011). Such a protection can be obtained by preventing access to the whole hive or to the bottom board. Hive protection can be achieved by using a stand with feet smeared with grease or resting in containers containing a liquid over which ants cannot walk (water or oil). Here it is important to regularly verify that dirt does not accumulate in the container or on the grease, allowing ants to reach the hive. Blades of grass can also form bridges and should be cut in the surrounding of the hives. Alternatively, the varroa board itself can be protected against ants. This is achieved by covering the board with sticky material (e.g. Vaseline, glue, absorbent paper impregnated with vegetable oil). Such 'sticky boards' can be purchased or homemade.

All three methods (using acaricides, monitoring natural mite fall and assessing infestation levels) were found to provide comparable results (Branco *et al.*, 2006). For the adult infestation rate estimate, the sample size in relation to the level of precision required by the experimenter has been determined by Lee *et al.* (2010a). Their study provides methods with different workloads permitting to achieve several levels of precision. We present here the method with optimal time and effort investment ratio that is necessary to reach the precision necessary to researchers. Since researchers are mostly interested in the infestation rates of particular colonies rather than of whole apiaries, we do not describe the latter method here, but refer to Lee *et al.* (2010a) for the number of colonies to sample from in order to obtain a representative figure at apiary level.

The methods based on mite fall or on evaluating infestation rates from adult or brood samples are only reliable for colonies with medium to high infestation rate. The methods show imprecision when colonies have less than 3,000 brood cells, when the brood infestation rate is < 2% (unless very large samples are taken, see the *BEEBOOK* paper on statistics (Pirk *et al.*, 2013)) or when the colony is collapsing (due to decreased amount of brood) (Branco *et al.*, 2006; Lee *et al.*, 2010a). In these cases, the acaricide treatment can be used. Using synthetic acaricides to estimate parasite population size in the host is reliable provided a product with high efficiency (> 95%, taking possible resistance by the mite into account) is used. However, it is destructive and can only be used for a quantification /diagnostic purpose. The mites being killed by the treatment and the hive being contaminated with acaricide residues, the treated colony cannot be used as source or host of mites.

4.2.1. Acaricide treatment

Use an effective acaricide > 95% product as per manufacturer recommendation. Beware of resistance of mites to this product, see section '3.6.3. Bioassays to quantify the susceptibility of the varroa mite to acaricides ' for methods on how to test mite susceptibility to acaricides.

1. A protected bottom board should be used to prevent bees removing the fallen mites.
   The protection is typically a wire screen with 3-4 mm holes covering the whole surface of the board, leaving no access for bees to the fallen mites.
2. Ant protection should be put in place to prevent their access to the hives and predation on fallen mites and therefore biasing the number of mites counted.
3. Given the rapid action of efficient acaricides and to ease counting, mite fall should be assessed daily.
   See sections 4.2.4. 'Natural mite fall' and 4.2.5. 'Sub-sampling mites', to count mites on a bottom board.

If the active ingredient used is persistent enough (i.e. the treatment still in place or if residues persist in the hive) and do not penetrate in the cell through the capping (e.g. most synthetic acaricides), the mites that entered cells just before the treatment

become exposed upon their emergence with their bee host and die within a few days. Mite fall should thus be counted for 3 weeks, this period covering the development times of pupae and the time necessary for mite fall to decrease to pre-treatment levels. The same counting period should be covered if a non-persistent acaricide is used that also kills mites in the cells (e.g. formic acid). Indeed, mites dead in the cells will only be released and fall on the bottom board to be counted upon emergence of their host bee. In case the product is not persistent and does not affect mites in cell (e.g. oxalic acid), colonies without capped brood must be treated. Absence of capped brood can be obtained by caging the queen 22 days before the planned treatment. All mites being in the phoretic phase, mites should fall for a shorter period (since none are trapped in cells). Mite fall count can therefore stop when it decreased to pre-treatment levels.

Pros: efficient, relatively low workload.

Cons: slow, dead mites, and in case of use of persistent acaricides, contaminated colonies cannot be used further; in case of queen caging, the development of varroa population before treatment can be slightly affected by the interruption of brood rearing.

4.2.2. Whole colony estimate

This method requires killing the whole colony. This is necessary when the real infestation rate of a colony is needed. Indeed, the use of acaricides is under these circumstances not appropriate since their efficiency is not 100%.

1. When all foragers are in the colony (early in the morning, late in the evening or at night) close the hive so that no bees can escape.
2. Place the whole colony in a freezer.
   Depending on nutritional status and size, colony survival in a freezer will vary. To determine when the colony died, workers from the centre of the cluster can be sampled and left to thaw. If they do not wake up, the whole colony can be considered dead and used for mite counts. In case the colony is of large size, gazing with CO_2 is required before freezing. This will prevent the bees thermoregulation and entering in the cells. Thermoregulation extends the duration needed to kill the colony and if bees get into the cells, they will be more difficult to collect for mite counting.
3. Refer to section 4.2.3. 'Measuring the infestation rate of brood and adult bees' for phoretic and brood infestation rate measurement.

If a measurement of total infestation rate is needed in summer, the colony can be made broodless by caging the queen for three weeks. When all the brood runs out (after 21 days if only worker brood was present or 24 days if drone brood was present), all mites have become phoretic. There is then no need to look for mites in the brood.

Pros: provides the exact total number of mites in a colony.

Cons: destructive, high workload, tedious.

4.2.3. Measuring the infestation rate of brood and adult bees

4.2.3.1. Infestation rates of adult bees

4.2.3.1.1. Sampling

Material: a rectangular graduated container in which 300 bees fit. Three hundred bees occupy a volume of 100 ml water. Fill this volume of water in a container and mark a line at the water surface (Lee *et al.*, 2010b, www.beelab.umn.edu). Given that bee sizes change with race, this volume should be verified and adapted for the particular bee under scrutiny.

1. Hold the frame at approximately 10 degrees from the vertical.
2. On the upwards facing side, slide the graduated container downwards on the back of the bees so that they tumble in it, making sure the queen is not one of them.
3. Rap the cup on a hard surface to be sure the bees are at the marked line; add or subtract bees as needed.
4. Collect 3 x 300 workers from any three frames in the first brood box.

Sampling such a large number of bees takes into account variations among frames to obtain an average infestation rate, and does not damage the colony if a non-destructive method is used to loosen the mites from the bees (see section 4.2.3.1.2.1. 'Powdered sugar'). Strong colonies (> 10,000 bees) are not dramatically affected by the removal of this amount of bees and will quickly recover. However, for analysing varroa population dynamics throughout the whole season with frequent and distructive sampling of bees (e.g. at 3-week intervals), lower numbers of individuals (300 bees per sampling date) should be used.

4.2.3.1.2. Dislodging mites from bees:

There are several ways to dislodge the mites from the bees. Some were already presented in section 3.1., but not all of them are adapted to estimating the infestation rate of the colony. Indeed, these methods must be standardised and deliver repeatable results.

4.2.3.1.2.1. Powdered sugar

After step five of section 3.1.2. describing how to dislodge mites from honey bees kept in a jar, perform the following steps:

6. Count the mites fallen out of the jar (e.g. 43).
7. Count the number of bees in the sample washed (e.g. 310)
8. Divide the number of mites counted by the number of bees in the sample (310) and multiply by 100 to determine the number of mites per 100 bees (e.g. (43/310)x100 = 14.3 mites per 100 bees).

Pros: practical, low cost, non-destructive (the bees can be reintroduced in the colony and will be cleaned by their nestmates), environmentally friendly.

4.2.3.1.2.2. Ether wash:

This method is modified from Ellis *et al.* (1988).

Material needed: a jar with a screen raised 2-3 cm above the bottom, automotive starter fluid

1. Spray the jar for two seconds with starter fluid to kill bees and mites.
   Dying bees regurgitate consumed nectar or honey that will make the wall of the jar sticky.
2. Shake the jar for 1 min to dislodge the mites from the bees.
3. Lay the jar sideways and roll three times completely along its vertical axis.
4. Count the mites stuck to the sides of the jar.
5. Count the number of bees in the sample washed.
6. Divide the number of mites counted by the number of bees in the sample to determine the proportion of infested individuals.
7. Multiply by 100 to obtain the number of mites per 100 bees.
   Caution: ether is highly flammable!

Pros: fast, low workload.

Cons: environmentally unfriendly, expensive, destructive, dangerous.

4.2.3.1.2.3. Warm/soapy water or ethanol (75%):

This method follows the protocol by Fries *et al.* (1991a). Since mites do not have to be collected alive as allows the method described in section 3.1. 'Collecting mites', soap can be added to water or ethanol can be used to improve the efficiency of mite dislodging.

1. Warm/soapy water or ethanol is added to jars to cover the 300 honey bees.
2. The jars are shaken for 20 s to dislodge the mites from the adult honey bees.
3. The content of the jar is poured over a first sieve (aperture: 3-4 mm) to collect all the bees.
4. Check the jar for mites sticking to the sides.
5. Place a second sieve (aperture < 0.5 mm) underneath the first to collect the mites.
6. Flush the bees and mites with large amounts of warm water. Strength of the water stream or volume of water used for rinsing as well as duration of rinsing should be standardised.
7. Count the mites remaining on the second sieve (e.g. 13).
8. Count the bees in the sample washed (e.g. 303).
9. Divide the number of mites counted by the number of bees in the sample to determine the proportion of infested individuals (13/303 = 0.043).
10. Multiply by 100 to obtain number of mites per 100 bees (4.3).

Pros: water based method: low environmental impact, low cost.

Cons: not practical on remote apiaries (large amount of water for rinsing and heat source needed); alcohol based: expensive, environmentally unfriendly.

None of these three methods is distinctly superior to the other and they can all be considered as reliable given that mite separation is done in a standardised manner (water always at the same temperature, or containing a standardized amount of soap etc.) and that the efficiency of the method is determined as described below.

4.2.3.1.2.4. Assessing the efficiency of dislodging method

It is important to dislodge mites in all samples in a standardized manner so as to being able to compare the infestation rates measured between samples. When comparisons of infestation rates between samples are aimed at, calculation of washing efficiency is not needed. In contrast, when the absolute number of mites is important, the efficiency of the washing method should be assessed to correct the figure obtained for errors. In addition, it is necessary to obtain absolute numbers in order to compare the figures obtained with other studies and therefore calculating efficiency in all cases is recommended.

1. Perform additional washes (with same or other solvent) until no more mites are found (optional).
2. Check bees manually for the presence of mites after the wash(es) or sugar treatment.
3. Add mites found after repeated washes and/or manually (e.g. 1) to those of the first wash/sugar treatment to obtain the total number of mites in the sample (e.g.10).
4. Divide number of mites of the first wash/sugar treatment by the total number of mites to obtain the method efficiency (10/11 = 0.91).
5. Repeat with 5-10 samples to obtain an average efficiency (e.g. 0.9).
6. Divide the number of mites obtained in samples of interest (X) by the average efficiency to obtain the corrected figure (Y) (Y = X/0.9).

4.2.3.2. Infestation rates of brood

1. Cut out 200 randomly selected capped cells from a brood frame. Sampling cells from several frames will account for the spatially irregular infestation by varroa.
2. Open each cell and examine it for mite infestation.
   Mite infestation can be diagnosed by observation of mites themselves or of their dejection (white rubbery material located most of the times on the two upper walls, towards the bottom of the cell, Fig. 3).
3. Count the total number of cells opened.

4. Count infested cells.
5. Divide the number of infested cells (e.g. 15) by the total number of opened cells (e.g. 212) to obtain the proportion of mite infested cells (0.071).
6. Multiply this figure by 100 to obtain the brood mite infestation rate in mites per 100 cells (7.1).

Depending on the question addressed, more detailed observation of cell infestation can be done (see section 4.3.3. 'How to measure reproductive success')

4.2.3.3. Evaluation of total mite population size in the colony

From the infestation rate of adult bees or from that of brood, it is possible to calculate the infestation rate of the whole colony (Martin, 1998). However, it is more accurate to assess both parameters based on samples and revert to the total for the colony after estimating the amount of adults and brood.

1. Measure colony strength (see the *BEEBOOK* paper on estimating colony strength by Delaplane *et al*. (2013)) on the same dates as sampling for determining mite infestation rates, so as to accurately calculate the total colony infestation rate (Fries *et al*., 1991b).
2. Multiply the total number of bees in the colony (e.g. 9,356) by the proportion of infested workers in the sample investigated (e.g. 0.107) to obtain the size of the varroa population in the phoretic phase in the colony (1,001).
3. Multiply the total number of sealed brood cells in the colony (e.g. 12,035) by the proportion of infested cells in the sample investigated (0.071) to obtain the size of the varroa population in the reproductive phase in the colony (855).
4. Add adult and brood infestation numbers to obtain mite population size in the colony (1,001 + 855 = 1,856).

With this information, the mite distribution between the phoretic and reproductive phases can be determined as the proportion of either mites on adults, or mites in brood, in relation to the total mite population within the colony (in our example 54 and 46% respectively). Mite distribution patterns can be used to determine the effect of brood attractiveness on varroa for example.

4.2.4. Natural mite fall

This method is based on the quantification of naturally dead mites. Counting can be exhaustive (more accurate when done with a guide, Fig. 13) when mite fall is low to medium or can be sub-sampled (using a checked pattern board; see section 4.2.5. 'Subsampling mites to count on a bottom board') when mite fall is high. In both cases, a formula needs to be applied to calculate the total infestation rate of the colony. Various studies gave contradictory conclusions regarding the accuracy of the natural fall method to determine total infestation rate since natural mite fall is largely determined by the amount of emerging infested brood (Lobb and Martin, 1997), but it is in general considered as a good indicator of colony infestation (see Branco *et al*., 2006).

Fig. 13. A guide is placed above the varroa bottom sheet to help guide the eye when counting and thus avoid double counts.

Photo: Swiss Bee Research Centre

Material needed: screened floor board, guide or sheet with checked pattern see 4.2.5. 'Sub-sampling mites to count on a bottom board'. Note: make sure the hives are inaccessible to ants or use a sticky board.

1. Pull the bottom board from underneath the colony.
2. When using non-sticky boards, shelter from the wind to count.
3. Place a guide above the board to avoid counting the same mites (Fig. 13) or count mites from the selected squares on a checkered board (see section 4.2.5. 'Sub-sampling mites to count on a bottom board').
4. If dead bees are present on the board, check them as they act as magnets to fallen live mites.
5. Adapt counting frequency to mite fall rate since many mites on the board are difficult to count and since increasing amount of debris accumulating over time makes counting difficult.
   If frequency of visit cannot be increased and high mite numbers must be counted, use the checkered board method (see section 4.2.5. 'Subsampling mites to count on a bottom board').
6. Collect data for 2 weeks, average the figure to obtain mean weekly mite fall.
   This period covers natural variation in mite fall due to population dynamics cycles within the host.
7. Calculate the total colony infestation rate from the weekly mite fall by multiplying the daily mite drop by 250–500 or 20-40 when brood is absent or present, respectively (Martin, 1998). These correction factors are valid for central European conditions.

Pros: non-destructive/non-invasive, fast, no need to open the hive depending on design; reliable for non-collapsing colonies with brood; this is a method providing relative quantification that can be used for comparison between colonies within an experiment, not across studies.

Cons: sometimes unreliable, death rate may vary according to colony status, season, bee race, climate (regional variations possible), amount of brood; little is known on the influence of these factors on mite death; specific equipment necessary.

4.2.5. Sub-sampling mites to count on a bottom board

This method is useful when a high number of mites have to be counted on the board. However, it provides reliable counts for all mite densities on the board. If more than 1,000 mites are present, it is sufficient to count 22% of the cells to obtain a highly reliable figure (Ostiguy and Sammataro, 2000).

Material needed: a grid with 2 cm cells printed onto a floor board or sheet fitting the size of the board. Note: make sure the hives are inaccessible to ants or use a sticky board.

1. Cells are grouped into blocks of nine.
2. Three cells per block are randomly selected and greyed (10% shade) to indicate the cells in which mites are to be counted.
3. The number of mites in the shaded squares are counted.
4. The total number of mites is then divided by the number of cells counted and multiplied by the total number of cells on the board.
5. The total number of mites is divided by the number of days the board was under the hive to obtain the mite fall per day.

Pros: can be more accurate than exhaustive counts when mite fall is high.

Cons: need to design / build / buy the checkered board.

4.3. Estimating reproduction parameters

4.3.1. Assessing reproductive success

Mite reproductive success is defined as the ability of a mother mite to produce at least one viable mated female offspring before the developing bee pupa hatches as an adult. Successful mite reproduction requires the maturation of at least two eggs laid by a reproducing mother mite inside the brood cell: a male mite and a sister female mite, which must mate together before emergence of the host bee. The male mite offspring will die when the bee hatches from the cell, but any mated mature daughter mites will enter the colony's mite population along with their mother to find a new brood cell for reproduction. A mother mite that lays no eggs, lays only one egg, produces no male offspring, or begins egg-laying too late in relation to larval development, will not contribute any progeny to the mite population. The fecundity (number of eggs laid) is an additional parameter that can determine variation in the number of viable

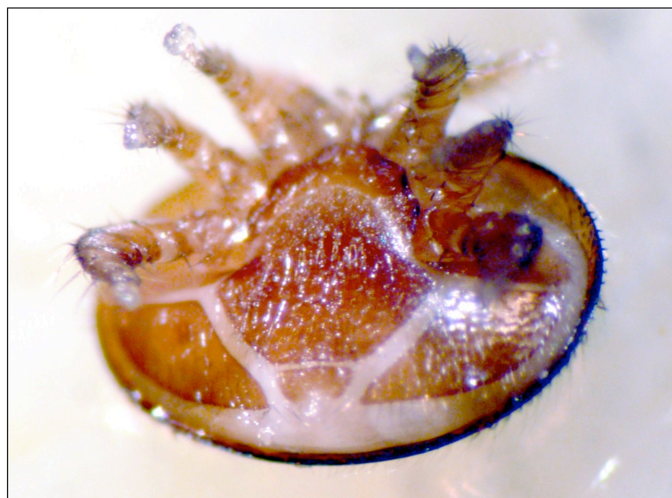

Fig. 14. The ventral side of a physogastric varroa mite. The intersegmental membranes are stretched due to the presence of oocytes in the mite's reproductive tract, which becomes partially visible.

Photo: Eva Frey

females each mother mite contributes to the population. Fecundity does not necessarily contribute to the mite's ability to reproduce successfully. Instead, it represents only the number of eggs laid without accounting for the age of the offspring or the likelihood of them reaching maturity. Therefore, fecundity may not be independent from the incidence of delayed egg-laying since any mother mite that begins laying eggs late may consequently lay fewer eggs.

Therefore, information on the following parameters are required to assess successful reproduction: the fertility (whether the mother mite laid eggs); the presence or absence of male offspring; the proportion of dead offspring; and the incidence of delayed egg-laying by mother mites (identified by relating the developmental stage of mite offspring to the developmental appearance and thus the determined age of the infested pupa; see the section 'Obtaining brood and adults of known age' in the *BEEBOOK* paper on miscellaneous methods (Human *et al.*, 2013)). These are important since relatively small differences in reproductive factors can have a large effect on the population dynamics at a population level (Martin and Medina, 2004).

4.3.2. When to measure reproductive success

The most accurate evaluation of mite reproductive success can be obtained just prior to worker emergence. However, the hours before emergence is not optimal since the last stage of bee development makes mite recognition difficult. It is therefore recommended to assess this parameter latest one day before emergence and to project in the future the development of the varroa offspring by comparing the developmental stage of the pupae to the developmental stage of the mite offspring. If direct comparisons between studies for several reproductive traits are required then the data must be collected over

the same time period. That is, fecundity cannot be accurately measured until 220 hours after cell capping in workers and 240 hours in drone cells, as it is only after this period that all the potential eggs may be laid. It is only possible to determine various abnormal reproductive patterns 60 hours post capping. Table 2 gives the time after capping for which several traits related to reproductive success can be accurately measured.

To obtain a high amount of brood of relevant age in which reproductive success of the mite can be evaluated, cage the queen of a highly infested colony on an empty frame for several hours at the appropriate time before measurement.

Pros: mites are easily recognisable on the pupae.

Cons: a drawback is that the male or the daughters might die before emergence and been counted as part of reproductive success; determining of pupal age based on appearance is approximate.

4.3.3. How to measure reproductive success

The reproductive success of a mite is assessed by reconstructing the mite family in infested cells (Martin, 1994, 1995a). See section 4.3.2. 'When to measure reproductive success' for the optimal time when to measure this parameter.

It is important to examine cells infested by single mother mites as opposed to multiply infested cells since multiple infestations add an additional effect on the success of mite reproduction. Whether only one 'mother' mite reproduces can be tricky to ascertain in cells with multiple red mites, but using the mite development guide (Figs. 11 and 12) will indicate what is expected and give a good idea of the number of mother mites present. For statistical significance, at least 30 single mite infested cells per colony should be examined.

Different aspects of mite reproduction can be measured. Within each pupal cell, the following information can be collected: sex, developmental stage and vitality. Live or dead mites are easily identified if fresh material is used. If frozen material is used, mite appearance must be relied on. A shrivelled individual or abnormal appearance means it was dead prior to freezing. This characteristic can also be used to distinguish a dead individual from an individual in an immobile developmental stage in live samples. In addition, death rate can be estimated indirectly by comparing the developmental stages obtained with the reference: the development of any dead individual is prematurely arrested and can therefore be identified with the help of Figs. 11 and 12.

1. Using forceps, carefully open each capped cell by peeling back its top, papery seal and push away the walls of the cell.
2. Remove the pupa from the cell.
   This is best done by sliding the forceps each side of the neck between head and thorax and gently lifting up. When getting the pupa out of cell, place it on a microscope slide next to the cell to avoid mites dropping in the comb.
3. Record its developmental stage based on the appearance description given in Fig. 12.
   It is important to examine the pupae under a stereomicroscope once it is removed from the cell to make sure that mite progeny are not discarded with the pupae. Cell walls should also be inspected meticulously for mites and exuviae. The use of an optic fibre light source is particularly suitable to direct the beam to the bottom of the cell and inspecting it after the removal of the pupa.
4. Remove complete mite families together with exuviae from their cells and pupae using a fine brush.
5. Examine under a stereomicroscope.
6. Classify all female offspring into developmental stage groups using Figs. 11 and 12 as a guide.
   Protonymphs can be distinguished from similar looking young deutonymphs by the number of hair in the intercoxal region (between the 4 pairs of legs on the ventral side). Male and female protonymph have 3 and 4 pairs of hair, respectively, whereas deutonymphs have 5-6 pairs (see drawings pp 55-57 in Fernandez and Coineau, 2007). This information allows the mite family to be reconstructed in birth order or to check for multiple infestation and normality of development.
   Protonymphs are usually not sexed, but the number of hairs on the intercoxal region and the patchy hair pattern on the dorsum of males compared to the homogeneous and dense pattern of females provide recognition traits (Fernandez and Coineau, 2007). For the number of eggs laid by mites and to allow a more accurate comparison between studies, only mites that laid two or more eggs are compared. Mites that produce no eggs (non-reproductive) or one egg (single male) are considered as distinct reproductive categories (see b and c below). The mortality of the mite offspring in worker and drone cells is calculated by comparing the number of live and dead offspring at each position in birth order, i.e. first offspring, second offspring, etc. Then the average number of surviving females (unfertilised and fertilised) is calculated using only the levels of offspring mortality.
7. All infested cells are analysed by placing the mother mites into one of the following six categories:
   a) mother dead
   b) mother (alive), no offspring
   c) mother plus only male offspring
   d) mother plus (live) mature male and female offspring so mating is assumed
   e) mother plus (live) female offspring and dead male offspring
   These female offspring might remain unfertilised since the male might have died before they were mated (Harris and Harbo, 1999). Dissection of the spermatheca and microscopic

examination of its content can help determine their mating status (see Steiner *et al.*, 1994 for pictures of reproductive tract with spermatheca).

f) mother without (live) female offspring.

When working with frozen material, beware of repeated freezing and thawing cycles since this damages the samples. Only take out of the freezer the amount of material can be dealt with at a given time.

Pros: if this type of data is collected it can be compared with previous studies (e.g. Martin, 1994, 1995a, b; Medina and Martin, 1999; Martin and Kryger, 2002), where the same data for reproductive parameters of susceptible mites were obtained by using the exact same methodologies.

Cons: tedious, time consuming.

4.3.4. Assessing oogenesis

For estimates of oogenesis in mites, refer to section on marking methods 3.4.1. 'Oogenesis'.

4.4. Estimating damage thresholds

This section describes methods used to measure varroa damage at the colony level and to associate that damage with economic damage thresholds.

One of the goals of varroa Integrated Pest Management (IPM) is the reduction of beekeeper reliance on pesticides in the bee hive. Mite eradication is not a necessary goal, as IPM philosophy recognizes that eradication may require practices that are excessively toxic, invasive, or impractical. Varroa IPM places a premium on non-chemical management practices that eliminate mites from a colony (such as drone brood trapping) or slow the rate of mite population growth (such as genetic host resistance). The most promising varroa IPM practices have been reviewed by Rosenkranz *et al.* (2010). Unfortunately, it has been shown anecdotally as well as in computer simulations (Hoopingarner, 2001; Wilkinson *et al.*, 2001) that few if any of these practices can by themselves or indefinitely keep mites at non-damaging levels. Thus at this point it seems best to think of IPM as a means to delay, not necessarily eliminate, the application of acaricides (Delaplane, 2011). Many benefits accrue if the time between chemical treatments is delayed – namely, reduced toxin delivery to bees and the environment, reduced chemical residues in honey, and relaxed selection pressure for chemical resistance and the conservation of susceptible alleles in the mite population that prolong the commercial life of an acaricide.

Since the focus of IPM is not mite eradication, but rather mite management, the whole system hangs on the existence of criteria that can distinguish mite densities that are tolerable from those that are approaching damaging levels. Classical IPM tenets (Luckmann and Metcalf, 1982) identify the economic injury level (EIL) as that pest density at which point the grower is experiencing economic loss. The goal is to prevent this level from happening, in other words, to identify an earlier and lower pest density at which point a treatment could prevent reaching the EIL. This lower pest density is named variously the economic threshold, treatment threshold, action threshold, or damage threshold. Implicit in the use of damage thresholds is the recognition that some pest levels are tolerable and do not warrant the use of a pesticide. A good damage threshold will not only distinguish damaging from non-damaging pest densities, but also accommodate local variations due to geography and biology of host and parasite. Thresholds are simply a form of applied population modelling, and like any model, they are only as good as the data, detail, and specificity applied to their construction.

Fig. 15. Adult bees with their phoretic mites from several colonies are mixed to homogenise the starting infestation level in the replicates.
Photo: Keith Delaplane

4.4.1. How to estimate damage thresholds

The following instructions are a synthesis from the field-derived damage thresholds of Delaplane and Hood (1997, 1999) and Delaplane *et al.* (2010) for the south-eastern USA, Strange and Sheppard (2001) for the north-western USA, and Currie and Gatien

(2006) for Manitoba, Canada. All of these studies used a design in which colonies of uniform strength and mite density are experimentally set up, applications of acaricide made at different times to create a spread of mite history and colony condition, and samples taken regularly to document mite levels and colony condition. With some, negative controls are present as colonies that are never treated, and positive controls present as colonies that are treated continuously. Thus, a range of mite densities exists across the experiment in space and time, and every colony has a history of known mite levels and colony strength at the time it was treated.

4.4.1.1. Colony establishment

1. Equalize field colonies in regard to bees, brood, food resources (see section 1.2.2.1 'Setting up experimental colonies of uniform strength' of the *BEEBOOK* paper on estimating colony strength (Delaplane *et al*., 2013)) and mites within units of higher-order experimental replication, i.e. blocks or whole plots, usually based on geography.
   At least two more source colonies than the target number of experimental colonies should be used to account for bee loss through death or flight.
2. Collect adult bees and phoretic mites for experimental set-up by shaking workers from a diversity of source colonies into one large, common cage, allowing workers (and mites) to freely mix (Harbo, 1993) (Fig. 15).
3. Maintain the cage in cool conditions to prevent bee death from over-heating for at least 24 hours to allow thorough admixing of bees and mites, resulting in a uniformly heterogeneous mixture.
4. Distribute worker cohorts of equal size (preferably by weight, *ca.* 1 kg) into hive boxes pre-stocked with near-equal amounts of brood, honey, pollen, and empty cells (Fig. 16).
5. Provide each colony a sister queen reared from the same mother and open-mated in the same vicinity to minimize variation due to bee genetics.
6. Collect a sub-sample of *ca*. 300 workers from each incipient colony.
7. Weigh the samples.
8. Count the number of bees to derive a colony-specific measure of average fresh bee weight (mg).
9. Divide initial cohort size (kg) by average fresh weight of individuals (mg) to obtain initial bee population.
10. Collect a sample of *ca*. 300 worker bees (can be the same sample as above after it is weighed fresh) to obtain a measure of initial density of phoretic mites.
11. Separate the mites from the bees, see section 4.2.3.1. 'Infestation rates of adult bees' (Fig. 17).
12. Count the number of bees and number of mites to derive a colony-specific measure of mite / bee density.

Fig. 16. Boxes are pre-stocked with near-equal amounts of brood, honey, pollen, and empty cells to host the experimental colonies.
Photo: Keith Delaplane

Fig. 17. Number of bees and mites are counted to obtain a measure of initial density of phoretic mites. Photo: Keith Delaplane

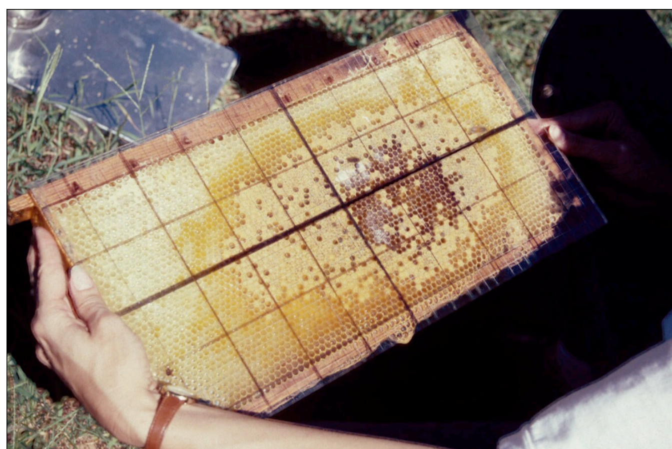

Fig. 18. Measuring the beginning quantity of sealed brood.
Photo: Keith Delaplane

13. Multiply this number by initial bee population to obtain phoretic mite population.
14. Store the samples in alcohol for future reference.
15. Collect brood for incipient experimental colonies from the same source colonies used to collect adults.

16. Assign a near-equal quantity of brood to experimental colonies without regard to source (see section 1.2.2.1.1. 'Classical objective mode' of the *BEEBOOK* paper on estimating colony strength (Delaplane *et al.*, 2013)).
17. Measure the beginning quantity of sealed brood to calculate beginning mite populations.
    This is done by overlaying on each side of every brood comb a grid pre-marked in cm^2 and visually summing the area of sealed brood (Fig. 18).
18. Convert the area (cm^2) of sealed brood to cells of sealed brood by multiplying cm^2 by the average cell density per cm^2 (see section 1.2.2.1.1. 'Classical objective mode' of the *BEEBOOK* paper on estimating colony strength (Delaplane *et al.*, 2013)).
    Cell density per cm^2 varies by geography; in the south-eastern USA conversion factors between 3.7 and 3.9 are used (Delaplane and Harbo, 1987; Harbo, 1993). This value should be verified with the honey bee lineage used for the experiment.
19. Measure incipient mite population in brood by opening at least 200 cells of sealed brood per experimental colony and examining under strong light and magnification to see and count sclerotized mites (see section 4.2.3.2. 'Infestation rates of brood').
20. Multiply the mite/cell density by cells of sealed brood to obtain the mite population in cells.
21. Sum phoretic mites + mites in cells to obtain the beginning total mite population.
22. Take corrective action should initial measures expose outliers in initial populations of bees, brood, or mites.
    In general, corrections aimed at minimizing experimental error are permissible until the point at which treatments are begun.
23. Once colonies are established and queens released and confirmed laying, it is recommended to collect one or more beginning relative mite measures. The most commonly recognized relative measures are mites recovered per 24 h on bottom board sheets (see section 4.2.4. 'Natural mite fall') and mites per adult bee recovered from samples (as described in section 4.2.3.1.2. 'Dislodging mites from bees'). It is to be hoped that these relative measures will correspond closely to real colony mite populations, and the investigator is encouraged to check the validity of the relative measures with regression analyses against total mite population.
24. Colony maintenance should include control of non-target diseases and disorders, swarm prevention, and feeding as necessary.
    The goal of these manipulations is to decrease experimental residual error.

4.4.1.2 Experimental treatments ,sample size, and colony arrangements

1. Create a range of varroa colony densities by treating sets of colonies with acaricide at different points of a season, bracketing as widely as possible the months of bee activity particular to one's region. At the very least, one treatment should be early in the season when bees are emerging from winter senescence; one treatment should be at the peak of the active season, and another should occur in autumn which is typically the period of highest ratio of mites : bees. More intervals will improve the resolution of the resulting model. It is imperative that the design include a negative control – a set of colonies left untreated, and it is highly recommended that the design include a positive control – a set of colonies treated continuously (Table 3). Including both controls will provide the widest range of varroa densities possible within which the investigator can retrospectively search for mite densities that are damaging or non-damaging.
2. Use an average sample size (initial number of colonies per treatment) of 11.
   The literature indicates a range 7-20. Studies using sample sizes within this range never failed to detect treatment effects for at least some dependent variables (Table 3).
3. Stick to one mite control product.
   This avoids the risk of experimental confounding error due to variation in acaricide efficacy or unknown sublethal effects on host bees (investigators sometimes included different acaricides in their treatments, apparently with a view to fine-tuning control recommendations for their region).
4. Take into account resistance of mites to acaricides when choosing the treatment.
   Whether the population is resistant to a particular product can be tested following the method described in section 3.6.3. 'Bioassays to quantify the susceptibility of the varroa mite to acaricides'.
5. Control or at least monitor colony spatial arrangement.
   Varroa mites can spread horizontally through infested workers and drones drifting between colonies (Greatti *et al.*, 1992; Frey *et al.*, 2011) and exert a strong influence on results. Depending on one's objectives, one can set up apiaries to encourage drift (assign treatments within the same apiary) or discourage drift (assign treatments by apiary). The first scenario acknowledges that immigration may confound results, yet this condition is presumed more "real world" because modern beekeeping often encourages mite horizontal transmission with high-density apiaries. This option is however not relevant if treatments are made with persistent active ingredients that are distributed by contact between bees (e.g. fluvalinate). Drifters could contaminate colonies with different

Table 3. Experimental treatments, sample size, and colony spatial arrangements recommended and found in the literature on field-derived damage thresholds. [a] All studies cited here were performed in the Northern Hemisphere, so early season is Feb-May and autumn Sep-Oct; [b] initial colonies per treatment; [c] Delaplane and Hood (1997); [d] Delaplane and Hood (1999); [e] fluvalinate year 1 and thymol year 2; [f] Delaplane *et al.* (2010); [g] Strange and Sheppard (2001); [h] 10 each for May, 7 for Sep; [i] Currie and Gatien (2006).

Experimental treatments[a]	***n*[b]**	**Colony spatial arrangement**	**Reference**
1. acaricide X early in the season 2. acaricide X at peak of season 3. acaricide X end of season 4. untreated colonies (negative control) 5. acaricide X continuous treatment (positive control)	12	according to objective (see step 5, section 4.4.1.2.)	recommended
1. fluvalinate Jun. 2. fluvalinate Aug. 3. fluvalinate Oct. 4. no treatment	18	divided equally between 2 states (ca. 120 km apart), treatments applied by apiary within state (minimize drift effect)	c
1. fluvalinate Feb. 2. fluvalinate Aug. 3. fluvalinate Feb.+Aug. 4. continuous fluvalinate 5. no treatment	12	divided equally between 2 states, treatments applied within apiary within state (maximize drift effect)	d
1. fluvalinate Feb. 2. fluvalinate Aug. 3. fluvalinate Feb.+Aug. 4. continuous fluvalinate 5. no treatment	8	divided equally between 2 states, treatments applied by apiary within state (minimize drift effect)	d
1. continuous treatment[e] beginning Jun. 2. treatment Aug. 3. treatment Oct.	20	divided equally between 2 states, treatments applied within apiary within state (maximize drift effect)	f
1. fluvalinate Apr. 2. fluvalinate Aug. 3. fluvalinate Oct. 4. fluvalinate Apr.+Oct. 5. continuous fluvalinate 6. no treatment 7. coumaphos Apr.	8	divided equally among groups (circles) of 8, each circle 15 m apart (minimize drift effect)	g
1. fluvalinate May 2. fluvalinate Sep. 3. 4 formic 4 d apart May 4. 4 formic 4 d apart Sep. 5. 4 formic 10 d apart May 6. 4 formic 10 d apart Sep. 7. coumaphos May 8. coumaphos Sep. 9. no treatment	10 or 7[h]	divided equally between 2 apiaries 8 km apart, within apiary colonies further subdivided into "low" initial varroa density or "moderate" initial density	i
1. fluvalinate May 2. 5 formic 1 week apart May 3. 1 formic slow release May	7	treatments applied within 1 apiary of "high" initial varroa density	i

treatment regimes (Allsopp, 2006). The second scenario, in contrast, gives a more uncluttered description of the effects of delayed mite treatment. Including both conditions permits a test of the assumption that thresholds occur earlier under conditions of high mite immigration. In either case, it is recommended that the objective be explicit and the spatial arrangement designed accordingly: 1. if immigration is to be minimized then assign all colonies within one apiary the same treatment and space apiaries as widely apart as possible; or 2. if immigration is to be maximized then assign all treatments within the same apiary. Other drift-minimizing practices, such as painting symbols at hive entrances or arranging colonies in circles, will not substitute for wide spatial distances between colonies of different treatments.

4.4.1.3 Dependent variables and sampling protocols

A variety of parameters is measured to relate to damage thresholds of the colonies hosting different parasite population sizes (Table 4). For some of the most invasive measures, i.e., measures of bees or brood (Table 4), it is common during the mid-experiment sampling intervals to sample only a subset of colonies in each treatment. These numbers have ranged from 3 (Delaplane and Hood, 1999) to 4 (Delaplane and Hood, 1997) to 2 (Strange and Sheppard, 2001). It is important to remember that these mid-experiment measures are made not in pursuit of statistic differences, per se, but to provide a retrospective snapshot of colony condition at time of treatment or when threshold was achieved. They will also slightly increase within group variability of the parameters measured. All colonies are dismantled and fully measured at the end of the study when statistical rigor is desirable to identify the treatment regimens that optimized colony condition.

Fig. 19. The whole hive is weighed in the field to obtain final colony bee population. Photo: Keith Delaplane.

1. Place bottom board sheets under a wire mesh and positioned under the colony (see section 4.2.4. 'Natural mite fall').
2. Remove the sheet after 24-48 h.
3. Count number of mites and adjust number to mites recovered per 24 h.
4. Collect samples of 300-900 adult bees.
5. Count the number of mites and number of bees (see section 4.2.3.1. 'Infestation rates of adult bees').
6. Report the data as mites / 100 bees.
7. Derive fresh bee weight (mg) by sampling live bees off the comb into pre-weighed, or tared, containers, weighing the container, subtracting container weight to get net bee weight, counting bees, and dividing by net bee weight to get mg per bee.

   This sampling can be combined with sampling for mites per adult bee as described above.
8. Obtain ending colony bee population according to the methods described under section 1.2.2.2. 'Measuring colony strength at end of experiment: objective mode' of the *BEEBOOK* paper on estimating colony strength (Delaplane *et al.*, 2013), or from a variation from this method using net colony bee weight (kg) and average fresh bee weight (mg):
   - 8.1. Obtain net colony bee weight by first closing the colony entrance with a ventilated screen in the evening or early morning before sampling to trap all bees inside.
   - 8.2. Weigh the whole hive in the field (Fig. 19).
   - 8.3. Open the hive.
   - 8.4. Brush all bees off every comb and surface (usually into a temporary holding hive).
   - 8.5. Re-weigh the hive without bees.
   - 8.6. Calculate the difference in weight, which is the net weight of bees.
   - 8.7. Divide this number by fresh bee weight to derive colony bee population.
9. Count the number of sealed brood cells as described in section 4.4.1.1., steps 17 and 18.
10. Colony mite population is derived from the methods described in section 4.4.1.1.

Table 4. Dependent variables and sampling protocols recommended and employed in the literature on field-derived damage thresholds. Months have to be considered according to the season at the location of the study. [a] Delaplane and Hood (1997); [b] Delaplane and Hood (1999); [c] fluvalinate year 1 and thymol year 2; [d] derived from regression predictions based on sticky board mite counts; [e] Delaplane *et al*. (2010); [f] Strange and Sheppard (2001); [g] Currie and Gatien (2006).

Experimental treatments	**Dependent variables measured in each treatment group**	**Sampling intervals**	**Reference**
1. acaricide X early in the season 2. acaricide X at peak of season 3. acaricide X end of season 4. untreated colonies (negative control) 5. acaricide X continuous treatment (positive control)	1. mites / 24 h on bottom sheets 2. mites / 300 bees 3. fresh bee weight (mg) 4. colony bee populations 5. number sealed brood cells 6. colony mite populations 7. visible brood disorders and other diseases 8. colony honey yield (kg) 9. subjective "survivability" score at beginning of the season of following year	at regular intervals in the year at mid-season	recommended
1. fluvalinate Jun. 2. fluvalinate Aug. 3. fluvalinate Oct. 4. no treatment	1. mites / 18 h on sticky sheets 2. mites / 300 bees 3. fresh bee weight (mg) 4. colony bee populations 5. number sealed brood cells 6. colony mite populations 7. visible brood disorders (Dec only)	Jun., Aug., Oct., Dec.	a
1. fluvalinate Feb. 2. fluvalinate Aug. 3. fluvalinate Feb.+Aug. 4. continuous fluvalinate 5. no treatment	1. mites / 20 h on sticky sheets 2. mites / 300 bees 3. fresh bee weight (mg) 4. colony bee populations 5. number sealed brood cells 6. colony mite populations 7. visible brood disorders (Sep.-Oct. only) 8. subjective "survivability" score (following Jan only)	Feb., May., Aug., Sep.-Oct.	b
1. continuous treatment[c] beginning Jun. 2. treatment Aug. 3. treatment Oct.	1. mites / 100 bees 2. fresh bee weight (mg) (Dec only) 3. colony bee populations 4. colony weight (kg) (Dec only) 5. cm^2 brood (all stages) 6. colony mite populations[d] 7. % bees infected with *Acarapis woodi* (Dec. only)	Aug., Oct., Dec.	e
1. fluvalinate Apr. 2. fluvalinate Aug. 3. fluvalinate Oct. 4. fluvalinate Apr.+Oct. 5. continuous fluvalinate 6. no treatment 7. coumaphos Apr.	1. mites / 20 hr on sticky sheets 2. mites / 300 bees 3. fresh bee weight (mg) 4. colony bee populations 5. number sealed brood cells 6. colony mite populations	Jun., Aug., Oct., Nov., Apr.	f
1. fluvalinate May 2. fluvalinate Sep. 3. 4 formic 4 d. apart May 4. 4 formic 4 d. apart Sep. 5. 4 formic 10 d. apart May 6. 4 formic 10 d. apart Sep. 7. coumaphos May 8. coumaphos Sep. 9. no treatment	1. mites per bee 2. colony honey yield (kg) (Aug. only)	At pre-treatment (May and Sep.), then post-treatment weekly for 3 weeks (spring) then biweekly thereafter	g

Once the investigator knows ending bee population, phoretic mites per bee, number of sealed brood cells, and mites per sealed brood cell, then one can extrapolate to (phoretic mite population + mite population in brood) = total colony mite population.

11. Quantify visible brood disorders.
    Brood disorders are sometimes associated with varroa parasitism (Shimanuki *et al.*, 1994) and can contribute to colony damage.
    11.1. Select two relatively contiguous patches of brood in the late larval – capped stages (stages more likely to express visible symptoms).
    11.2. Overlay on each patch a 10-cm horizontal transect and 10 cm vertical transect intersecting at the centre (Fig. 20).
    11.3. Examine along each transect every cell of brood under strong light and magnification for visible disorders, i.e., symptoms typical of American foulbrood (see the *BEEBOOK* paper by de Graaf *et al.*, 2013), European foulbrood (see the *BEEBOOK* paper by Forsgren *et al.*, 2013), sacbrood (see the *BEEBOOK* paper on honey bee viruses by de Miranda *et al.*, 2013), or chalkbrood (see the *BEEBOOK* paper on fungal diseases by Jensen *et al.*, 2013).
    11.4. Report the parameter as percentage of brood expressing visible disorders.
12. When honey yield needs be considered as a parameter of the economic threshold, calculate colony yield (kg) by weighing honey supers before and after they are placed on colonies during a nectar flow.
13. Measure the infestation rate by *Acarapis woodi* in the colonies. This rate could be affected by the presence of varroa and its measure might be needed as economically relevant. For this sample collect 50 workers per colony and place in alcohol. See the *BEEBOOK* paper on tracheal mites (Sammataro *et al.*, 2013) for the method to detect the presence of these mites.

4.4.1.4 Analyses, interpretation, and pitfalls

The designs featured in this section lend themselves to a straightforward analysis of variance testing the effect of date of treatment on colony strength parameters at the end of the study (Table 4). Depending on the presence of higher-order replications such as blocks, the investigator should be alert to interactions between treatments and the blocking factor which, if present, prescribe that the investigator test main treatments separately by block. The number of surviving colonies by the end of the study (n) may differ across treatments, so it may be necessary to accommodate unequal sample sizes through use of harmonic means transformation or lsmeans. Treatment means are separated ($\alpha \leq 0.05$) by a conventional test such as Tukey's or Student-Newman-Keuls. If the investigator procured whole colony mite populations (see step 10 of section 4.4.1.3.) along with more user-friendly relative measures such as varroa board counts (see section 4.2.4. 'Natural mite fall') and mites per 100 bees (see section 4.2.3.1.2. 'Dislodging mites from bees'), then it is desirable to test the rigor of the relative measures at predicting real mite populations through the use of regression analyses testing linear, quadratic, and cubic terms. Ultimately, the investigator would like to deliver to beekeeper clients a user-friendly relative measure that accurately predicts real colony mite populations.

Fig. 20. Selecting and inspecting brood cells for diseases.
Photo: Keith Delaplane

This analysis will permit the investigator to compare end-of-season colony condition across the various treatment regimens (times of acaricide application). The damage threshold is determined retrospectively as the highest average colony mite density at time of treatment associated with colony condition significantly non-different from positive controls at season's end. In one real example, the threshold was defined as conditions that prevailed when colonies were treated in August because August-treated colonies fared as well statistically at season's end as colonies treated continuously (Delaplane and Hood, 1999). The mid-season samplings permit the investigator to describe mite populations, user-friendly relative mite measures, and colony strength parameters that prevailed at the time thresholds were achieved. The highest, rather than lowest, retrospective mite density is used because of the conservative emphasis of IPM on prolonging the interval between treatments as long as possible. A low or zero pest tolerance is rare, unnecessary in the varroa / *Apis mellifera* IPM system, and more commonly associated with cropping systems for which pest-induced cosmetic damage is a problem with consumers.

This analysis will likewise identify mite densities that are irrecoverably damaging, in other words, mite densities at which point in time treatment does not prevent comparative colony deterioration by the end of the study. In another real example, it was shown that mite densities that prevailed in October exceeded a recoverable level because at season's end the October-treated colonies were in

significantly worse condition than continuously-treated colonies (Delaplane and Hood, 1997).

Alternatively, Strange and Sheppard (2001) defined damage threshold as: 1. the mite levels corresponding to colony treatment groups at season's end with weight of bees less than initial starting levels (0.92 kg in this case); and 2. colonies with < 1150 cm^2 sealed brood – a number derived from regression analyses predicting the amount of brood that should be present in colonies with 0.92 kg bees. With these boundary conditions the authors were able to retrospectively identify legacy mite levels that were either tolerable or irrecoverably high.

One pitfall in the field studies described here is a confounding effect, inherent to the design, between season (time of treatment) and colony mite densities. Mite population growth is regulated by length of brood-rearing season, ratio of worker brood to drone brood, and number of brood cells (Fries *et al.*, 1994) and tends to increase over the course of the active season. Delaplane and Hood (1997) pointed out this confounding issue when they said, "Thus, our treatment threshold is reliable for August colonies meeting the conditions described in [the table showing retrospective colony descriptions], but may not be reliable for August colonies with significantly different amounts of bees or brood." A more highly-resolved field model would replicate each of these terms independently within month of treatment.

Another pitfall comes from the emerging realization that honey bee morbidity is not always the product of a simple linear process or one factor, but rather a web of interacting factors (vanEngelsdorp *et al.*, 2009). More sophisticated damage thresholds are needed that can integrate more than one morbidity factor and account for their possible interactions.

Pros: allows the definition of damage thresholds as basis of IPM implementation.

Cons: high workload, tedious.

4.4.2. Regional variations in reported damage thresholds.

Table 5 gives some published field-derived damage thresholds and their geography of origin.

4.5. Standardising field trials

4.5.1. Starting conditions

4.5.1.1. Obtaining mite free colonies

Mite free colonies can be obtained from varroa free areas. These colonies will also be residue free since acaricides are not used. Obtaining such colonies is usually not possible so the varroa population of the experimental colonies needs to be removed using a highly effective control method adapted to the region in which the study takes place. The occurrence of resistant populations needs to be taken into account when choosing an acaricide for this purpose (see section 3.6.3. 'Bioassays to quantify the susceptibility of the varroa mite to acaricides' for methods to test for resistance). The efficacy of the treatment should be checked, as well as putative re-infestations from neighbouring apiaries (Greatti *et al.*, 1992).

Depending on the experiment planned, residues left behind by such treatment could bias the results by provoking delayed mortalities of mites. In such cases, residue free oxalic acid treatment can be used on swarms. No capped brood or frames with L5 larvae (see the section 'Obtaining adults and brood of known age' of the *BEEBOOK* paper on miscellaneous methods (Human *et al.,* 2013)) should be carried to the experimental hives in order not to bring in mites. Formic acid treatment can also be used on entire colonies with brood since the acid affects mites under the cell capping (Adelt and Kimmich, 1986; Koeniger *et al.*, 1987; Fries, 1991; Calis *et al.*, 1998). However, these two methods are only 95% efficient on average, which can influence the planed experiment. Oxalic and formic acid based products are available on the market and should be used as per manufacturer recommendations.

In such experiment, a control group of colonies treated continuously might be necessary. Such colonies need be separated from the experimental group since drifting and robbing bees could contaminate the test apiary (especially and mostly with synthetic acaricides; Allsopp, 2006). For the same reason, the control and experimental apiary need be separated by the same distance (~2km) from neighbouring uncontrolled apiaries. However, a compromise distance between control and experimental apiaries needs be found so that both are still subjected to equivalent environmental conditions.

4.5.1.2. Obtaining residue free hives

The presence of long lasting acaricide residues (Bogdanov *et al.*, 1998) in wax combs or honey can influence the results of experiments in which the survival of mites is a parameter of importance. Several methods to replace contaminated wax by residue free wax exist for beekeepers to switch to biological apiculture (Imdorf *et al.*, 2004). The method described here allows the decrease of residues below detectable levels within 1 year. This does not mean that all residues have disappeared, but that they have been diluted enough not to represent a problem for the quality of hive products. Whether the minimal quantities still present in the wax affect mite survival is however uncertain. This method based on comb removal can nevertheless be used to decrease the amount of residues in the wax for research purpose.

Complete removal of combs is best done at beginning of the bee season when comb is rapidly built by workers.

1. Split the colony.
2. Out of a split, create a broodless swarm with the old queen.

Table 5. Field-derived varroa damage thresholds and month of occurrence at various locations. [a]Delaplane and Hood (1999); [b]Delaplane *et al.* (2010); [c]Strange and Sheppard (2001); [d]Currie and Gatien (2006); [e]Gatien and Currie (2003).

Varroa damage thresholds and month of occurrence		**Location**	**Reference**
Feb.	Aug.		
1. colony mite population: 7-97	1. colony mite population: 3172-4261	Southeastern USA	a
2. 0.4 mites per 100 bees	2. 13 mites per 100 bees		
3. mites on overnight bottom board sheets: 0.6-10	3. mites on overnight bottom board sheets: 59-187		
	1. colony mite population: 1111 2. 20 mites per 100 bees	Southeastern USA	b
Apr.	Aug.		
1. 1 mite per 100 bees 2. mites on 48 hr bottom board sheets: 24	1. 5 mites per 100 bees 2. mites on 48 hr bottom board sheets: 46	Northwest USA	c
May	Late Aug. – mid Sep.		
1. 2 mites per 100 bees (to prevent honey loss)	1. 4 mites per 100 bees (to prevent winter loss)	Manitoba, Canada	d
	1. 5-8 mites per 100 bees	Manitoba, Canada	e

3. Scrape all propolis and wax, wash with soda and surface burn with a flame used hive parts to remove residues before introducing the colony; alternatively, use new hive parts.
4. Place swarm on residue free foundation (originating from location where no persistent miticides are used and originating from wax correctly sterilised by melting >121°C for >30 min).
5. Feed this first split.
6. In the other split, let the brood run out and a new queen be produced.
7. When the majority of the brood has emerged and the queen has started laying, remove all old combs.
8. Replace with residue free wax foundation.
9. Feed the second split.
   It is also possible to let bees build new combs from their own wax production rather than giving wax foundations.

Alternatively, the following can be done at the end of the bee season on whole colonies.

1. Trap the queen in a large cage made out of queen excluder allowing for the passage of workers.
2. Let the brood run out.
3. Scrape all propolis and wax, wash with soda and surface burn with a flame used hive parts to remove residues before introducing the colony; alternatively, use new hive parts.
4. Remove old combs.
5. Replace with residue free wax foundations.
6. Feed the colony.

Pros: decreases acaricide residues below detectable levels.

Cons: whether putative, but minimal residues remaining have a biological effect is unknown.

4.5.2. Artificial mite infestations

4.5.2.1. How many mites to introduce

The number of mites to introduce in colonies depends on the experiment performed. There are several factors to take into account:

- The statistical relevancy: a minimum number of successful infestations must be obtained (see the *BEEBOOK* statistics paper (Pirk *et al.*, 2013)).
- A higher number of mites introduced decreases the importance of resident residual/local mites.
- The infestation level depends on how long the colony should survive: the more mites are introduced, the quicker susceptible colonies might collapse.
- The method of introduction: introducing mites on the top of frames might result in high losses, but is easy. Alternatively, placing them on bees decreases this loss and allows a reduction in the number of mites used. Introducing mites in cells is a highly controlled method that requires few mites, but it is tedious.

- The rejection rate of mites by workers by grooming or hygienic behaviour.
- The sterility of some mites.
- The old age of mites of uncontrolled origin.
- The availability of mites.

In general, the number of mites to be introduced in experimental colonies should be overestimated to guarantee a sufficient sample size.

4.5.2.2. How to introduce varroa mites in colonies

There are two ways to obtain infested colonies: mites obtained from other colonies can be introduced or the existing mite population can be measured and the colony manipulated to obtained the desired infestation level. Bees can be taken out of a colony and the mite directly placed on its host. This can be done by pouring the collected mites on top of the workers in a cage or by picking mites up one by one with a paintbrush and placing directly on a worker. Time should be allowed for the mite to take refuge under the bees' abdominal plates before placing the latter back in its colony. This method is more efficient than dropping the mites onto the top of the frames since more mites can get attached on their host. Alternatively and if the level of infestation desired is not too different from the initial level of the colony, the latter can be split to obtain the desired level. If the level of infestation is above the desired level, brood combs (in which mites are trapped) can be removed.

4.5.2.3. How to introduce varroa mites in cells

An advantage of introducing mite directly in cells is to be able to monitor the events occurring in this particular cell. Cells can be manually infested or can be left to natural infestation if the infested cells can later be recognised. We here describe such artificial infestation methods.

4.5.2.3.1. Manual infestation

1. Using recently capped brood i.e. within 6 hours (see section ‘Obtaining brood and adults of known age’ of the *BEEBOOK* paper on miscellaneous methods (Human *et al*., 2013)) make a small hole in the side of the capping.
2. Introduce the mite using a fine wetted paint brush.
3. Close and reseal the hole by pushing the capping down. Workers will seal the hole when the frame is reintroduced in the colony. Using melted wax to prevent the mite escaping is not recommended since it could damage the fragile larva.
4. Mark the location of the cell on a transparent sheet placed above the comb.

This method needs practice. From an initial 20% acceptance of artificially infested brood, one can rapidly reach 80%. This rate is however variable according to colony and experimenter. The success rate can be checked by removing frame after few hours and verifying the status of the cell. Important: bees covering the combs used for artificial transfers must be carefully removed with a brush and not by shaking, which could damage pupae and mites. An opened and empty marked cell means that the workers removed the larva and the mite. Workers might also discard the old capping and reseal the cell without removing the larva. This can be recognised by a fresh capping deprived of cocoon layer. In this case the mite might have escaped or have been removed before resealing.

4.5.2.3.2. Natural infestation

Boot *et al*. (1992) designed a method that allows locating naturally infested cells. It is based on a one sided comb of which the cell walls where cut away from the bottom. The walls were then melted on a transparent sheet. These combs are consolidated by workers when replaced in the colonies and were accepted for oviposition by the queens. It might be necessary to cover the exposed side of the transparent sheet to prevent the bees building on it. Beetsma *et al*. (1994) also describe single rows of cells with two transparent walls that help locating and observing natural infestations.

4.5.3. Field bioassays of semiochemicals

Semiochemicals for which an effect on mite behaviour or physiology is proven in laboratory assays need to be tested under natural conditions in the hive. For example, semiochemicals involved in cell invasion and reproduction were tested with such method (Milani *et al*., 2004).

4.5.3.1. Cell invasion

In the case of the compounds affecting cell invasion (either attractants or repellents), field testing involves treating brood cells with the chemical under study and evaluating the number of mites that entered the cell after it has been sealed.

1. Dissolve the compound to be tested in 1 µl of de-ionised water or other appropriate solvent.
   The dose used for the field bioassay is normally the most active in the laboratory bioassay. Beware that the solvent might dissolve the wax of the cell walls.
2. Select a highly infested colony.
3. Identify cells containing L5 larvae (see the section on obtaining brood and adults of known age in the *BEEBOOK* paper on miscellaneous methods (Human *et al*., 2013)).
4. Apply the solution to these cells' walls with a 10 µl Hamilton syringe.
5. Treat an equal number of cells with 1 µl of solvent as a control.
6. Mark the position of the cells on a transparent sheet for subsequent tracking.
7. Open the sealed cells 18 h after treatment.
8. Inspect the cells for the presence of mites and count mites.

4.5.3.1.1. Data analysis

The proportion of treated and control cells that were infested are compared using the Mantel-Haenszel method after testing the homogeneity in the odds ratios of the replicated 2 × 2 tables. Any test that is suitable for comparing proportions could be used instead. However, if there are more replicates, using a certain number of cell each time, it is recommended to use a test that allows the analysis of strata. The number of mites in treated and control brood cells, in the hive bioassay, can be compared by a stratified sampled randomization test.

4.5.3.2. Mite reproduction

In the case of the compounds affecting mite reproduction, field testing involves treating brood cells with the chemical under study and evaluating both the fertility and fecundity of the mites reproducing in the cell.

1. Chose combs containing brood close to being capped.
2. Mark all the capped cells on a transparent sheet placed over the comb.
3. Replace the comb in the colony for two hours for workers to carry on capping cells.
4. Bring the combs to the laboratory after the two hours.
5. Dissolve the compound in an appropriate solvent.
   The dose used for the field bioassay is normally the most active in the laboratory bioassay.
6. Treat groups of freshly capped (unmarked) worker cells by injecting 1 µl of the solution with a 10 µl Hamilton syringe under the capping.
   Do not insert the syringe too deep into the cell to avoid hurting the larva. Beware that the solvent could dissolve the wax of the cell walls.
7. Treat an equal number of cells with 1 µl of the solvent as a control.
8. Choose groups of control and treated cells on both side of the comb, separated by at least one cell, which is left untouched to avoid contaminations.
9. Mark the position of the control and experimental cells on a transparent sheet placed over the comb.
10. Return the combs to the hive within 3 h.
11. Bring the comb to the laboratory 11 days later, when the bees are about to emerge.
12. Identify treated and control cells using the transparent sheet.
13. Count, uncap and inspect intact cells.
14. Note the condition of the infested pupae.
15. Collect mother mites and their offspring.
16. Mount on microscope slides and identify developmental stages as described in section 4.3.3. 'How to measure reproductive success'

In particular, the number of offspring and the number of mated daughters (i.e., the number of adult daughters in cells containing an adult male), are considered.

The effects of the solvent on the reproduction of *V. destructor* are studied by comparing the reproduction of mites in cells injected with 1 µl solvent and in sham-treated infested cells (syringe was introduced, but no solvent was injected). Proportions of reproducing mites out of the total mites found in cells are compared using G-tests (with the Williams' correction). The number of offspring and that of mated daughters per mother mite in treated and control groups can be compared using a two-sample randomisation test. The randomization distribution should be resampled an adapted number of times (e.g. 10^6 times).

4.5.4. Testing varroacides in the field

The European medicines agency has issued recommendations for the development of anti varroa treatment. These guidelines have been built on the knowledge accumulated by the Concerted Action 3686 (Commission of the European Communities European), which developed the commonly named 'alternative varroa control methods' based on the use of organic acids and essential oils. The aim of the guideline is to test and demonstrate the efficacy and safety of new miticides with the purpose of facilitating homologation. The original document (EMA/CVMP/EWP/459883/2008) should be consulted for legal issues and test for applicability of the treatment in various climatic regions. We here summarize and adapt the experimental design for research purposes at the local scale. Acaricides are considered efficient if the proportion of mites killed is at least 95% for synthetic substances and at least 90% for non-synthetic substances.

4.5.4.1. Preliminary tests

To facilitate and optimize efficacy test, it is recommended to perform dose finding and tolerance test on caged bees under controlled conditions in the laboratory. See section 3.6.3. 'Bioassays to quantify the susceptibility of the varroa mite to acaricides' and the *BEEBOOK* paper on toxicology methods (Medrzykci *et al.*, 2013). The highest concentration/quantity tolerated by the honey bees can be used as an indication for concentrations or quantities that can be used in subsequent dose-titration as well as dose-confirmation or field studies. Dose-titration studies should aim at identifying the minimum effective and maximum tolerated levels of active substance reaching bees and parasites and thus help establishing the dosage and dosing interval of the product. Implementation of dose-finding studies, carried out under controlled laboratory conditions is preferred, e.g. using 10 workers per cage, 3 cages per concentration, 3 untreated controls and one replicate, i.e. the studies should be carried out twice. See the *BEEBOOK* paper on toxicological methods (Medrzycki *et al.*, 2013).

Small scale outdoor pilot studies to confirm dose, efficacy and tolerance should be considered before large scale field studies are

performed. It is thus possible to validate the results obtained in the laboratory in a situation closer to that of the field, but with a high reproducibility since variables can be better controlled in these small units compared to full size colonies. It also allows for troubleshooting before the investment in the full scale test is done. A minimum of five untreated control and five treated test colonies should be used. To ensure reproducibility in the pilot studies, colonies should be comparable with respect to environment, type and size of hive, level of varroa infestation, treatment history, age of queen, relatedness of queens (sister queens can be used to decrease variability between replicates, in contrast, unrelated queens can be used to consider a wider range of genotypes), presence of brood, and age distribution of worker bees.

4.5.4.2. Efficacy tests

4.5.4.2.1. Statistical analysis

Primary and secondary outputs, hypotheses, and statistical methods should be specified and justified in a protocol before beginning the experiments. Sample sizes, in terms of hives per area for climatologically different regions (when relevant), should be large enough to provide sufficient statistical power. Whenever possible, results of the analyses should be accompanied by confidence intervals. Refer to the *BEEBOOK* paper on statistics (Pirk *et al.*, 2013) and to the CVMP guideline on Statistical Principles for Veterinary Clinical Trials (EMEA/CVMP/816/00).

4.5.4.2.2. Hives

1. Type of hives should be homogenized.
2. Ant protected varroa boards should be installed under the hives for mite counting (see section 4.2.4. 'Natural mite fall').
3. Temperature and relative humidity inside the hive(s) as well as exposure to solar radiation can be recorded, if they can influence the performance of the product.

4.5.4.2.3. Colonies

The following parameters should be taken into account:

1. Do not include weak colonies or colonies affected by diseases other than varroa in the study.
2. Equalize or randomize bee breed depending on the aim of the test regarding genetic diversity.
3. Select sister queens or unrelated queens of same age.
4. Measure and equalize colony strength (see the *BEEBOOK* paper on estimating colony strength (Delaplane *et al.*, 2013)).
5. Measure and equalize the amount of brood (see the *BEEBOOK* paper on estimating colony strength (Delaplane *et al.*, 2013)). Presence and type of brood is determined by the mode of action of the product. The tests should thus be performed in the absence of sealed brood, unless the product is intended to be effective on mites in capped cells.
6. Initial varroa infestation level should be high enough (> 300 mites per colony) to be able to measure mite drop. It should however be below damage thresholds (e.g. for central Europe: < 3,000 mites per colony, see also Table 5 and section 4.4. 'Estimating damage thresholds') and comparable between hives included in the study.
7. Treatment history should be similar for all colonies to equalize the effect of past treatments on the results (e.g. type and amount of acaricide residues present in the wax); when possible use residue free wax.

4.5.4.2.4. Location

1. Apiaries tested should be sufficiently distant from neighbouring apiaries to avoid disturbance and reduce risk for re-infestation. Type and availability of food sources should be recorded.
2. As a general principle, if studies are carried out at different apiaries, habitats should be comparable (access to similar forage and exposure to similar climatic conditions). If these conditions are not met the sample size should be adapted (number of apiaries increased) to take these differences into account.
3. Depending on the mode of dispersion of the product, control and test apiaries should be distant enough to prevent the contamination of control groups by the tested product by drifting foragers and drones or robbers. Synthetic acaricides have been shown to contaminate control hives placed in the same apiary (Allsopp, 2006)

4.5.4.2.5. Treatment

The following parameters should be defined, as determined in the preliminary tests:

1. Treatment period. Treatment should preferably be carried out at outdoor temperatures > 5°C.
2. Number of treatments, if more than one treatment is carried out.
3. Treatment intervals, if more than one treatment is carried out.
4. Include an untreated control group in the study to establish the effect of handling and of natural variations on the level of infestation and thus to confirm that a decrease in mite population size observed is indeed due the product under investigation.

4.5.4.2.6. Observations and parameters

Studies should encompass a pre-treatment, a treatment and a post-treatment period. Monitoring begins with the pre-treatment 14 days before the first treatment is carried out. The post-treatment period should extend > 14 days after the last treatment. These periods

encompass the pupal development time and allow taking into account the mites that are enclosed in the cells. The post-treatment period might need being prolonged, depending on the mode of action and persistence of the product tested.

4.5.4.2.6.1. Assessment of efficacy

1. Count dead mites on the bottom boards at regular intervals before, during and after treatment.
   The primary variable is mite mortality. During the treatment period dead mite counts should be carried out every 1-2 days given the high mortality expected. Pre- and post-treatment counts should be made 1-2 times per week depending on amount of mites falling, see section 4.2.4. 'Natural mite fall' and 4.2.5. 'Sub-sampling mite fall'. This allows the verification of efficacy since mite drop should peak during the treatment period.
2. Determine the amount of mites surviving the treatment with the product under investigation using a follow-up treatment with a chemically unrelated substance with > 95% documented efficacy.
   Follow-up treatment should be carried out in tested and control groups at the same time. This follow-up treatment should take place shortly after treatment with the test product, in order to keep the re-infestation level (and therefore the biasing of results) low when test apiaries and groups are not isolated by enough distance from neighbouring apiaries or hives. However, it is necessary to wait until mite drop returned to pre-treatment level in order to measure the full effect of the treatment and dissipation of the delayed mite mortality. This period is at least 14 days if the product kills mites in the cells or not. It is only after adult bee emergence that these mites will be released and fall on the bottom board or that they will get into contact with the product if the latter did not penetrate into the cell.
3. Count dead mites every 1-2 days in the week after follow-up treatment and 1-2 per week until mite drop returns to pre-treatment values.
4. Calculate treatment efficacy as follows:
   % mite reduction = (number of mites in test group killed by treatment x 100) / (number of mites in test group killed by treatment + number of mites killed in test group by follow-up treatment)
   Do not use data from colonies with abnormally high bee mortality in the efficacy evaluation.
5. Compare mite fall after treatment with untreated control to verify that the fall measured was not a natural phenomenon.

4.5.4.2.6.2. Assessment of safety of product for honey bees

1. Record bee mortality inside and adjacent to the hive daily or at least three times a week throughout the three stages of the experiment.
   The use of dead-bee traps is recommended (see section 2. 'Estimating the number of dead bees expelled from a colony' of the *BEEBOOK* paper on miscellaneous methods (Human *et al.*, 2013)).
2. Monitor the morbidity, mortality, as well as the size and development of surviving colonies at the time of the first flight in spring and thereafter (see the *BEEBOOK* paper on estimating colony strength (Delaplane *et al.*, 2013)) if applicable (envisaged therapeutic use in autumn or winter).
3. Measure flight activity of bees during the trial (see the *BEEBOOK* paper on behavioural methods.
   This verifies whether the product influences foraging activity of the treated colonies.
4. Measure honey production.
   This verifies whether the product influences the productivity of the treated colonies
5. Quantify brood area of test colonies during the three phases and compare to the control group (see the *BEEBOOK* paper on estimating colony strength (Delaplane *et al.*, 2013)).

In cases in which the product is intended for use in colonies with brood, the demonstration of safety for all stages of brood should be carried out (see the *BEEBOOK* article on toxicology methods by Medrzycki *et al.* (2013)). An additional method to determine effect of the product tested on brood is to determine which of feeding incompetence of worker bees and direct adverse effects on eggs and larvae occurred. For this:

1. Leave frames with eggs and larvae to develop in the hive until a chosen stage of the larval stage after applying therapeutic doses of the test product.
2. Monitor feeding behaviour of these larvae by measuring the amount of food found in their cells.
   By comparing development of brood and amount of larval food and taking into account the ratio between quantity of brood and number of worker bees between control and test groups, it is possible to differentiate between effects of feeding incompetence of worker bees and direct adverse effects on eggs and larvae after application of the product.
3. Verify the presence of the live queen at the end of the experiment.
   A significant difference in queen survival between test and control groups indicates an effect of the treatment.

4.5.4.3. Resistance pattern

The possibility of resistance emerging after several treatments should be monitored. The product applications should cover several reproductive cycles of the parasite to show the development of resistance and the rate of such development. Such studies can be performed under laboratory (see section 3.6.3. 'Bioassays to quantify the susceptibility of the varroa mite to acaricides') and/or field

conditions. Not only mites, but also bees might develop resistance against miticides after regular use for several bee generations. This translates in a change in dose-lethality relationship of the product or active substance(s) and therefore affects the safety of the product for bees which increases. See the *BEEBOOK* paper on toxicology methods (Medrzycki *et al.*, 2013) to evaluate acaricide toxicity for honey bees.

4.6. Breeding mites in colonies

A common problem for varroa research is obtaining mites in sufficient quantities for experiments. It is desirable to obtain mites already early in the season when their numbers in the colonies is still low and in large quantities for as long as possible thereafter. The method described here allows, within a short time, the regular harvesting of a high number of phoretic mites early in and throughout the season. The method is based on the trapping comb originally designed to control the mite (Fries and Hansen, 1993; Maul *et al.*, 1998). It consists in caging the queen from an infested colony, and letting all the brood emerge. Once the colony is broodless and all mites are in the phoretic stage, a comb of open brood is introduced. Just before capping, most of the phoretic mites looking for reproduction opportunities will enter the cells provided. Once the brood is capped, the comb is removed and placed in an incubator until the bees emerge. The newly emerged non-flying and non-stinging bees will be highly infested with varroa, making mite harvesting easy and fast. The infested comb can also be retrieved at any time to obtain mites at a particular developmental stage. This method is further developed to optimise logistical aspects according to the following protocol:

1. Prepare several hives as breeder colonies during the season preceding the experiments.
2. Adjust varroa treatment during the season preceding the experiments to ensure the survival of the colonies, but to also allow the survival of a relatively high number of mites over the winter.
   This makes it possible to keep a starter mite population for a fast growth in parasite numbers following the winter.
3. In the next year, when the colonies are well developed, the weekly natural mite fall is counted over a brood cycle (3 weeks).
4. Rank the colonies according to their mite load and strength. The most infested hives should be used first since they are susceptible to collapse before less infested colonies. The parasite population can still be left to grow in the less infested colonies until they are used for mite collection. Among several hives with the same range of infestation, those closer to swarming stage can be used first. This makes it possible to prevent swarming and the loss of mites.

The breeding cycle can start:

5. Day 1: cage the queen from the colonies selected for mite rearing.
   At day 22, all the brood present at day 1 will have emerged.
6. Day 12: prepare the trapping comb:
   6.1. Select a strong colony (brood provider) with an actively laying queen.
   6.2. Cage the queen on an empty dark comb (that queens prefer for egg-laying) and placed in the brood nest of her colony.
7. Day 13: after 24 h, remove the queen from the cage, but leave the comb in the cage to prevent further egg-laying by the queen.
   This comb contains brood of similar age in which varroa mites will later be trapped. To increase chances of obtaining enough brood for trapping, queens of several colonies can be caged and the comb with the most brood is used.
8. Day 19:
   8.1. Transfer the trapping comb that now contains 7 days old brood to the varroa rearing colony, of which the brood has emerged.
   8.2. Release the queen of the rearing colony so that she can resume her egg-laying activity.
9. Day 22: the brood cells of the trapping comb have been capped.
   9.1. Remove the comb from the rearing colony.
   9.2. Transport to the laboratory.
   9.3. Place in a well-ventilated bee tight box.
   9.4. Keep in an incubator at 34.5°C with 60-70% relative humidity until adult worker emergence.
   The comb should contain sufficient pollen and honey supplies so that the emerging bees can feed. Is it not the case, food should be supplied.
   Work on the rearing apiary should end with the collection of the trapping comb so that it does not remain for too long outside the colony before being placed in the incubator. To avoid damage to the brood transport should be done in a thermoregulated and moist container.
10. Day 33: Start collecting mites from the infested workers emerging this and the following day.
    For mite collection, bees can be held with forceps and the mites caught with a size 00 paintbrush or a mouth aspirator.

During the rearing cycle, the colony experiences 2 to 3 weeks without brood. After two subsequent brood cycles, the colony has usually regained strength and the varroa population will have increased again. Given that the varroa natural fall indicates a sufficiently large varroa population, the same colony can be used again to harvest mites. Furthermore, depending on the amount of mites needed, several colonies can be used at a time to increase the harvest. Breeding cycles on new colonies can be started every week. Thus, after 5 weeks, batches of mites can be harvested weekly. The

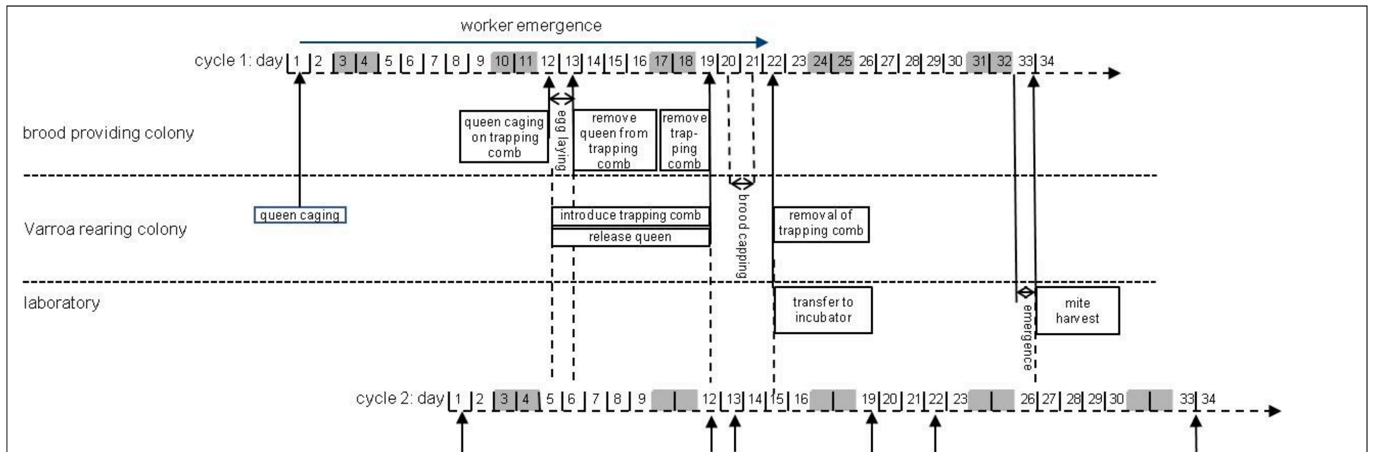

Fig. 21. Timeline of the rearing cycle. An additional cycle is depicted below the main timeline to illustrate how the various tasks (symbolized by arrows) can be combined between different rearing cycles to optimize the process.

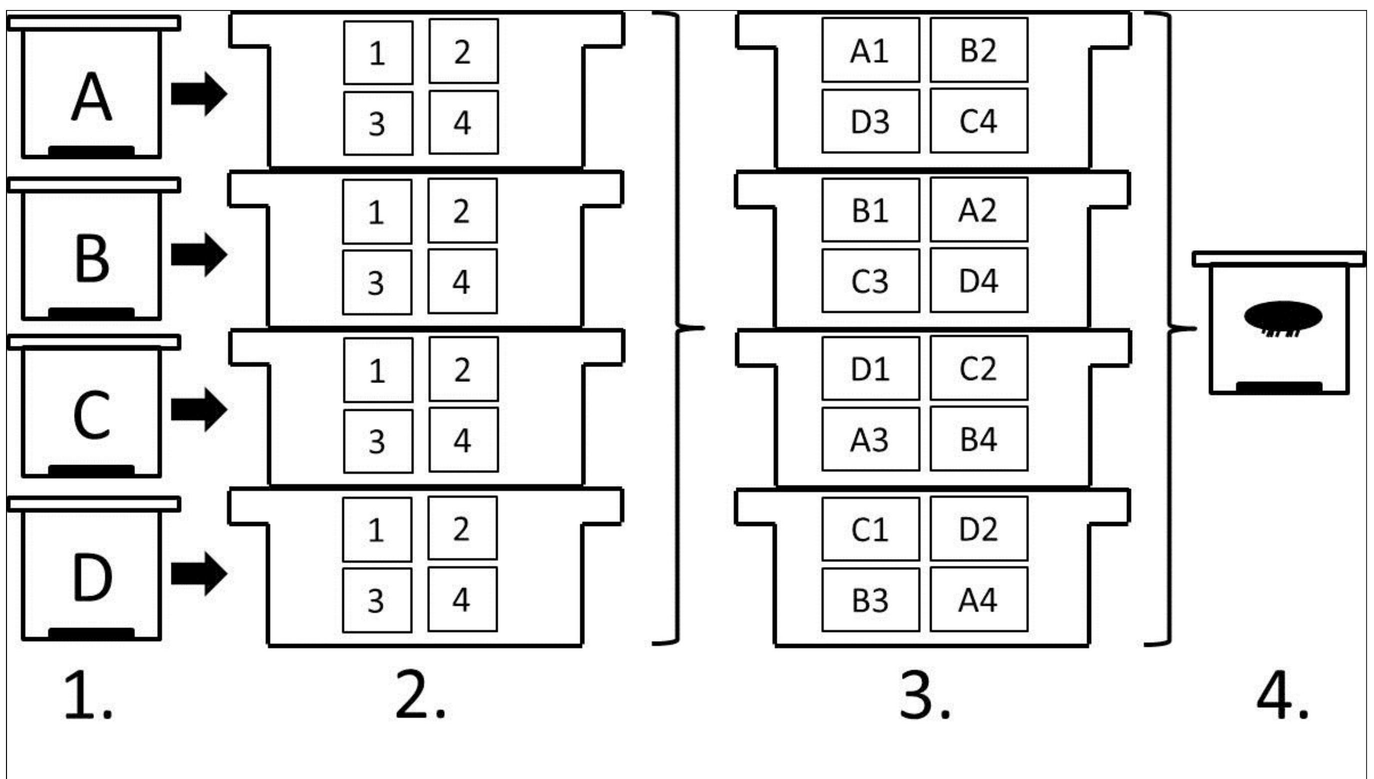

Fig. 22. A schematic of a method used to appraise varroa attraction to brood from different queen lines (modified from Ellis and Delaplane, 2001).

additional time axis shown at the bottom of Fig. 21 illustrates how most of the working days can be combined for colonies or groups of colonies at different stages in the cycle. By starting on a Thursday for example, no work on a Wednesday, Friday or weekend day is necessary and mite collection always occurs on a Tuesday.

Pros: the method allows the collection of mites indoors rather than on the apiary, prevents the danger of robbing by neighbouring colonies since the colonies do not remain open for mite sampling, necessitates few visits to the breeding apiary, allows the collection of mites on a particular day, facilitates sampling as the density of mite per emerging bee is high.

Cons: logistic intensive; if mites are collected from emerged workers older mother and young daughter mites are not separated.

4.7. Brood attractiveness

Varroa mites prefer drone over worker brood (Fuchs, 1990; Rosenkranz, 1993; Boot *et al.*, 1995). Honey bee lineages also vary in the attractiveness of their brood for the mites (Büchler, 1990; De Guzman *et al.*, 1995; Guzman-Novoa *et al.*, 1996). We here present a bioassay destined to compare brood attractiveness in comparable condition, i.e. in the same colony. This method is adapted from those of Ellis and Delaplane (2001) and Aumeier *et al.* (2002).

4.7.1. Procedure to test brood attractiveness

1. Queens from four different lines (Fig. 22, Step 1) tested are placed individually on a drawn, empty comb contained in a queen excluder cage.
2. Allow queens to oviposit for 24 h.
3. Twenty four hours later, the queens are removed from the combs, but the combs are left in the cages.
   This limits further queen oviposition in the target combs.
4. Leave each comb with eggs is in its respective line colony for 6 or 7 days (for worker / drone brood respectively).
5. After this, cut out of the comb squares or circles of comb containing L4 larvae (see the section on obtaining brood and adults of known age in the *BEEBOOK* paper on miscellaneous methods (Human *et al.*, 2013)) using a sharp knife or a metal spatula (Fig. 22, Step 2).
6. Take one section of larvae from each of the lines to be tested and randomly combine the sections of different lines in the centre of a fully drawn comb in which squares or circles of fitting sizes have been removed.
   Each frame now contains one brood section from each of the four genetic line colonies (Fig. 22, Step 3).
7. Prepare a varroa receiver colony (Fig. 22, Step 4) by removing the open brood in the colony to decrease target brood (the frame just created having the various queen lines represented on one comb) competition with the colony's own brood for the varroa present in the colony.
   Alternatively, the queen in the receiver colony could be caged 22 or 25 days earlier to let all worker and drone brood, respectively, emerge.
8. Introduce each reconstituted comb holding eggs from each genetic line into a receiver varroa infested colony (Fig. 22, Step 4).
   The colony can receive up to four frames generated from the procedure if the varroa populations are high enough. Mites invade worker cells from 15–50 h preceding cell capping (depending on the sex of the larvae).
9. Fifty hours post cell capping, remove the sections of capped pupae from the colonies.
10. If necessary chill in a freezer.
11. Assess varroa infestation rates (see section 4.2.3. 'Measuring the infestation rate of brood and adult bees').

Pros: brood attractiveness is assessed in comparable condition, i.e. in the same mite provider colony.

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