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TRACHEAL MITES
Tracheal mite, *Acarapis woodi* (Rennie) (Acari: Tarsonemidae)

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Countries that export bees and bee products are required to conduct apiculture surveillance programs to meet disease reporting and sanitary control requirements of the OIE (Office International des Epizooties) to facilitate international trade. A surveillance program also aids in early detection of honey bee pests and diseases including any new introductions. This is quite critical to initiate eradication or control measures. One pest in this surveillance program is the Honey Bee Tracheal Mite (HBTM) *Acarapis woodi*, an obligate endoparasite of honey bees. First described from the Western (European) honey bee *Apis mellifera* L, these mites were initially observed when bees on the Isle of Wight were dying between 1904 and 1919. In 1921 the tracheal mite was first described by Rennie as *Tarsonemus woodi*, but later changed to *Acarapis woodi* (Lindquist, 1986; Wilson et al., 1997; Sammataro et al., 2000). Its detection led to the restriction of all live honey bee imports into the United States in 1922 (Phillips, 1923). Despite this, the first report of colony losses from HBTM in the United States came from beekeepers in Texas in 1984. Thereafter, *Acarapis* spread to all of the states, facilitated by commercial beekeepers transporting bees for pollination, and from the sale of mite-infected package bees. The real cause of the loss of colonies during this time is still unknown and may have been the result of several diseases or other factors causing the symptoms.

In addition, infected swarms, drifting bees, and the distribution of *A. mellifera* around the world have contributed to the spread of this mite. Although its current range is not well known, HBTM has successfully invaded most countries, including Europe, Asia, parts of Africa, North and South America, but is not known to occur in Australia, New Zealand or Scandinavia (Denmark *et al*., 2000; Hoy, 2011). Recent work by Kojima et al. (2011) reported *A. woodi* on Asian honey bees, *Apis cerana japonica* in Japan. It is fairly safe to say, wherever *A. mellifera* has been introduced, HBTM will most likely be found.

In addition to *A. woodi*, there are two external species in the genus *Acarapis*, namely *A. externus* Morgenthaler (infesting the neck region) and *A. dorsalis* Morgenthaler (in the dorsal groove of the thorax) (Ibay and Burgett, 1989; see Figure 1). They were considered to be harmless by Eckert (1961) and Delfinado-Baker (1982), but that is probably due to a lack of information on these two *Acarapis* species.

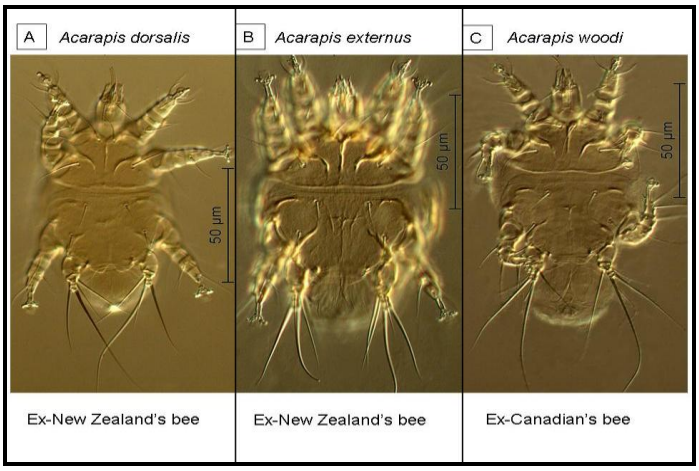


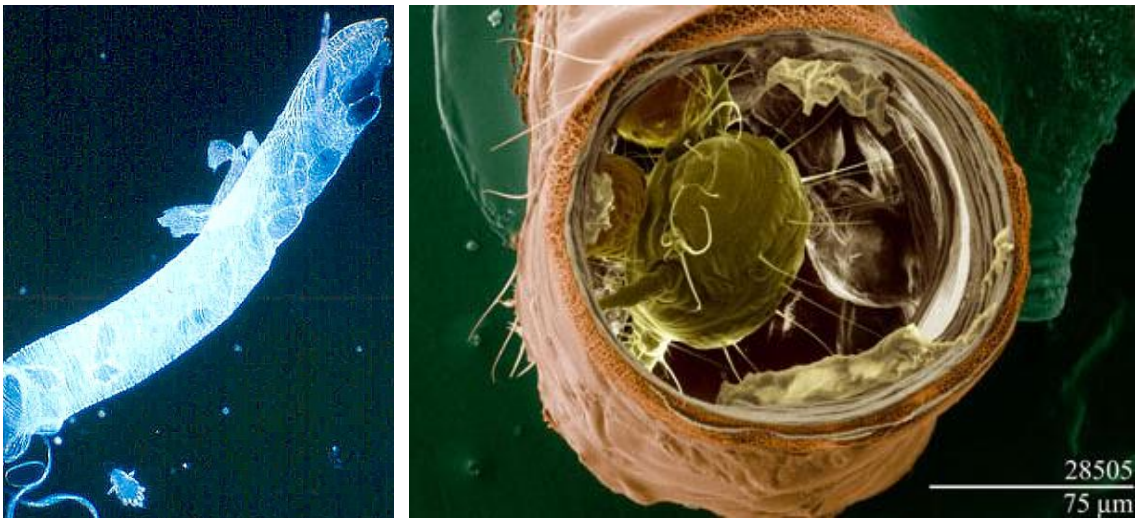
Figure 1: Ventral view of (A) *Acarapis dorsalis*, (B) *A. externus*, and (C) *A. woodi* adult female taken at a 400x magnification under light microscopy (Photos by Dr Qing-Hai Fan).

Unfortunately, HBTM is now overshadowed by the ectoparasitic mite, *Varroa destructor*Anderson & Trueman. As a result, the presence of this mite in some instances, is not now regularly investigated or is found in very low levels, perhaps due to the treatments used to control Varroa.

Effects on Bees: Tracheal mites affect the overwintering capability of bee colonies and have been associated with paralyzed bees displaying disjointed wings (called 'K-wing') and crawling on the ground near hives. A heavy HBTM load causes diminished brood area, smaller bee populations, looser winter clusters, increased honey consumption, lower honey yields and, ultimately, colony demise. In temperate regions, mite populations increase during the stress of cold winter temperatures, when bees are confined to the hive; this stress and the inability of bees to keep the winter cluster warm may be the cause of colony loss.

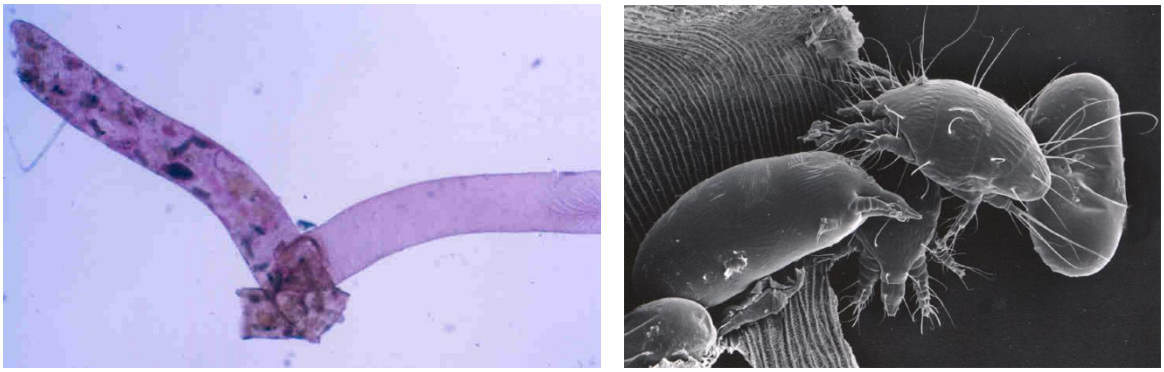
Life Cycle: Adult female tracheal mites measure 120 to190 µm long by 77 to 80 µm wide; adult males are 125 to 136 µm by 60 to 77 µm. The mites can hide under the flat lobe that covers the bee's first thoracic spiracle, accessing the main pro-thoracic tracheal trunk(see Figure 2). The life stages are, egg, larva, and adult; the nymphal instar remains inside the larval skin. 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 stages of HBTM feed on bee hemolymph, which they obtain by piercing the tracheal walls 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 the mites suck bee hemolymph through the short tube into the pharynx.

All mite instars live within the tracheae (see Figures 2 and 3), except during a brief period when adult, mated females disperse to search for callow (less than four days old) bee hosts. Reproduction can also occur at the wing axillaries. Mites are attracted to the outflowing air from the prothoracic spiracle and to specific hydrocarbons from the cuticle of bees (Phelan et al., 1991; McMullan et al., 2010) and immature stages may move in the trachea with the air currents during breathing by the bee (Ochoa et al., 2005). HBTM females are less attracted to older bees, which will not live long enough for the mites to complete their cycle.



A. B.

Figure 2. A. Pro-thoracic trachea of a honey bee filled with HBTM (Photo by D. Sammataro, 400X). B. LT-SEM micrograph of the interior of a tracheal tube with female mite, eggs and debris inside.(Photo by E. Erbe and R. Ochoa).



A. B.

Figure 3. A. *A. woodi* infested (L) and clean (R) trachea tubes, dyed for clarity (Photo by. D. Sammataro, 400X). B. Larval mite (L) adults and egg viewed by LT-SEM from inside a trachea (Photo by E. Erbe and R. Ochoa).

Once a suitable host is found, preferably drones, the female mite enters the trachea via the spiracle to lay eggs. Queens, even those commercially reared, often have HBTM and 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, and can be found externally at the base of the bee's wings; the fate of the mites found in these areas and their effect on the host is unknown.

Female mites 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; see Fig. 4). During this questing period, mites are vulnerable to desiccation and starvation, and their survival depends on the ambient temperature and humidity and mites have a higher dispersal rate at night (Pettis et al., 1992). 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 molt to adulthood and guard them in advance of mating (Ochoa et al., 2005). The males do not attach to the immatures as is common in other genera in the family Tarsonemidae (Ochoa et al., 2005). The female HBTM are the ones that go deep in the tracheal system, measuring the walls of the trachea branches with their dorsal and ventral seta and using the leg IV seta (see Fig 2B). The eggs are 5 to 15 microns longer that the length of the females (see Fig 3B).

Figure 4. A female tracheal mite questing on bee seta (Drawing by D. Sammataro).

Sampling Methods to Detect HBTM

Field methods

Because these mites are microscopic, it is impossible to tell whether or not a bee is infested with HBTM by just looking at it. In general, the bees do not show symptoms that can be used as a reliable indicator of their presence. However, as mentioned above, highly infested worker bees can sometimes be seen crawling in front of colonies. These crawlers may or may not have K-wings; this symptom is only apparent during winter or early spring, particularly when HBTM infestations are very high. With the widespread distribution of nosemosis, which may show the same symptom, the presence of crawlers in front of colonies should not be used as a reliable indicator.

Sampling Colonies

Best time to sample - When trying to detect tracheal mites, sampling time is very important to consider. Infestation by tracheal mites varies through time; see Figure 5. 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 bees is present in the colonies. The tracheal mites have a longer time reproducing in older, overwintering bees and thus, more mites actively feeding can cause the tracheal trunk to turn black. Infestation of HBTM decreases in summer due to the dilution of mite population because of the emergence of new hosts.

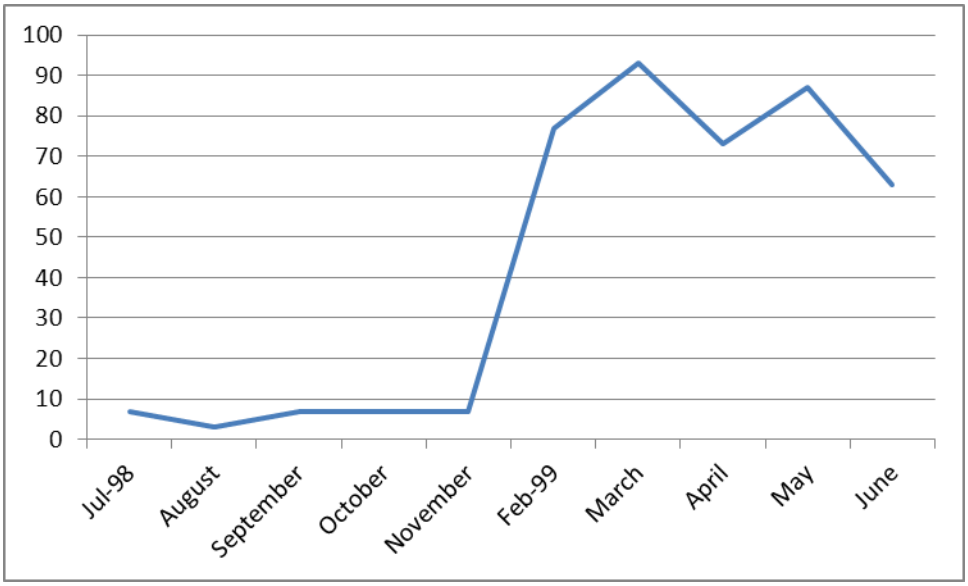


Figure 5. Growth of tracheal mite populations in bee colonies over one year. Data from de Guzman et al. 2002.

The genotype of honey bees and the location of the colonies also influence the levels of HBTM infestations. The Buckfast, ARS-Y-C-1 (Yugoslavian) and Russian honey bees (Danka et al., 1995; de Guzman et al., 2002, 2005) are known to be resistant to tracheal mite infestations. Heat is also associated with HBTM mortality (Harbo, 1993), a similar observation made with Varroa mites. Colonies exposed to direct sun impedes HBTM mite growth and shade tends to accelerate it (L. de Guzman, unpub. data). A similar observation was made with Varroa mites, where it was found that the growth of mites in colonies that were exposed to direct sun was impeded, whereas shady conditions tended to accelerate mite growth (Rinderer et al,. 2004).

Collecting bee samples: HBTM infestations are influenced by the age of bees, therefore the location within the hive from which the sample bees are collected should be considered. Because queens can be on honey frames, it is recommended to examine all the combs for the presence of the queen before sampling. Adult drones should also be collected, since they are more susceptible to mite infestations than worker bees (Royce and Rossignol, 1991). Because drones are seasonal, adult worker bees are often sampled for detection or surveillance purposes. Collect about 50 bees from frames in the honey super or from inner covers where older bees congregate. Although mites have usually left the trachea to find younger hosts, highly infested older bees have blackened trachea which can easily be noticed. In contrast, very young bees, which are more attractive for transfer of foundress (or mother) mites, may only have foundress mites that may have just started reproducing. The presence of one foundress or two mites (a foundress and an egg) near the opening of the trachea may be difficult to detect. Thus, if mite load or number of mites per infested bee is also of interest, sample bees from honey frames in the brood chamber where a good mixture of young and old bees are generally found.

Bees can be collected by using portable insect vacuums (see Figure 6) or by scooping bees with a plastic cup directly from the frames or inner cover. Samples can be placed into vials or

plastic bags. Label each container or plastic bag with location, colony number and the date the samples were collected. Although bees can be preserved in 70% alcohol, fresh or frozen bees are easier to dissect, and also examination of tracheae is easier when no alcohol is inside them. If molecular techniques are used for mite detection, bees should remain frozen.

Figure 6. Sampling bees for HBTM using a modified portable car vac, which collects bees into a plastic vial (Photo of S. Cobey by D. Sammataro).

Number of bees to be examined: In general, about 30-50 bees are examined per colony. However, there are different ways of determining 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 level of significance $\alpha = 0.10$ and 0.20, and precision level $\beta = 0.05$ and 0.10. This improved technique can save time and money since it only requires fewer than 50 samples to reach a decision. However, users of this technique are cautioned with the selection of alpha and beta. The values for alpha should be small (0.05 or 0.01) and value of beta large (0.95) to have a rigorous assessment.

The following equation developed by Cochran (1963) is also another way of finding the number of bees needed to be sampled for each colony:

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

Where:

$\mathbf{n_0}$ is the sample size,

$\boldsymbol{Z^2}$ is the abscissa of the normal curve that cuts off an area at the tails (1 equals the desired confidence level, e.g., 95%). 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 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. 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 not be recommended for scientific reporting.

Interpretation of Results. Count the numbers of bees infested and bees examined to determine levels of infestation. Tracheal mite infestations lower than 20% do not require treatment.

Other Detection Methods:

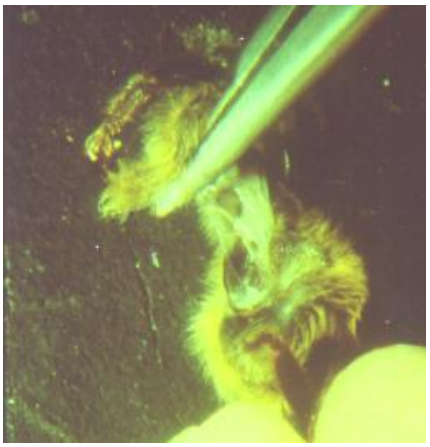
Since these parasitic mites reside inside the trachea, their detection requires specialized techniques, such as thoracic disc preparation and examination under a microscope which makes it a laborious procedure. Molecular techniques are currently being developed for processing the bees in bulk which is expected to provide increased sensitivity, specificity and speed to the screening of bees for tracheal mites.

Laboratory detection

1. Microscopic detection of *Acarapis woodi*

The morphological technique involves examining the prothoracic trachea under a microscope. Beekeepers often use unreliable bee stress symptoms, such as dwindling populations, abandoned overwintered hives full of honey, or weak bees crawling on the ground as symptoms of HBTM. Detection of low level infestation by *A. woodi* requires careful microscopic examination of the

trachea, whereas when the infestation is heavy, the trachea will turn opaque and discolored and can be noticed without the aid of a microscope (see Figure 6). One method is to pull off the head and collar of a bee and examine the trachea (Sammataro, 2006 and see video of bee dissection at: *http://www.ars.usda.gov/pandp/docs.htm?docid=14370*).



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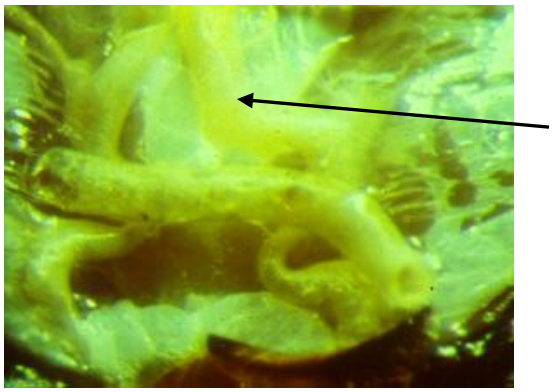

C.

Figure 6. A. Pulling off head and first pair of legs of bee to expose prothoracic trachea. B. View of trachea after prothoracic collar is removed and also exposing spiracle; exposed darkened trachea on right has mites, removed and enlarged in C. C. Shadows and round objects can be seen through tracheal wall compared to a clean tube, above (arrow). Photos by D. Sammataro.

Screening individual bees:

When the level of infestation is low, trachea from an individual bee needs 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. According to this technique, the bee is placed under a dissecting microscope, held prone with forceps (across abdomen) and the head and the first pair of legs are scraped off using a scalpel or razor blade. The ring of prothoracic sclerite (collar) is also removed using a fine forceps. The exposed tracheae of both sides are removed after carefully detaching it from the thoracic wall. The tracheae are 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 from the thoracic wall and transferring to the slide. Lorenzen and Gary (1986) modified this technique where the thoracic tergite was removed as a flap to look at mites in situ. This technique, though it requires no further treatment, is also time-consuming as the bees need to be examined individually.

Liu (1995) developed a rapid technique to distinguish live mites from dead by staining with thiazolyl blue tetrazolium which makes the live mites purple.

Screening large number of bees:

For screening tracheae of many bees together a number of methods have been developed. Colin et al. (1979) developed a technique where the bee thoraces were placed in a blender with water and ground for several seconds at 10,000 rpm 3 times to suspend the mites. The liquid was then strained to remove larger particles and then centrifuged to deposit the suspended particles at the bottom of the tube, which was then examined for mites. The advantage of this technique is that a large number of bees (100-200) can be processed together, but will potentially collect other *Acarapis* species such as *A. dorsalis* and *A. externus* that reside on the thorax thorax and wing axillaries as well. The morphological separation of these species is very time consuming. Washing bees prior to grinding was not found to be effective in getting rid of *A. externus* or *A. dorsalis* (Lorenzen and Gary, 1986; S. George, pers. obs. in NZ). The 'tracheal flotation technique' developed by Camazine (1985) reduced this risk by examining individual trachea after grinding the thoraces and floating them in water. But again would be optimal to detect very a low level of infestation.

The Thoracic Disc Method (TDM) was another technique developed for screening large number of bees together. The technique involves cutting a thoracic disc containing the prothoracic trachea which are then heated in 10% potassium hydroxide (KOH) to dissolve the surrounding tissue and then individually mounted on slides and examined under a microscope (Shimanuki and Cantwell, 1978; Delfinado-Baker, 1984).

A **modified** version of the thoracic disc method is used in surveillance program for detection of tracheal mite in New Zealand. Samples are frozen for at least 24 hours to facilitate processing.

- Thoracic discs are prepared as described before, placed in labelled Petri dishes and suspended in 10% KOH solution.
- The thoracic discs are heated on hot plate (approximately 60ºC for a minimum of 2 hours). The contents are passed through a standard strainer over a sink and rinsed with cold water to remove dissolved matter.
- The samples are returned to a hot plate to digest further for another hour after adding fresh KOH.
- 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.

- The discs are returned to the Petri dish and suspended in distilled water and a few drops of aqueous methylene blue (1%).
- Tracheae are then examined for tracheal mites (inside trachea) under magnification (ca. 20×) using a dissecting microscope with lit base. Even small number of mites can be detected through this method.

Serological detection of *Acarapis woodi*

Enzyme-linked Immunosorbent Assay (ELISA).

Ragsdale and Furgala (1987) developed antiserum against *A. woodi* where tracheae infested with the mite were detected using a direct enzyme-linked immunosorbent assay and Ragsdale and Kjer (1989) further modified this technique. This assay was sensitive enough to detect very low level of tracheal mite infestation but was found to cross-react with other proteins present in the hemolymph 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 analyzed for tracheal mite detection but the sensitivity of the test was found reduced when the level of infestation falls below 5%.

Guanine visualization

This is an indirect method of tracheal mite detection 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 negligible amount in bee excretions. In this method, bee tracheae are individually homogenized and their guanine content is visualized on TLC plates. Bees need to be individually tested and low level of infestation may go undetected (Mozes-Koch and Gerson, 1997).

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

The very small size of the mite and its concealed positioning inside the trachea poses challenges to its detection. Moreover, since the morphological technique is time consuming, requiring detailed attention of the screener, the chances of missing detection of low level population is possible. 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 FERA (The Food and Environment Research Agency, United Kingdom) which amplified a section of the internal transcribed spacer region 2 (ITS2); but when tested, was found to also amplify ITS sequence from other *Acarapis* species.

Evans et al. (2007) developed a nested PCR for *A. woodi* designed to sequence in the Cytochrome oxidase1 gene (CO1). The PCR was designed to pick up a low infestation of *A. woodi* mites from the entire thorax of bees. At the time the assay was not tested against other

Acarapis spp., but subsequent testing has shown that these primers also amplify sequence from the other *Acarapis* spp. (Delmiglio et al., 2012, MS under submission)

Delmiglio et al. (2012, MS under submission) obtained sequences from CO1 region for *A. woodi*. *A. externus* and *A. dorsalis* and designed real time PCR primers and a LNA TaqMan probe for *A. woodi* within a single variable region of the CO1 gene. The authors could amplify *A. woodi* DNA from a single mite (obtained from Canada & UK) and the primers did not cross react when tested against DNA from *A. externus* and *A. dorsalis*. This test has been validated in detail with conventional thoracic disc method too.

Controlling Tracheal Mites

Control: Treatments for HBTM include using vapors from menthol crystals, chemical acaricides and oil or grease patties, made from vegetable shortening and sugar. However, today there are lines of honey bees, including Varroa Sensitive Hygiene, Russian honey bees and other lines that have been developed for resistance to HBTM (see below).

A cautionary note should be added. Many non-commercial beekeepers are opting not to treat for mites or diseases, allowing survivor stock become established. HBTM could reappear if treatments for Varroa mites are suspended; sampling for this mite should therefore continue.

Chemical: The overriding constraints for chemical control of mites are that the chemicals must be effective against the target 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 tracheae, be inhaled by the bee, and be lethal only to the parasite. A single registered treatment in the United States was pure menthol crystals, originally extracted from the plant *Mentha arvensis*. However, in cold conditions menthol sublimation is ineffective because an insufficient amount of vapor is released from the crystals. Conversely, at high temperatures the vapors may repel bees from the hive. An effective pesticide (sold as Amitraz) was used for HBTM, but its current availability is doubtful. Formic acid has also been used against *A. woodi*.

Cultural: An alternate, environmentally safe control is to apply a vegetable shortening and sugar patty at peak mite populations. A quarter-pound (113 g) patty, placed on the top bars at the center of the broodnest where it comes in contact with the most bees, will protect young bees (which are most at risk) from becoming infested over winter. The oil appears to disrupt the questing female mite searching for a new host (Sammataro and Needham, 1996). Because young bees emerge continuously, the patty must be present for an extended period. The optimal application season is in the fall and early spring, when mite levels are increasing.

Resistant Bees: Several lines of bees resistant to HBTM have been developed; resistance seems to be accomplished by the increased grooming behavior of bees (Pettis and Pankiw, 1998;

Danka and Villa, 2005; Villa, 2006; de Guzman et al., 2002, 2005; Lin et al., 1996). Such lines include Varroa Sensitive Hygiene and Russian bee stock.

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