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REVIEW ARTICLE

Standard methods for tracheal mite research

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Summary

The honey bee tracheal mite (HBTM) *Acarapis woodi* (Rennie) (Acari: Tarsonemidae) is an obligate endoparasite of honey bees. First described from the Western (European) honey bee *Apis mellifera* L., this mite species was initially observed when honey bee colonies on the Isle of Wight, UK were dying between 1904 and 1919 (Rennie, 1921). Since then, this mite has been found in Europe, North and South America and parts of Asia, but its global distribution is not well understood. In this chapter, we outline protocols for collecting, detecting, identifying, diagnosing and measuring the infestation rates of *A. woodi*. We also describe methods to determine the damage threshold, outline several control measures, and describe methods for studying live mites.

Métodos estándar para el estudio del ácaro traqueal

Resumen

El ácaro traqueal de la abeja de la miel (HBTM son sus siglas en inglés) *Acarapis woodi* (Rennie) (Acari: Tarsonemidae) es un endoparásito obligado de la abeja. Fue descrito por primera vez en la abeja occidental europea *Apis mellifera* L. Esta especie se describió inicialmente en la mortandad de las colonias de abejas de la isla de Wight, Reino Unido, entre 1904 y 1919 (Rennie, 1921). Desde entonces, este ácaro ha sido encontrado en Europa, en el Sur y Norte de América y en zonas de Asia, aunque su distribución global aún no se comprende bien. En este capítulo, se describen los protocolos para la recolección, detección, identificación, diagnóstico y medición de las tasas de infestación de *A. woodi*. También se describen métodos para determinar el umbral de daño, se esbozan una serie de medidas de control, y se describen métodos para el estudio de ácaros vivos.

气管螨研究的标准化方法

摘要

蜜蜂气管螨（*Acarapis woodi* ）(Rennie) (Acari: Tarsonemidae)是一种蜜蜂的专一性体内寄生虫。该螨最早发现于西方蜜蜂（*Apis mellifera* L）上，当时正值1904至1919年间英国怀特岛上蜂群死亡期（Rennie, 1921）。此后，该螨发现于欧洲、南北美洲和部分亚洲地区，但它在全球的分布情况尚未充分探明。本章我们概述了气管螨研究中样品采集、检测、鉴定、诊断和感染率测定的实验方法。同时，我们介绍了测定损伤阈值的方法，概述了多种防治措施，并介绍了研究活体螨的方法。

Keywords: COLOSS *BEEBOOK*, *Acarapis woodi, Apis mellifera,* honey bee, research methods, protocol

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1. Introduction

Countries that export bees and bee products are required to conduct apiculture surveillance programmes to meet disease reporting and sanitary control requirements of the OIE (Office International des Epizooties) to facilitate international trade. A surveillance programme also aids in early detection of honey bee pests and diseases including any new introductions. One pest is the Honey Bee Tracheal Mite (HBTM) *Acarapis woodi* (Acari: Tarsonemidae), an obligate endoparasite of honey bees. This microscopic mite was discovered in 1919 in the UK (Rennie *et al.*, 1921). The identification and detection of the mite led to a law from the US Department of Agriculture restricting all live honey bee imports into the USA in 1922 (Phillips, 1923). Despite this restriction, HBTM was first seen in the USA by beekeepers in Texas in 1984 and the prairie provinces of Canada in 1985. Thereafter, *A. woodi* spread throughout the USA and most Canadian provinces, facilitated by commercial beekeepers transporting bees for pollination, and the sale of mite-infested package bees.

In addition, infested swarms, drifting bees, and the worldwide distribution of *A. mellifera* have contributed to the spread of this mite. Although its current range is not fully known, the HBTM has successfully been established in many countries in most continents, including Europe, Asia, parts of Africa, and North and South America (Ellis and Munn, 2005). To date, it is not known to occur in Australia, New Zealand or Scandinavia (Denmark *et al.*, 2000; Hoy, 2011). Recent work by Kojima *et al.* (2011a) reported *A. woodi* was found on Asian honey bees, *A. cerana japonica,* in Japan.

From what we currently know, *A. mellifera* is the original host of HBTM, as this mite has only been recorded on other *Apis* species following the introduction of *A. mellifera* to Asia. The exact causes of the loss of colonies infested with HBTM are still unknown. This problem is exacerbated by the lack of unique symptoms associated with tracheal mite.

2. Taxonomy and systematics

2.1. Taxonomy

Honey bee tracheal mites were first described by Rennie as *Tarsonemus woodi* in 1921, as parasites of the Western (European) honey bee *Apis mellifera* L. This discovery followed extensive colony mortality on the Isle of Wight and elsewhere in the UK between 1904 and 1919 when bee colonies began to die from unknown causes (Rennie, 1921). The initial suspicion that the "Isle of Wight Disease" was caused by tracheal mites (Hirst, 1921; Rennie, 1921) was never confirmed (Bailey, 1964). Hirst (1921) reclassified the species as *Acarapis woodi,* the name by which it is known today (Lindquist, 1986).

Table 1. Differential diagnosis of *Acarapis* species (Ritter, 1996).

Character	***A. dorsalis***	***A. externus***	***A. woodi***
Notch of the coxal plate	Deep	Flat to Short	Short
Space between stigmata	16.7 µm	16.8 µm	13.9 µm
Length of tarsal limb (IV leg pair)	7.6 µm	11.4 µm	7.5 µm

Its detection led to the restriction of all live honey bee imports into the USA in 1922 (Phillips, 1923). Despite this, the first report of colony losses from HBTM in the USA came from beekeepers in Texas in 1984. Thereafter, *A. woodi* spread to all states of the USA and most Canadian provinces. Their range expansion was facilitated by commercial beekeepers transporting bees for pollination, and from the sale of mite-infested package bees.

In addition to *A. woodi*, there are two external mite species in the genus *Acarapis* that infest honey bees. *A. externus* Morgenthaler is found on the cervix (the neck region) and *A. dorsalis* Morgenthaler is found on the dorsal groove of the thorax (Ibay and Burgett, 1989; Fig. 1. A, B; Table 1). They were considered to be harmless by Eckert (1961) and Delfinado-Baker (1984), but that may reflect a lack of information on these two *Acarapis* species. A third external species, *A. vagans* (Schneider), described from central Europe and New Zealand, was found principally on drones, on the basal part of the hind wing (Lindquist, 1986). However, other researchers considered this species to be *nomen dubium* and to date this issue has not been resolved (Delfinado-Baker and Baker, 1982; Lindquist, 1986).

Unfortunately, HBTM is now overshadowed by the ectoparasitic mite, *Varroa destructor* Anderson and Trueman. As a result, the presence of HBTM in some instances is not regularly investigated. When found, they are often at very low levels, perhaps due to the treatments used to control *V. destructor* (see Dietemann *et al.*, 2013).

The current taxonomy of HBTM, based on Krantz *et al.* (2009) is:

Kingdom: Animalia
Phylum: Arthropoda
Class: Arachnida
Subclass: Acari
Superorder: Acariformes
Suborder: Prostigmata
Order: Trombidiformes
Cohort: Heterostigmatina
Superfamily: Tarsonemoidea
Family: Tarsonemidae
Genus: *Acarapis*
Species: *woodi*

2.2. Identifying tracheal mites

Also see Section 3 for methods of collecting and identifying mites.

2.2.1. Mite appearance

Adult female tracheal mites have a pyriform (pear-shaped) body (Fig. 2), measuring 120 to190 µm long by 77 to 80 µm wide. Female mites can be distinguished by their stubby form and the presence of five setae on leg IV (Fig. 1C). Adult males are 125 to 136 µm long by 60 to 77 µm wide (Fig. 2). Leg IV of the males lacks all the tarsal structures. Both

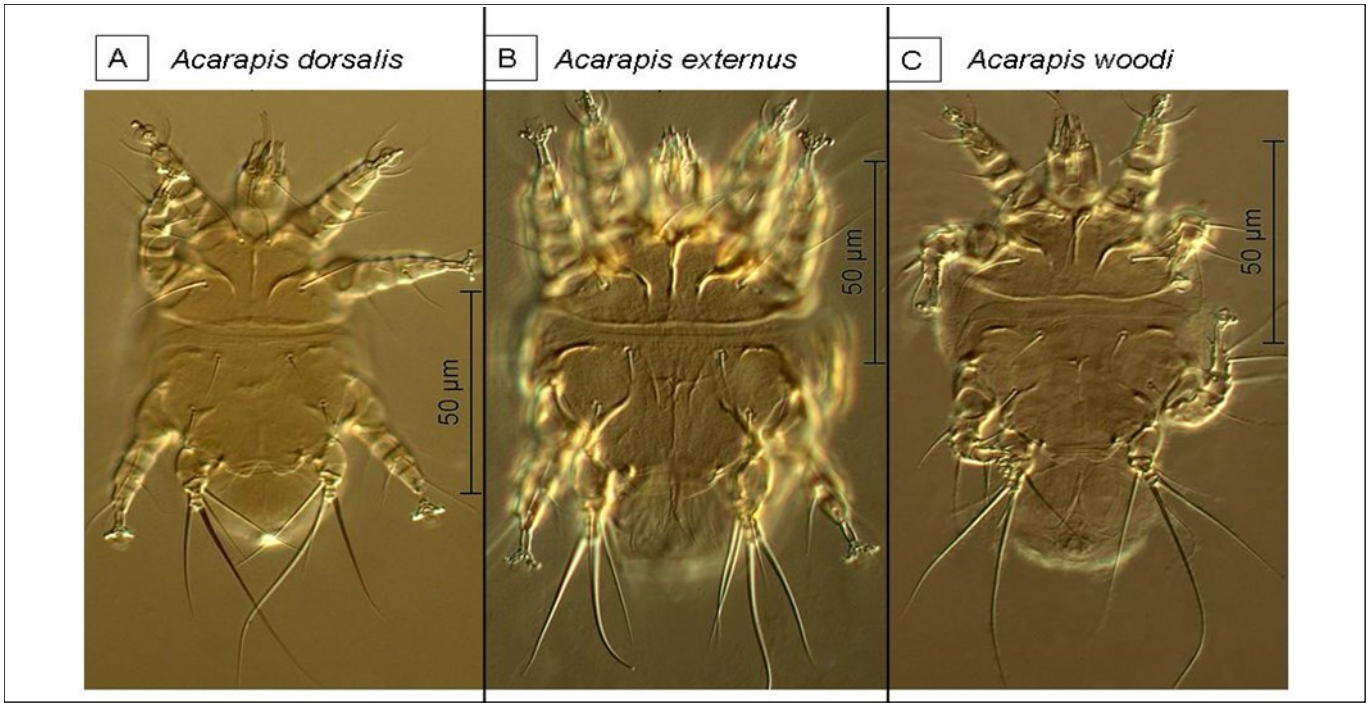

Fig. 1. A. Ventral view of *Acarapis dorsalis* (ex. New Zealand honey bee); ***B.*** *A. externus* (ex. New Zealand honey bee) and; ***C.*** *A. woodi* adult female (ex. Canadian honey bee) taken at a 400x magnification under light microscopy. Photos: Dr Qing-Hai Fan.

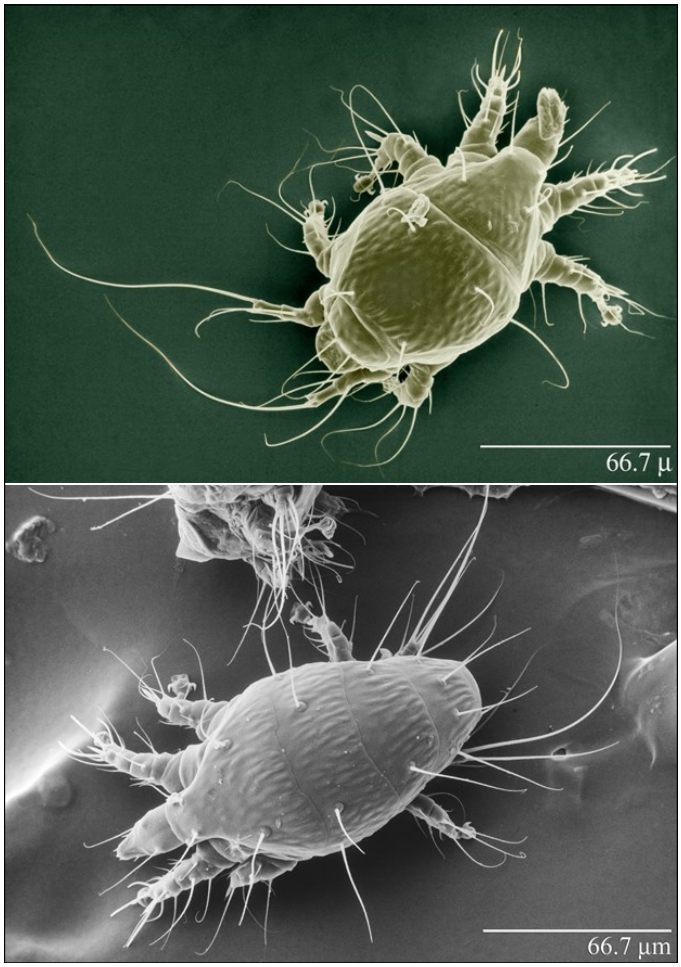

Fig. 2. LT-SEM micrographs of the dorsal view of a male (top) and female (bottom) of *A. woodi.* Photos: E Erbe and R Ochoa.

sexes have the dorsum covered with round to elongated bumps and an absence of bothridial setae on the prodorsal area (Fig. 2). The gnathosoma contains the mouth parts, palps and the chelicerae. Ventrally, the adults have an anterior apodeme forming a Y-shaped juncture with the prosternal apodeme (Fig. 1C). All tracheal mites are a semi glossy white colour. Immature mites have 3 pairs of legs and are bigger than the adult female (Figs 3 and 6C). Delfinado-Baker and Baker (1982), Lindquist (1986) and Ochoa *et al.* (2005) present detailed descriptions of the genus *Acarapis,* and in particular of *A. woodi.*

2.2.2. New tarsonemid mite associations

Other mites in the family Tarsonemidae have been reported in association with *Apis* bees. *Tarsonemus apis* was reported on *Apis* spp. by Rennie (1921) and *Pseudacarapis indoapis* was reported on *Apis cerana* Fabricius by Lindquist (1986). In 2003, Ochoa *et al.* reported a new mite, *Pseudacarapis trispicula* (Fig. 4), collected from the comb of live *A. mellifera* in Mexico. Females of *P. trispicula* are similar in size (length 173-214 µm, width 85-93 µm) to *Acarapis* mites. Studies in India found *P. indoapis* was only associated with colonies of *A. cerana* in areas where colonies of *A. mellifera* were also present (Sumangala, 1999; Sumangala and Haq, 2001). Abou-Senna (1997) reported the presence of *P. indoapis* on *A. mellifera* in Egypt. However, based on the information and drawing by Abou-Senna (1997), Ochoa *et al.* (2003) considered this association and the identification of the mite incorrect. The males, immatures and the feeding habits of *P. indoapis* have been described by Sumangala and Haq (2002), while males, immatures, and the feeding behaviour of *P. trispicula* remain unknown. Recently, *P. indoapis* were reported from both *A. cerana* and *A. mellifera* from Vanuatu and from China (Q-H Fan and S George, pers. comm. 2013; see Fig. 4).

2.2.3. Where to find mites

Acarapis woodi can hide under the flat lobe that covers the bee's first thoracic spiracle, accessing the main pro-thoracic tracheal trunk (Fig. 5). To look for or collect mites, bees must be dissected, see sections 3 and 4.

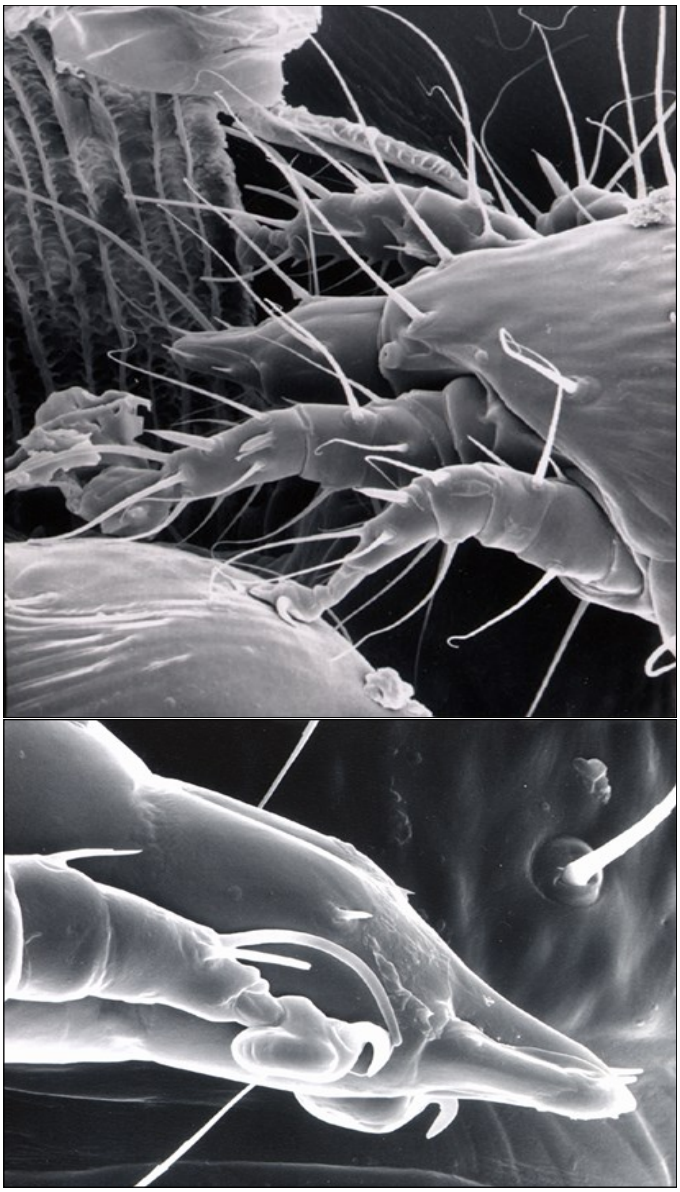

Fig. 3. SEM micrographs of anterior region of *A. woodi* female (top) and close-up of the larva (bottom). Photos: E Erbe and R Ochoa.

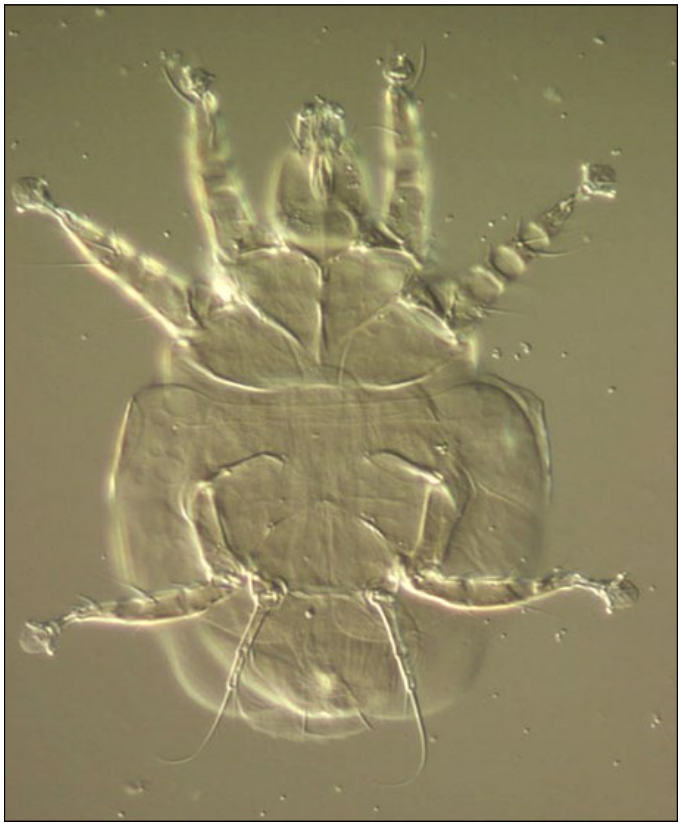

Fig. 4. *Pseudoacarapis trispicula* on *Apis mellifera*.

Photo: R Ochoa (400×).

2.2.4. Life cycle

The life stages are, egg, larva, and adult. The nymphal instar remains inside the larval skin (Fig. 5). Males complete their development in 11 to 12 days, females in 14 to 15 days; therefore, a new generation of mites can emerge in two weeks (Pettis and Wilson, 1996). All larval and adult stages of HBTM feed on bee haemolymph, which they obtain by piercing the walls of the trachea and air sacs with their sharply pointed stylets that move by internal chitinous levers (Hirschfelder and Sachs, 1952). Once the bee trachea is pierced, the mites' mouth presses close to the wound and bee haemolymph is sucked through the short tube into the pharynx.

All mite instars live within the tracheae and associated air sacs (Figs. 5, 6, 7, 8), except during a brief period when adult, mated females disperse to search for young (generally less than four days old) bee hosts. Mites are attracted to the outflowing air from the prothoracic spiracle and to specific hydrocarbons from the bees' cuticle (Phelan *et al.*, 1991; McMullan *et al.*, 2010) and immature stages may move into the trachea via air currents during bee respiration (Ochoa *et al.*, 2005). HBTM females are less attracted to older bees, which during the summer will usually not live long enough for the mites to complete their life cycle.

2.2.5. Mite dispersal

Once a suitable host is found (queens, workers or drones), the female mite enters a trachea via the spiracle to lay eggs. Drones have been found to have more mites than workers (Royce and Rossignol, 1991; Dawicke *et al.*, 1992), perhaps due to their larger tracheal trunks. Workers, however, which are more abundant through the year, are the prime host and reservoir for HBTM in bee colonies. Queens, even those commercially reared, often have HBTM. Camazine *et al.* (1998) found that infested queens weighed less, however, queens with completely black thoracic tracheae have been observed laying eggs and otherwise acting normally (D Sammataro, pers. obs.). Mites will also infest the air sacs of the bees' abdomen and head (Giordani, 1965), and can be found externally at the base of the bee's wings (Royce and Rossignol, 1991); the fate of the mites found in these areas and their effect on the host is unknown.

Female mites can disperse when the host bee is more than 12 days old, peaking at 15 to 25 days by questing on bee setae (Pettis and Wilson, 1996; Fig. 8); mites have a higher dispersal rate at night (Pettis *et al.,* 1992). Eggs and female HBTM have been found at the wingbase of the bee's thorax (Royce *et al.,* 1988). During this questing period, mites are vulnerable to desiccation and starvation, and their survival depends on the ambient temperature and humidity

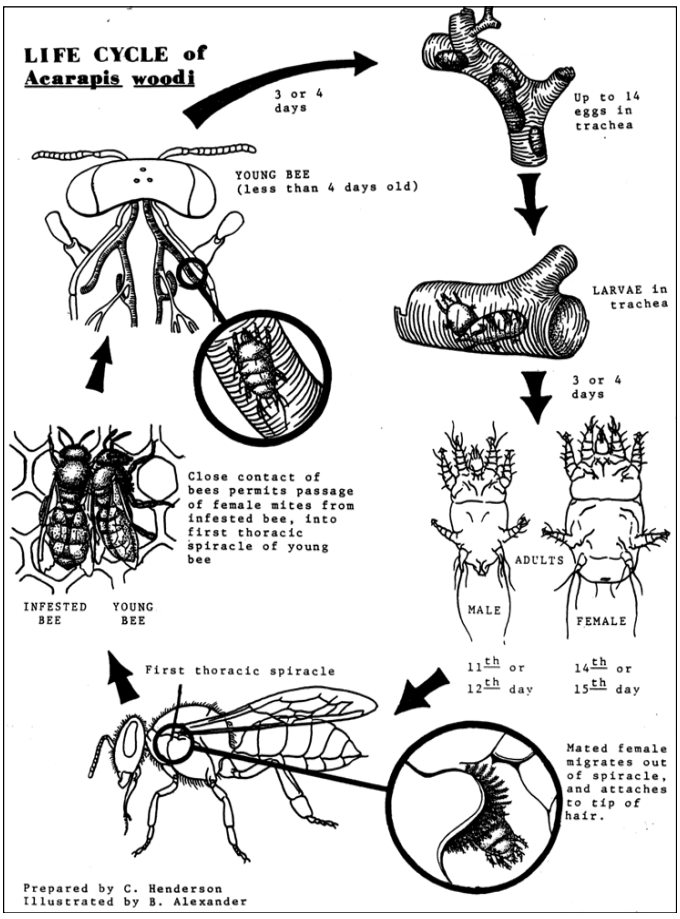

Fig. 5. Life cycle of tracheal mites. (Morse, 1991)

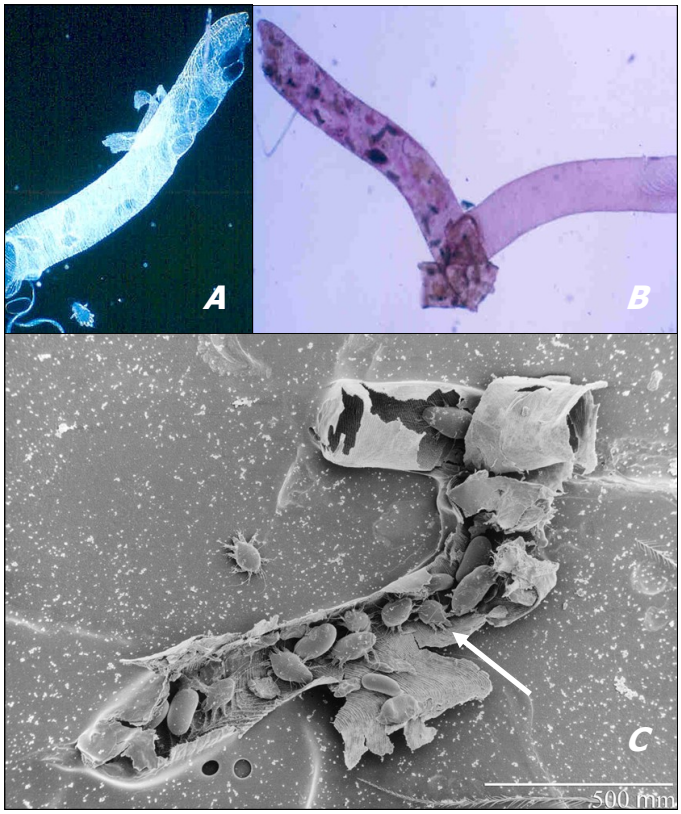

Fig. 6. A. Pro-thoracic trachea of a honey bee, filled with HBTM; ***B.*** Micrograph of stained tracheal tubes with mites (left) and clean, no mites (right) (Photos: D Sammataro, Light Stereoscope 400×); ***C.*** LT-SEM micrograph of an open tracheal tube showing female, male and immature mites and eggs. Note the smaller males further inside the trachea, arrowed. Photo: E Erbe and R Ochoa.

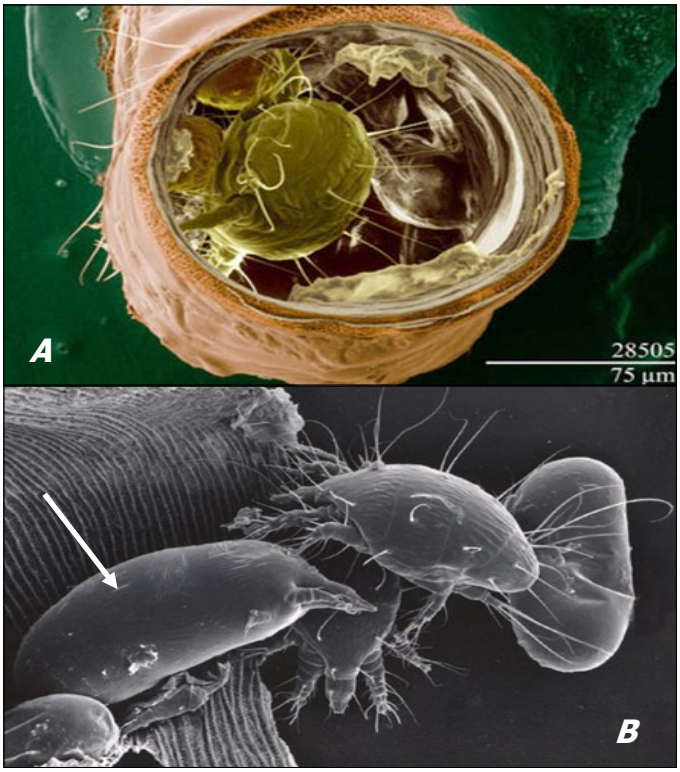

Fig. 7. A. View of interior of tracheal tube containing a female HBTM; the long setae on Leg IV are thought to be used to help measure the interior walls of tracheae (Ochoa *et al.*, 2005); ***B.*** Larval mite (arrow), adults and egg (far right). LT-SEM photos: E Erbe and R Ochoa.

(Giordani, 1962). An exposed mite will die after a few hours unless it enters a host; they are also at risk of being dislodged during bee flight and grooming (Sammataro and Needham, 1996; Sammataro *et al.*, 2000). In infested and crowded tracheal tubes, males move about and locate pharate nymphal females that are about to moult to adulthood and guard them in advance of mating (Ochoa *et al.*, 2005). The males do not attach to the immature stages as is common in other genera in the family Tarsonemidae (Ochoa *et al.*, 2005). Only the female HBTM go deep into the tracheal system, measuring the walls of the tracheal branches with their dorsal and ventral setae and the leg IV seta; see Fig. 7A (Ochoa *et al.*, 2005). The eggs are 5 to 15 µm longer than the adult females (see Fig. 7B).

The genotype of honey bees and the location of the colonies influence the levels of HBTM infestations. Buckfast, ARS-Y-C-1 (Yugoslavian) and Russian honey bees are known to be resistant to HBTM (Lin *et al.*, 1996; Danka *et al.*, 1995; de Guzman *et al.*, 2002, 2005). Heat is also associated with mite mortality (Harbo, 1993). Exposing hives to direct sun impedes HBTM mite population growth and shading them tends to accelerate it (L. de Guzman, unpub. data).

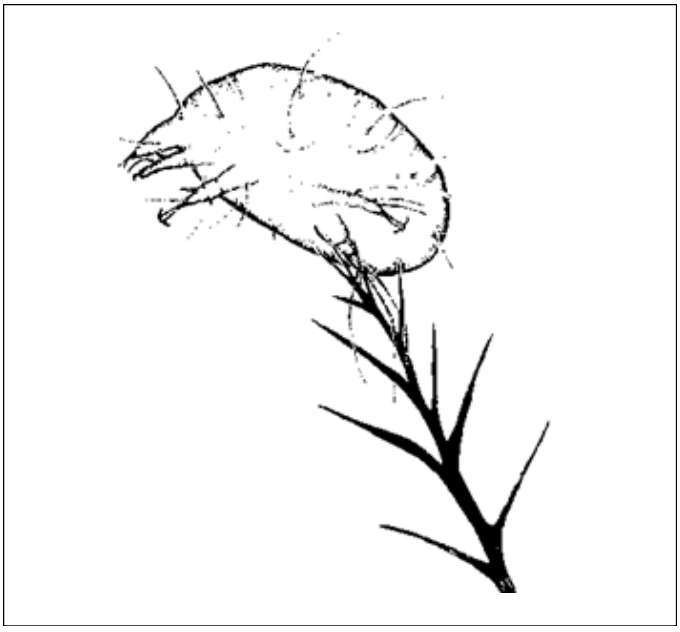

Fig. 8. A female tracheal mite questing on bee seta. Drawing: D Sammataro (Sammataro and Avitabile, 2011).

3. Collecting mites

Beekeepers often use unreliable bee and colony symptoms, such as dwindling populations, abandoned overwintered hives full of honey, or weak bees crawling on the ground, as symptoms of HBTM infestation. These symptoms are not dependable and are not recommended.

3.1. Field methods

In general, bees do not show symptoms that are reliable indicators of mite infestation. Tracheal mites affect the overwintering capability of bee colonies and have been associated with bees displaying disjointed wings (called 'K-wing') or crawling on the ground near hives. Crawling bees are only apparent during winter or early spring months, particularly when HBTM infestations are very high. With the widespread distribution of nosemosis (see Fries *et al.*, 2012) and some viral infections which may cause similar symptoms (de Miranda *et al.*, 2013), the presence of crawlers in front of colonies should not be used as a reliable indicator of HBTM infestation (Bailey, 1961; Bailey & Ball, 1991).

The only way to identify HBTM is to physically examine honey bees for mites. This section describes several sampling techniques to look for mites inside bees.

3.2. Sampling colonies

3.2.1. When to sample

When trying to detect tracheal mites, sampling time is crucial. Infestation by tracheal mites varies through time. For detection of HBTM in colonies, bees should be collected in winter or early spring when HBTM populations are highest because of the reduced bee brood production. During this time, a high proportion of older, overwintering bees is present in the colonies, and the mites have had a long time to reproduce. The large number of actively feeding mites can cause the tracheae to turn black. Infestation of HBTM decreases in summer due to the dilution of mite populations as they enter the large population of emerging host bees.

3.2.2. Collecting bee samples

Because HBTM infestations are influenced by the age of bees, the location within the hive from which bees are sampled should be considered. Since queens can be found on honey frames, it is recommended to examine frames of the entire colony to find the queen before taking any samples; this will ensure that the queen will not end up in the sample jar. Collect adult drones for sampling as well, as they tend to have higher mite abundance than worker bees (Royce and Rossignol, 1991; Dawicke *et al.*, 1992). However, because drones are seasonal, adult worker bees are most often sampled for detection or surveillance purposes.

Procedure:

1. Collect about 50 bees from frames in the honey super or from the inner covers where older bees congregate. Highly-infested, older bees will have darkened trachea and many stages of mites, although some of the younger female mites may have migrated out of the tubes.
2. If young nurse bees (found in the broodnest) are present, these young bees may only have foundress mites that just started reproducing. The presence of one foundress or a foundress and an egg near the opening of the trachea may be difficult to detect. Thus, to determine mite abundance (number of mites per bee) or mite intensity (number of mites per infested bee), it is best to sample bees from the honey and pollen regions of brood frames, where a good mixture of young and old bees is generally found.
3. Collect bees by using portable insect vacuums (Fig. 9) or by scooping bees with a plastic cup directly from the frames or inner cover.
4. Place samples into vials or plastic bags or sample directly into a wide-mouth jar containing 70% ethanol. Label each container or plastic bag with location, colony number and the date the samples were collected.

Fig. 9. Sampling bees for HBTM using a modified portable car vacuum, which collects bees directly into a plastic vial.

Photo of S Cobey by D Sammataro.

5. Although bees can be preserved in 70% alcohol, fresh or frozen bees are easier to dissect. Examination of tracheae is easier when no alcohol is inside them. Bees stored for a long time in alcohol will have darker muscle and tracheal tissues, making the mites harder to detect.
6. If molecular techniques are used for mite detection, bee samples should be stored in a refrigerator at 4°C or frozen at -80°C. Storage containers are similar to those used for varroa (see Dietemann *et al.*, 2013).

3.2.3. Shipping samples

If bees are to be used for molecular analysis, samples should be shipped overnight on dry ice. Otherwise, alcohol wet samples can be shipped through postal services (see similar for varroa: Dietemann *et al.*, 2013).

3.2.4. Number of bees to be examined to detect the presence of HBTM

About 30-50 bees are examined per colony in most studies. Because the tracheal networks on the two sides of the bees do not interconnect, this represents independent samples of 60-100 tracheae. There are different ways of determining the sample size needed to accurately detect tracheal mite infestation of a colony. Frazier *et al.* (2000) developed a sequential sampling technique which they validated twice by using two levels of significance (α = 0.10 and 0.20), and a precision level of β = 0.05 and 0.10 (Table 2). This technique allows one to classify low-infested (< 10%) and highly infested (> 10%) colonies. Such information is needed before deciding when to treat or not to treat a colony, and also if further sampling is necessary. This improved technique can save time and money since it requires fewer than 50 bees to reach a decision.

For example, when 3 bees are found infested after examining 3-7 bees, stop sampling and decide to treat the colony because it is highly infested. However, if only 1 or 2 bees are infested with the first 7 examined, continue dissecting samples. If after dissecting 17 bees only 1 bee is found infested, also stop sampling and declare the colony to be low-infested and therefore, no treatment is required. However, if 5 bees are infested out of 17 bees examined, then the colony is highly-infested and needs to be treated. If only 2, 3 or 4 bees are infested out of 17 bees, continue sampling (see Table 2).

The following equation developed by Cochran (1963) is another way of finding the number of bees that need to be sampled for each colony in order to get an accurate number of mites per bee and therefore if the colony needs to be treated:

$$n_0 = \frac{Z^2 pq}{e^2}$$

Where:

n_0 is the sample size needed,

Z^2 is the abscissa of the normal curve that cuts off an area at the tails (1 equals the desired confidence level). The value for Z is found in statistical tables which contain the area under the normal curve.

e is the desired level of precision (for example, setting it at 0.05 means that the sample size provides 95% certainty of detecting a 5% tracheal mite infestation level),

p is the estimated proportion of bees infested with tracheal mites,

q is 1-p.

Example: A colony has an expected infestation of about 5%. Using this equation to determine a sample size, we will have:

Z = 1.96; α (Alpha) = 0.05 (significance level)

p = 0.05 (5%, estimated proportion of bees that are infested)

q = 0.95 (1-0.05)

e (Beta, β) = 0.05 (95% precision level)

Substituting the values:

$$n_0 = \frac{1.96^2 * 0.05\ (0.95)}{0.05^2} = \frac{3.8416 * 0.0475}{0.0025} = 72.99 \text{ or } 73 \text{ bees}$$

If, on the other hand, infestation is estimated to be 10%, about 17 bees should be examined; an estimated 20% infestation only requires about 4 bees to be examined, since there is a higher percentage of bees infestated. This method as well as the sequential sampling technique may be useful for detection purposes (to determine when to apply treatments or for regulatory purposes) and is not recommended for scientific reporting. In that case, a full sample should be analysed (e.g. 50 or 100 bees) to determine mite prevalence (percentage of hosts infested) and/or mite abundance (number of mites per host bee). In general, tracheal mite infestations lower than 20% do not require treatment, but this depends on the severity and length of the winter months (when bees are confined in their hives).

3.3. Detection methods

Since these parasitic mites reside inside the tracheae, their detection requires specialized techniques, such as thoracic disc preparation and examination under a dissecting microscope. This is a laborious procedure. Molecular techniques are currently being developed (see section 3.4.3.) for processing bees in bulk, and should provide increased sensitivity, specificity and speed of screening bees for tracheal mites.

3.3.1. Laboratory detection: microscopic detection of *Acarapis woodi*

The morphological technique most frequently used involves examining the prothoracic tracheae under a microscope. Detection of low level infestation by *A. woodi* requires careful microscopic examination of tracheae. When the infestation is heavy, the trachea will turn opaque and discoloured and mites can be noticed without the aid of a microscope. One method is to pull off the head and collar of a bee and examine the trachea (Sammataro, 2006; see video of bee dissection at: http://www.ars.usda.gov/pandp/docs.htm?docid=14370).

Table 2. How to make decisions using the sequential sampling technique (modified from Frazier *et al.,* 2000); Calderone and Shimanuki 1992; see text 3.2.4. for explanation.

No. of bees examined	**Number of infested bees**		
	Low infestation (stop, don't treat)	High infestation (stop, treatment)	Moderate infestation (continue sampling)
1	-	-	0,1
2	-	-	0,1,2
3	-	3	0,1,2
4	-	3	0,1,2
5	-	3	0,1,2
6	-	3	0,1,2
7	-	3	0,1,2
8	-	4	0,1,2,3
9	-	4	0,1,2,3
10	-	4	0,1,2,3
11	0	4	0,1,2,3
12	0	4	1,2,3
13	0	5	1,2,3,4
14	0	5	1,2,3,4
15	0	5	1,2,3,4
16	1	5	1,2,3,4
17	1	5	2,3,4
18	1	6	2,3,4,5
19	1	6	2,3,4,5
20	1	6	2,3,4,5
21	1	6	2,3,4,5
22	1	6	2,3,4,5
23	2	6	3,4,5
24	2	7	3,4,5,6
25	2	7	3,4,5,6
26	2	7	3,4,5,6
27	2	7	3,4,5,6
28	3	7	4,5,6
29	3	8	4,5,6,7
30	3	8	4,5,6,7
31	3	8	4,5,6,7
32	3	8	4,5,6,7
33	4	8	5,6,7
34	4	8	5,6,7,8
35	4	9	5,6,7,8
36	4	9	5,6,7,8
37	4	9	5,6,7,8
38	4	9	5,6,7,8
39	5	9	6,7,8
40	5	10	6,7,8,9
41	5	10	6,7,8,9
42	5	10	6,7,8,9
43	5	10	6,7,8,9
44	6	10	7,8,9
45	6	11	7,8,9,10
46	6	11	7,8,9,10
47	6	11	7,8,9,10
48	6	11	7,8,9,10
49	6	11	7,8,9,10
50	7	11	8,9,10

3.3.2. Screening individual bees

When the level of infestation is low, tracheae from an individual bee need to be examined. Bees may be anesthetized or killed by freezing before examination. Milne (1948) developed a technique to locate the internal mites on individual bees (see Figs. 10 and 11 for details).

1. The bee is placed under a dissecting microscope, held prone with forceps (across abdomen) and the head and the first pair of legs is scraped off using a scalpel or razor blade.
2. The ring of prothoracic sclerite (collar) is also removed using a fine forceps.
3. The exposed tracheae of both sides are removed after carefully detaching them from the thoracic wall.
4. The tracheae are removed and placed on a glass slide and examined under a microscope for mites; this technique is very time-consuming and also has the possibility to lose mites while separating tracheae from the thoracic wall and transferring them to the slide.
5. Lorenzen and Gary (1986) modified this technique where the thoracic tergite was removed as a flap to look at mites *in situ* (see also Ritter *et al.,* 2013).

Liu (1995) developed a rapid technique to distinguish live mites from dead by staining with thiazolyl blue tetrazolium, which stains the live mites purple. The tracheae, after mounting on glass slides, are perfused with thiazolyl blue tetrazolium solution (5 mg stain in 5 ml distilled water). The cuticle of live mites picks up the stain immediately and turns purple, dead mites turn greenish yellow.

3.3.3. Sample preparation: morphological analysis and slide preparation

HBTM specimens should be preserved carefully for microscopic examination. Since these mites are weakly sclerotized, no clearing agents are need. The mounting medium listed below is recommended for mounting specimens.

Mounting medium (Hoyer's medium from Kranz and Walter, 2009)

50 ml of distilled water
30 g gum Arabic
200 g chloral hydrate
20 ml glycerol

Note: The ingredients should be mixed in the sequence listed above. Allow each solid ingredient to dissolve before adding the next one. The mixture needs to be warmed and stirred gently so the gum arabic can melt. Filter and store in airtight containers with rubber stoppers. Do not use a screw top container. ***Caution:*** chloral hydrate is a toxic chemical, a mutagen and a chromosome-damaging agent.

3.3.4. How to mount specimens

1. Place a drop of Hoyer's medium (section 3.3.3.) in the centre of a clean microscope slide.
2. Using the tip of a fine needle, minute pin or a wire loop, pick up a mite.

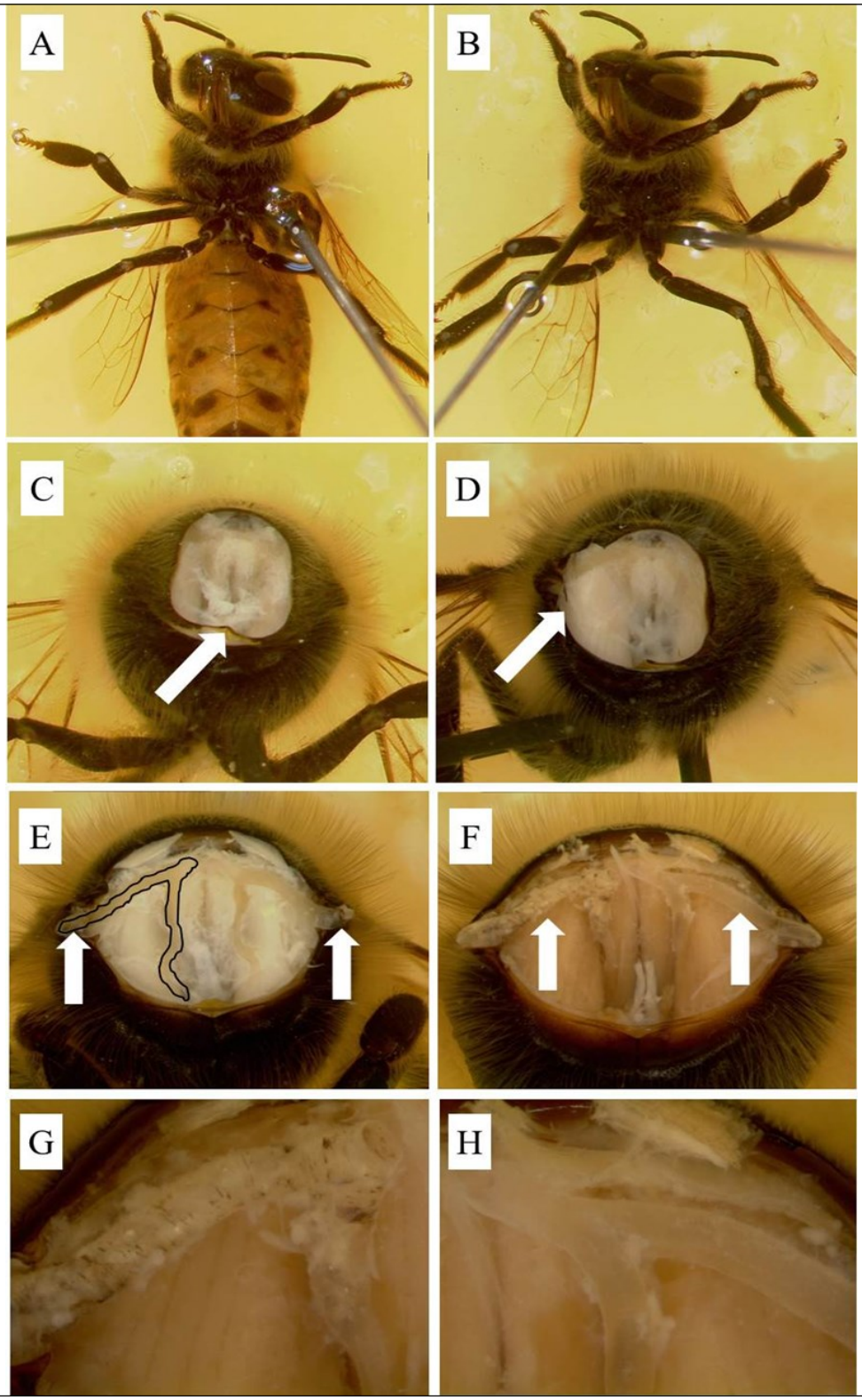

Fig. 10. Dissecting a bee to determine tracheal mite infestation. ***A.*** Pin a worker bee through the thorax using two insect pins (the bee's body will pivot in the dish if pinned with only 1 pin). The bee in this figure is pinned into a petri dish of hardened beeswax. The bee is covered in 70% ethanol to facilitate dissection. ***B.*** Remove the abdomen. This is a helpful technique to limit contents from the bee's digestive systems from emptying into the field of view. ***C.*** Remove the head and front pair of legs. The "collar" junction is arrowed. ***D.*** The beginning of collar removal. The area where the collar has been removed is arrowed. ***E.*** The collar has been removed, including the part that covers the spiracles (arrowed). The bee's right trachea (the one on the left in the figure) is outlined to show shape and position. ***F.*** Trachea infested with mites (on left, arrowed) and not infested with mites (on right, arrowed). ***G.*** Close-up of infested trachea and ***H.*** close-up of uninfested trachea.

Photos: Lyle Buss and Tricia Toth, University of Florida, USA.

3. Touch the tip of the tool to which the mite adhered to the droplet of Hoyer's medium.
4. Gently press the mite to the bottom of the droplet and position the mite on a vertical axis.
5. Using a pair of forceps, pick up a cover slip and gently place it on top of the droplet.
6. Mark the mite's location by drawing a ring around the specimen using a permanent marker, to facilitate locating the specimen later.
7. Label slides with date of collection, place of collection, species name, host species and name of collector.
8. Dry slides at 45ºC for 48 h to one week.
9. Slides can be sealed with a ring of glyptal (Glyptal, Inc.; Chelsea, MA, USA)

Fig. 11. Mites seen through tracheal tube.
Light Microscope photo: L de Guzman (140x).

3.3.5. Screening a large number of bees

For screening tracheae of many bees together to look for HBTM, a number of methods have been developed. Colin *et al.* (1979) developed the following technique.

1. Place bee thoraces in a blender with water
2. Grind for several seconds at 10,000 rpm 3 times to suspend the mites.
3. Strain the liquid through a screen mesh to remove larger particles and then centrifuge it to deposit the suspended particles at the bottom of the tube, which is then examined for mites.

The advantage of this technique is that a large number of bees (100-200) can be processed together. However, other *Acarapis* species such as *A. dorsalis* and *A. externus* that reside on the neck, thorax and wing bases will also be extracted with this method; see Section 2.2.

The morphological separation of these species is very time consuming. Washing bees prior to grinding was not found to be effective in removing *A. externus* or *A. dorsalis* (Lorenzen and Gary, 1986; S George, pers. obs. in New Zealand). A 'tracheal flotation technique' developed by Camazine (1985) reduced this risk somewhat (of mixed *Acarapis* species) by grinding the bee thoraces in water; however, this method would not be suitable to detect low levels of infestation.

3.3.6. Thoracic disc method (TDM)

TDM is a technique developed for detailed assessment of mite infestations. TDM involves cutting a thoracic disc that contains the prothoracic tracheae.

1. The bee is placed, dorsal side down and pinned in place and a razor blade or scalpel is used to cut off the head; then a thin transverse section is cut from the anterior face of the thorax, resulting in a 1 to 1.5 mm section, which includes the trachea (Fig. 12).
2. The discs are then heated on a hot plate (approximately 60ºC for a minimum of 2 h) in 5-10% potassium hydroxide (KOH) to dissolve the surrounding tissues.
3. The contents then are passed through a fine strainer over a sink and rinsed with cold water to remove dissolved matter.
4. The samples are returned to a hot plate to digest further for another hour after adding fresh KOH, if the muscle tissue has not been completely cleared.
5. When the thoracic discs become transparent in the middle, leaving only the sclerotized tergites around the outside, they are sieved and gently rinsed with cold tap water.
6. The discs are returned to the Petri dish and suspended in distilled water containing a few drops of aqueous methylene blue (1%) (Peng and Nasr, 1985).
7. Tracheae are then examined for tracheal mites under magnification (*ca.* 20-40×) using a dissecting microscope with lighting from below. Even a small number of mites can be detected through this method.

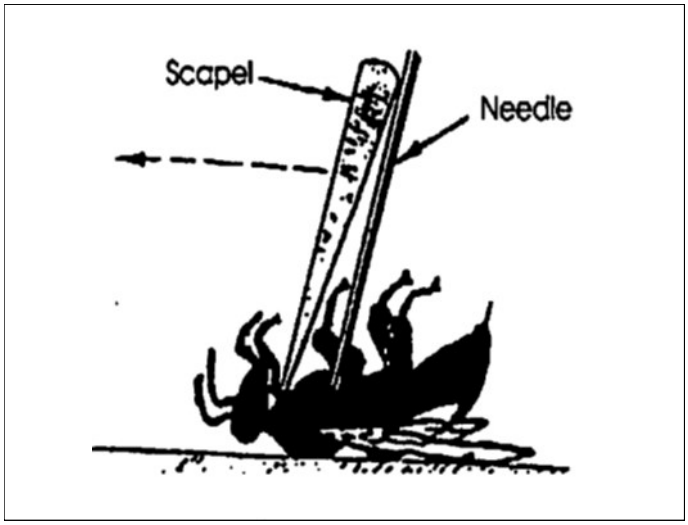

Fig. 12. Positioning a bee to cut thoracic disks. The head is first removed, then a thin section of the thorax is sliced. From Shimanuki and Knox, 1991.

8. Once cleared, the tracheae are then individually mounted on slides and examined under a microscope (Shimanuki and Cantwell, 1978; Delfinado-Baker, 1984; Shimanuki and Knox, 1991). A modified version of the thoracic disc method is used for detection of HBTM in New Zealand. Sampled bees are frozen for at least 24 h to facilitate cutting the thorax.

3.4. Serological and molecular detection of *Acarapis woodi*

3.4.1. Enzyme-linked Immunosorbent Assay (ELISA)

Ragsdale and Furgala (1987) developed antiserum against *A. woodi* where infested tracheae were detected using a direct enzyme-linked immunosorbent assay (ELISA); this method was further modified by Ragsdale and Kjer (1989). This assay was sensitive enough to detect a very low level of tracheal mite infestation but was found to cross-react with other proteins present in the haemolymph and thoracic muscles. The lack of specificity limits the application of this test to tracheal preparations. A practical ELISA test was developed by Grant *et al.* (1993) where whole bee samples could be analysed for HBTM detection, but the sensitivity of the test was reduced when mite prevalence fell below 5%.

3.4.2. Guanine visualization

This is an indirect method based on detecting Guanine (2-amino-6-oxypurine), which is the main end product of nitrogen metabolism in mites and other arachnids. It is present only in a negligible amount in bee excretions. In this method, bee tracheae are individually homogenized and their guanine content is visualized on thin layer chromatography (TLC) plates. Since bees need to be individually tested and low level infestations may go undetected (Mozes-Koch and Gerson, 1997), this method is not usually recommended.

3.4.3. Molecular detection of *Acarapis woodi* in *Apis mellifera*

Because morphological detection is time -consuming and requires detailed and sustained attention by the screener, detection of *A. woodi* using a molecular technique is currently being developed by various laboratories for routine screening and quarantine checking. A real-time PCR assay for *A. woodi* was designed by Giles Budge at the UK Food and Environment Research Agency (Fera) which amplifies a section of the internal transcribed spacer region 2 (ITS2); however when tested, it was found to also amplify the ITS sequence from other *Acarapis* species. Evans *et al.* (2007) developed a nested PCR to amplify part of the cytochrome oxidase1 gene (CO1) of *A. woodi*. The PCR was designed to detect a low level of infestation of *A. woodi* from the entire thorax of a bee. At the time of development, the assay was not tested against other *Acarapis* species, but subsequent testing has shown that these primers amplify sequences from them also (Delmiglio *et al.*, in prep.).

Kojima *et al.* (2011b) published a conventional PCR method shown to amplify *A. woodi* DNA and not *A. externus,* but this method has not been tested on *A. dorsalis* DNA. Although their test was able to detect *A. woodi* when four mites were present in a single bee, it was not able to detect the presence of a single mite unless a nested-PCR was performed. Furthermore, Kojima *et al.* (2011b) did not test the sensitivity of detection at different bulking rates for extractions and the need for post amplification handling (i.e. electrophoresis) increases the length of the detection procedure.

Delmiglio and Ward (unpublished) obtained sequences from the CO1 region for *A. woodi*, *A. externus* and *A. dorsalis* and designed real-time PCR primers and a TaqMan probe for *A. woodi* within a single variable region of the CO1 gene. The assay was able to amplify *A. woodi* DNA from a single mite (specimens obtained from Canada and UK).

Tests showed that the assay was able to detect *A. woodi* down to 1% and 2% prevalence in bees, and 200 copies of the target DNA when using plasmid standards. The real-time assay was not found to cross-react with mites of other genera associated with bees; however a very low level of cross-reaction occurs with the other two *Acarapis* species when sequence from these species are present at high concentrations in the form of plasmid DNA, or when there are a very high number *Acarapis* mites. To counter this, bees are externally washed before testing and a lower C_t cut-off of 35 cycles is used.

Nucleic acid extraction:

Note: the extraction method is based on the semi-automated system Thermo Kingfisher & InviMag® DNA Mini Kit (Invitek GmbH; Germany):

1. Using this semi-automated extraction method, 15 bees maximum can be sampled per extraction to allow reliable detection of *A. woodi* from a single infected bee.
2. Prepare bees by removing the abdomens using a clean scalpel, and place bee heads and thoraces into a filter grinding bag (Bioreba; Switzerland) of suitable size.
3. Add lysis Buffer P (Invitek GmbH; Germany) to the grinding bag at a rate of 0.5 ml buffer per bee (i.e. use 7.5 ml for 15 bees), and using a Homex grinder (Bioreba, Switzerland) grind the bees to form a lysate.
4. Decant 600 µl of the lysate into a clean micro-centrifuge tube, add 30 µl of proteinase K (Invitek GmbH; Germany) and incubate at 65°C for 30 min in a thermomixer (Eppendorf; Germany).
5. Centrifuge the resulting lysate at 8000 *g* for 1 minute to pellet debris.
6. Extract DNA from the cleared lysate using a KingFisher® ml Magnetic Particle Processor system (Thermo Fisher Scientific Inc.; USA) using the protocol and program recommended by the manufacturer, and using buffers and magnetic beads

supplied in an InviMag® DNA Mini Kit (Invitek GmbH; Germany).

7. The resulting DNA is eluted in 260 ml of elution buffer D (Invitek), transferred to a fresh 1.5 ml tube and stored at -80°C prior to use.

3.4.4. Real-time PCR

1. qPCR reactions are run in final volume of 10 µl using Platinum® qPCR SuperMix-UDG (Invitrogen-Life Technologies)
2. Each reaction contains a final concentration of:
   - 1x qPCR SuperMix
   - 3.5 mM of Mg ions
   - 0.3 µg of Bovine Serum Albumin (BSA)
   - 300 nM each of forward and reverse primer (Table 3) and
   - 100 nM of LNA probe (Table 3)
3. The cycling conditions used are (as optimized in a BioRad CFX -BioRad Laboratories) - 50°C for 2 min (UDG incubation-single hold)
   - 95°C for 2 min (initial denaturation-single hold)
   And 35 cycles of:
      - 95°C for 10 sec (denaturation)
      - 59°C for 45 sec (anneal and extension)
      - Reading of signal at end of each cycle.

Note: The PCR competency and success of nucleic acid extracted from bees is assessed using the internal control real-time PCR assay which amplifies part of the 18s rRNA gene of *A. mellifera* (Ward *et al.,* 2007). The 18s real-time reactions were set-up and cycled as described for the *A. woodi* assay.

4. Experimentation with live mites

Mite populations tend to fluctuate seasonally, in patterns that are moderately consistent at the regional level. However, individual colonies frequently experience unexpected increases or decreases in mite abundance, thereby complicating experimentation with them. There is no record of experimentation with individual mites until after they were discovered in North America in the 1990s. Because little has been published on techniques for experimenting with mites, most of the researchers who have studied mites in recent years provided information that is synthesized below.

4.1. Rearing HBTM in colonies

Tracheal mite prevalence (the proportion of infested bees in a sample; Margolis *et al.*, 1982) generally decreases during periods of the year when large numbers of young bees are emerging (due to extensive brood rearing and relatively short-lived adult bees). Prevalence increases during fall (when brood rearing is declining) or periods of confinement (e.g. rainy weather), presumably because there is extensive contact between older infested bees and young susceptible bees (Bailey & Ball, 1991). In cold temperate climates, mite prevalence usually increases rapidly from late summer until early winter (Otis *et al.*, 1988; de Guzman *et al.*, 2002). However, HBTM prevalence in individual colonies in summer is not correlated with mite prevalence in late fall; only when bee brood has largely disappeared can mite prevalence in wintering colonies be predicted with any assurance (r = ~0.8, Dawicke *et al.*, 1989). Due to variable bee mortality during the winter as well as brood production in infested colonies in early spring, mite prevalence in the fall is uncorrelated with mite prevalence the following spring (Dawicke *et al.*, 1989). This makes experimentation difficult because heavily infested colonies cannot be identified until shortly before they are needed for experiments.

Once the wintering population of bees has developed (Mattila *et al.*, 2001), mite prevalence tends to remain relatively constant over the winter months in the absence of newly emerging worker bees (Bailey, 1958); however, in some situations mite prevalence has increased over winter (Otis *et al.*, 1988; McMullan, 2011). It is possible that mite emigration from tracheae is stimulated by high or increasing titers of juvenile hormone (JH). This speculation would explain observations that although mite abundance (i.e. the mean number of mites per bee) continues to increase in bee tracheae during the winter months due to continuing mite reproduction, mite prevalence (percent of bees infested of the total number of bees in the samples) generally does not. J McMullan (pers. comm.) indicated that when there is little or no brood present in autumn and winter, it is difficult to influence mite infestations experimentally.

4.1.1. Removing sealed brood

During spring and summer, when brood rearing is continuous and extensive, it is possible to increase mite infestations in colonies by removing sealed bee brood (i.e. pupae). This has been done regularly

Table 3. Real-time PCR (qPCR). Sequence of primers and probe used for the detection of *Acarapis woodi.* Areas of sequences in bold (column 2) are non-complementary flaps; [+] Locked nucleic acid bases.

Primer/Probe name	Sequence 5'-3'	Amplicon length
aw_F1-flap	**AATAAATCATAA**TGATATCCCAATTATCTGAGTAATG	113 bp
aw_R3	AATATCTGTCATGAAGAATAATGTC	
aw-LNAProbe	6FAM-ACC[+T]GT[+C]AA[+T]CC[+A]CCTAC-BHQ1	

by numerous researchers (McMullan and Brown, 2005; G W Otis, pers. obs.; J Villa, pers. comm.). Confining bees in hives may also increase infestation of young workers, but this has not been tested. One challenge is to increase mite prevalence, yet not so much that winter survival is jeopardized. Because many heavily infested colonies die over winter, studying live mites often requires sampling hives in spring to find heavily infested colonies for experimentation. Several researchers have moved heavily infested colonies, only to subsequently find that the mite infestations declined drastically (J Pettis, pers. comm.; J McMullan, pers. comm.; G W Otis, pers. obs.). It is not known why this occurs, but suggested causes are overheating or chilling during transport, loss of infested bees due to their failure to return to their hives after orientation flights in new locations (J Pettis, pers. comm.), or emigration of infested bees from the nest (J McMullan, pers. comm.).

4.1.2. Artificial rearing of HBTM

Bruce *et al*. (1991) experimented with *in vitro* rearing of *A. woodi*. They encased modified insect tissue culture medium, Medium MD1 (Whitcomb, 1983) developed for rearing *Pyemotes tritici* mites (Bruce, 1989) in tiny parafilm® tubes with a thickness of approximately 10 µm, made by stretching the film several times. Live mites were placed on these tubes of medium within a closed Petri dish. Although only a small number of mites were studied, all of them successfully pierced the artificial membrane and fed on the medium. There has been no further research on artificial rearing methods for HBTM.

4.2. Infesting bees with HBTM

1. Allow worker bees to emerge in incubators.
2. Mark bees with paint on their abdomens or numbered tags glued to their thoraces shortly after emergence. Paint applied to the bees' thoraces may interfere with migration of mites into their tracheae.
3. Introduce them to infested colonies, preferably at dusk. Otherwise, screen the hive entrances for 1-2 h to prevent their rejection. Giordani (1962) referred to this method as *infestation expérimentale*. It has served as the method of obtaining infested bees to study the life history of the mite (Pettis and Wilson, 1996), host age-preference (Morgenthaler, 1931; Lee, 1963; Gary *et al*., 1989), caste preference (Dawicke *et al*., 1992), relative resistance of bees from different genetic sources (Page and Gary, 1990; Danka and Villa, 1999; Nasr *et al*., 2001), and the effects of mites on adult bees (Bailey and Lee, 1959; Gary *et al.,* 1989).

4.3. Collecting live mites

1. Anesthetize a bee with CO_2 (or place bees in refrigerator until immobile).
2. Grab the paranotal lobe that covers the metathoracic spiracle with fine forceps and pull the lobe posteriorly at a modest angle to the bee's surface (Smith *et al*., 1987)
3. The lobe, spiracle, and most of the main tracheal trunk will be removed together. This technique was subsequently used by Phelan *et al.* (1991), Bruce *et al.* (1991), and Sammataro and Needham (1996) to obtain living mites.
4. Other researchers have removed the bee's head and pronotum to expose the large thoracic tracheal trunks. These are either grasped at the spiracle with fine forceps (J Pettis, pers. comm.; vanEngelsdorp and Otis, 2001b) or a cut is made around the spiracle before removing the tracheal trunk (McMullan *et al*., 2010).
5. Allow the section of the trachea to dry onto a glass slide for ~ 2 min.
6. Tear open trachea with a minuten pin and pick up the mites with an eyelash attached to a wooden dowel (Phelan *et al.,* 1991; vanEngelsdorp and Otis, 2001b; McMullan *et al*., 2010).
7. Young, lightly bronzed female mites can be selected; males, darker (older) females and those obviously gravid with an egg can be avoided because they have reduced mobility (McMullan *et al*., 2010; J Pettis, pers. comm.).
8. A female mite placed externally near the paranotal flap of a bee will crawl towards and enter the spiracle, (D Sammataro, pers. obs.). When drones are present, they are preferred because mite intensity is generally higher in drones than in workers (Dawicke *et al*., 1992).
9. Exposed mites are sensitive to desiccation, so they should be kept at high humidity.
10. vanEngelsdorp and Otis (2001b) could perform bioassays with living mites only at night, perhaps because of a circadian rhythm in mite activity that influences their nocturnal dispersal to new hosts (Pettis *et al*., 1992).

5. Controlling tracheal mites

5.1. Effects on bees

HBTM can cause diminished brood area, smaller bee populations, looser winter clusters, increased honey consumption, lower honey yields and frequently, colony demise (Komejli *et al.,* 1989). In temperate regions, mite populations increase during the winter, when bees are confined to the hive in the winter cluster. Heavy mite infestation affects bee metabolism and the ability of colonies to regulate the cluster temperature (Skinner, 2000); chilling may be a significant cause of colony death. In North America, colony losses increased shortly after first exposure to HBTM (Wilson *et al*., 1997). Colonies with 40-50% tracheal mite infestation or higher frequently die during the winter in northern USA and Canada (Furgala *et al*. 1989; Otis and Scott-Dupree, 1992). The colder the winter temperatures, the greater the probability of mortality at any mite prevalence value.

Treatments for HBTM include using vapours from menthol crystals, synthetic acaricides and oil or grease patties made from vegetable shortening and sugar. Additionally, resistant lines of bees have been developed.

5.2. Resistant bees

Some races of bees are less susceptible to tracheal mite infestations. Resistance is accomplished in part by the increased autogrooming behaviour of bees (Lin *et al.*, 1996; Pettis and Pankiw, 1998; Danka and Villa, 2005; Villa, 2006; de Guzman *et al.*, 2002, 2005). Russian and Buckfast queens are among the more resistant lines of bees. Fig. 13 illustrates steps needed to select and propagate bees that show resistance to HBTM. Colonies with 15-25% of workers infested experience increased winter mortality. Bee stock can be screened for HBTM resistance to develop resistant queen lines. In general, untreated colonies that survive the winter with low mite numbers should be selected for a breeding programme.

1. First, select colonies to be tested for mite resistance and collect sealed brood frames from them.
2. Brush off the bees clinging to the frame and place it into a frame cage (Fig. 13, insert) and then into an incubator set at 35ºC and 50% RH.
3. Mark each frame to identify the test colony it was removed from
4. After 12-24 hours in the incubator, remove and mark any emerging bees. Bees identified by coloured plastic tags or model paint can be separated by colony of origin and placed into an inoculation colony, where HBTM prevalence is high (> 50%). (see section 4.2)
5. After a week or more, the marked bees that were released into host colonies can be retrieved and dissected to determine mite prevalences (percent of mite-infested bees in the samples) or mite abundances (average number of mites per bee/total number of individuals of the host bees in the sample) for the bees from each test colony source. Original colonies that tested lowest for HBTM can then be selected for a breeding program. For more information on breeding and selecting traits in queen bees, see Büchler *et al.*, 2013.

5.3. Chemical control

The overriding constraints for chemical control of mites are that the chemicals must be effective against the target (i.e. mites) and harmless to bees, and they must not accumulate in hive products. Because bees and mites are both arthropods, many of their basic physiological processes are similar, narrowing the possibilities for finding suitable toxicants. To control HBTM, the material must be volatile to reach the bee, inhaled, and lethal only to the parasite.

Based on early research by Giordani (1977), menthol was widely tested and registered for treatment of hives in the United States and Canada. Menthol is extracted from the mint plant *Mentha arvensis* and is sold as crystals, which can be applied inside hives (usually 50 g of crystals in a nylon mesh sac are placed on the top bars of frames). In cold conditions, menthol is ineffective for mite control because the rate of vaporization is too low to provide concentrations lethal to the mites. Some Canadian beekeepers obtained good results under cool conditions by applying pieces of cardboard saturated with a mixture of menthol (dissolved in alcohol) and vegetable shortening to hives. If the air temperatures outside are too high, menthol vapours may drive bees out of their hives. If bees are hanging outside the colony entrance in treated colonies, crystals should be removed until temperatures drop.

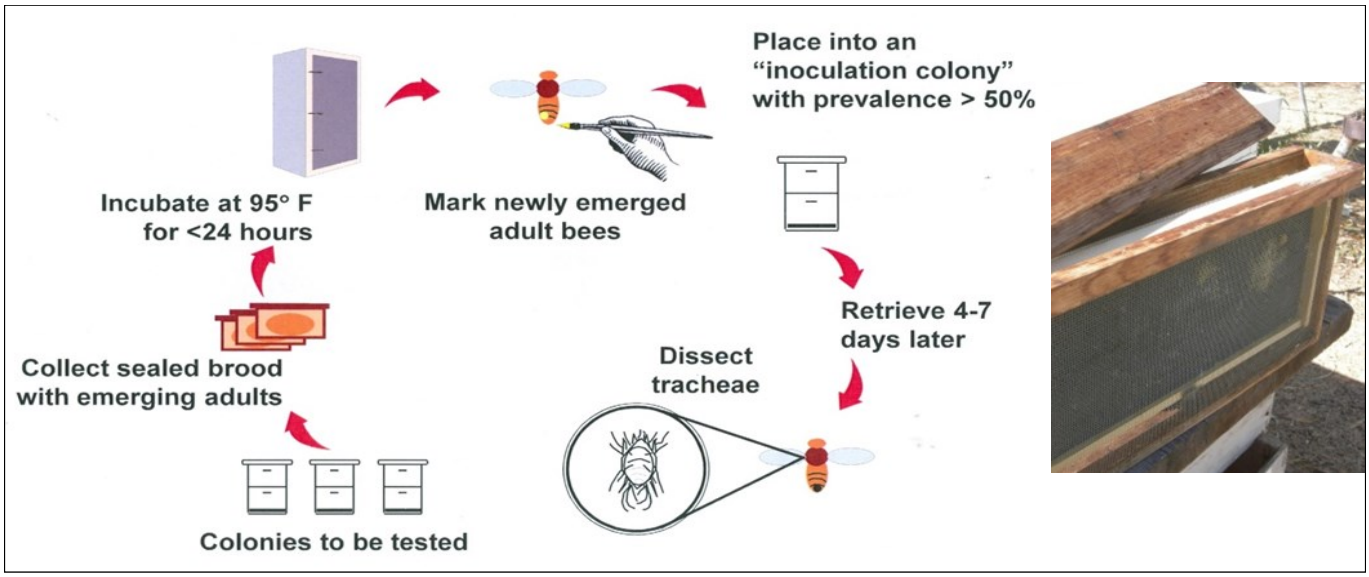

Fig. 13. Schematic diagram outlining a procedure to determine the relative susceptibility of honey bee colonies to HBTM infestation. Brood combs with emerging bees are brushed free of adult bees and then placed into frame cages (insert) or other containers to isolate bees; frames are placed into an incubator. After a week, emerging bees are marked and then placed into inoculation colonies. The marked bees are retrieved after a week and dissected to determine mite prevalences or mite abundances for the bees from each test colony source (from Danka, 2001). See text for explanation.

The synthetic pesticide amitraz, (sold as Apivar®) has been used for HBTM control. Check current regulations to determine its registration status. Formic acid has also been used against *A. woodi* (Hoppe *et al.*, 1989; Amrine and Noel, 2006; Underwood and Currie, 2009; Hood and McCreadie 2001). Widely used as a treatment for varroa mites, formic acid (along with other acaricides used against varroa) may be contributing to current lower populations of HBTM in North America. As many of these chemicals could have a negative impact on both the bees and the honey, they are not recommended as both non-toxic vegetable shortening and resistant queen stock are better alternatives.

5.4. Cultural control

An alternate, environmentally safe control is to use a 'grease' patty. More accurately, this is a vegetable shortening and sugar patty (2:1 sugar: shortening by volume, or sufficient shortening that the mixture does not break apart). Vegetable shortening (solid, hydrogenated vegetable oil, not animal fat) and white granulated sugar will keep mite prevalence to 10%, well below typical economic thresholds (< 20%). Animal fat will go rancid over time and is not recommended. Liquid vegetable oil can be used, but the patty will be looser and may not hold up in the hive, unless mixed with ample amount of sugar.

A 113 g patty, placed on the top bars at the centre of the broodnest where it comes in contact with the most bees, will protect young bees (which are most at risk) from becoming infested. Patties can be prepared ahead of time if they are wrapped in waxed paper and stored in the freezer. The shortening appears to disrupt the questing female mites as they search for new hosts (young bees) (Sammataro and Needham, 1996; Sammataro *et al.,* 1994). Because young bees are emerging continuously, the patty must be present for an extended period. The optimal application seasons are fall and early spring, when mite levels are increasing.

Requeening colonies is likely to alter HBTM infestations once offspring of the new queens begin to emerge; those new offspring may be more or less resistant to mites than the worker offspring of the original queens. Apiary location may also affect tracheal mite infestations (vanEngelsdorp and Otis, 2001a; L de Guzman, unpubl. data), perhaps due to temperature or humidity effects. Harbo (1993) demonstrated that hives exposed to the sun in Louisiana had reduced mite infestations. Integrated Pest Management (IPM) techniques have been successful in keeping HBTM under control; however, no biological controls currently exist (Fig. 14).

A cautionary note should be added. Many beekeepers are opting not to treat for mites or diseases, allowing survivor stock to become established. HBTM may reappear if treatments for varroa mites are suspended; sampling for HBTM is therefore recommended, especially if colony symptoms consistent with tracheal mite infestations are observed.

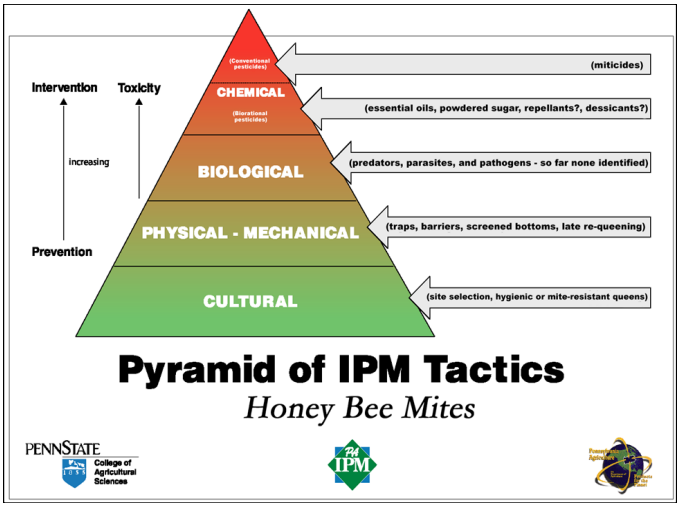

Fig. 14. Integrated Pest Management (IPM) tactics for bee mites. For HBTM, 'grease' patty should be listed with essential oils and powdered sugar (D Sammataro).

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