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Clarification of aspects of *Varroa* reproduction – first stage of a possible new control method

**A report for the Rural Industries
Research and Development
Corporation**

By Dr Denis Anderson

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Foreword

The varroa mite, *Varroa destructor*, is a serious quarantine threat to the Australian beekeeping industry and is categorised as an emergency animal disease in the AUSVETPLAN. If this mite became established in Australia it would radically change the face of beekeeping and cause economic hardship for local beekeepers. It is therefore imperative that measures are taken to safeguard Australian beekeepers against a possible varroa mite incursion.

Research conducted by CSIRO has identified a novel way to control the varroa mite that would safeguard Australian honey producers against losses in the event of a varroa mite incursion. It involves locating the bee signal that triggers the female varroa mite reproductive program, then manipulating or modifying the signal to produce varroa-resistant bee stock.

This project is the first stage in identifying that signal. The aim was to find a procedure for differentiating the various internal tissues and organs of female varroa mites. This procedure will be a crucial tool for second stage studies aimed at pinpointing the precise time when mite reproduction is first triggered. That, in turn, will allow for a detailed search for the signal.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to RIRDC's diverse range of over 1500 research publications. It forms part of our Honeybee R&D program which aims to improve the productivity and profitability of the Australian honeybee industry.

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Executive Summary

The invasive varroa mite (*Varroa destructor*) is a serious threat to Australian beekeeping. If this mite became established in Australia it would cause economic hardship for local beekeepers. This project is the first stage towards developing varroa-resistant honeybees (*Apis mellifera*) that will safeguard Australian beekeepers against a possible varroa mite incursion.

The pathway that will be followed to develop resistant bees stems from recent information that has come to light on the complex relationships that exist between *Varroa* mites and their native Asian honeybee hosts (strains of *A. cerana*), which are now known to have determined whether or not particular *Varroa* mites can utilize *A. mellifera* as an alternative host. In brief, recent research has shown that there are more than 20 different genotypes of *Varroa*, each a member of one of several different species and each a native parasite of a particular genotype of *A. cerana* in Asia. Only 2 of those genotypes, the so-called Korea and Japan genotypes of *V. destructor*, have become pests of *A. mellifera* because they recognize a signal released by *A. mellifera* larvae that allows them to produce offspring on the larvae. The other 90% of genotypes have not become pests of *A. mellifera* simply because they fail to recognize that signal. Finding the signal will allow it to be manipulated in *A. mellifera* so that the Korea and Japan genotypes, like all the other genotypes, do not recognize it. This could possibly even eliminate the varroa mite as a pest of *A. mellifera*.

Recent research has shown that the signal that triggers egg laying in females of the Korea genotype of *V. destructor* is released by honeybee larvae during the first 70 hours after their cells are capped. This project is the first step towards finding that signal. The aim was to find a procedure that could differentiate the different internal body tissues of female *Varroa* mites. This procedure is needed before second-stage studies, aimed at developing a model of the *Varroa* mite reproduction system, can begin. The model will reduce the field-of-search for the signal by pinpointing the precise time when mite reproduction is first initiated. The search for the signal will then become very directed.

A light microscope-based tissue-sectioning technique was found that clearly differentiates nerve, fat body, ovary and muscle body tissues of both *V. jacobsoni* and *V. destructor*. It involves embedding entire female mites in wax blocks, obtaining ultra-thin sections from them and staining those sections with Haematoxylin-Eosin. This procedure now allows for second-stage studies to develop a model of the *Varroa* mite reproduction system.

1. Introduction and Background

Over the past 50 years, the varroa mite, *Varroa destructor* (Anderson and Trueman, 2000), has spread to become the most serious pest ever of the Western honeybee, *Apis mellifera*. The mite is now present in most beekeeping countries of the world, including New Zealand. It is imperative that measures be taken to safeguard Australian beekeepers against a varroa mite incursion.

This project is the first stage of a longer term project aimed at producing varroa-resistant honeybees. Before outlining the strategy, stages and time frames involved to achieve this aim, some history and relevant information on *Varroa* mites is given as background to show how it is possible to produce resistant honeybees.

1.1 The discovery of *Varroa jacobsoni*, its switch to *Apis mellifera* and spread out of Asia

European honeybees are not the primary host of the varroa mite. All mites in the genus *Varroa* are indigenous parasites of Asian honeybees. *Varroa* mites were first discovered in Java, Indonesia, in 1904 on the Asian honeybee *Apis indica* (now known as *Apis cerana*) and named *Varroa jacobsoni* (Oudemans, 1904).

Following the Indonesian discovery, other similar-looking mites were discovered on different populations of *A. cerana* throughout Asia and these were assumed to be *V. jacobsoni* (Koeniger, *et al.*, 1983). Mites referred to as *V. jacobsoni* remained poorly studied until some 40-50 years ago when a mite, assumed to be *V. jacobsoni*, was reported to have shifted-host from *A. cerana* to *A. mellifera* following the introduction of that bee into Asia by humans. This shift provided a vehicle for the mite to be vectored out of Asia, first into Eastern and Western Europe, and then to other parts of the world, more recently to New Zealand (Matheson, 1996; Zhang, 2000). Australia is the only major beekeeping country that is still free of the mite.

1.2 The devastating effects of varroa mite on *Apis mellifera*

As it spread out of Asia, the varroa mite decimated *A. mellifera* populations in its path causing economic hardship for beekeepers. For those beekeepers now living with the mite, reliance on expensive acaricides to keep honeybee colonies alive has become part-and-parcel of everyday beekeeping.

All *Varroa* mites are highly specialised external parasites that feed exclusively on the blood (hemolymph) they take from the capped brood or adult bees of their honeybee hosts. On their primary hosts, strains of *A. cerana*, all *Varroa* mites are relatively harmless. However, the mites that switched host to *A. mellifera* are lethal to that bee. This difference in susceptibility is mostly due to the ways that adult female mites reproduce on the two bees.

On *A. cerana* female mites reproduce exclusively on the capped brood stages of drones after entering the cells prior to capping. They do not reproduce on worker brood even though they attempt to if no drone cells are present or if they enter worker brood cells by accident. The reason why this form of reproduction is harmless is due to several factors. Firstly, *A. cerana* colonies tend to produce drones only when needed and, then, in relatively low numbers. Hence, female mites often find susceptible drone cells hard to find. Secondly, at the time drone cells are produced, mite populations are small, having been decimated during the period when no drone cells were present through the well documented grooming behaviour of *A. cerana* workers (Peng *et al.*, 1987). Hence, by the time mite populations have built up to damaging threshold, enough drones have escaped parasitism as brood for the colonies' reproductive needs.

Thirdly, and most importantly, the exclusive restriction of mite reproduction to *A. cerana* drone brood means that the brood stages of the more productive worker bees escape the damaging feeding effects of the female mites and their offspring (Koeniger *et al.*, 1983).

On *A. mellifera*, the varroa mite encounters few defences and it can reproduce on both drone and worker brood. An ever-abundant worker brood population allows mites to build-up to large numbers and debilitate the adult worker bee population through their feeding action. This, coupled with the effects of viruses that the mites spread or activate, can be lethal to colonies (Fries, 1993; Ball, 2001).

1.3 Identity of *Varroa jacobsoni* questioned

Until 2000, it was assumed that the mite that switched host to *A. mellifera* was *V. jacobsoni*. However, the real identity of *V. jacobsoni* was first questioned in the 1980's following the discovery by US researchers that female *V. jacobsoni* infesting *A. cerana* in Asia were smaller than those infesting *A. mellifera* in the US (Delfinado-Baker and Houck, 1989). In the following decade other researchers found genetic differences between *V. jacobsoni* infesting *A. cerana* and *A. mellifera* (Kraus and Hunt, 1995; Anderson and Fuchs, 1998; De Guzman *et al.*, 1998; De Guzman and Rinderer, 1999). However, it was Australian research in Java and New Guinea during the early 1990's that raised the most serious questions on the identity of *V. jacobsoni*.

Honeybees are not indigenous to New Guinea but the island is now inhabited by both *A. mellifera* and *A. cerana*. Regular introductions of *A. mellifera* have been made into the eastern (Papua New Guinea) and western (Papua) parts of the island since the 1940's. *A. cerana* was also introduced to the western part from Java during the 1970's. This introduction introduced *V. jacobsoni*. Hence, the *Varroa* mites that were introduced to New Guinea would have been descendents of mites that Dr Oudemans first described as *V. jacobsoni* from Java in 1904.

In New Guinea the introduced mites were observed to spread at low levels from *A. cerana* to *A. mellifera* colonies. Once there, they proceeded to enter worker and drone brood cells to prepare for reproduction, favouring drone over worker brood. However, they could not go on to produce eggs or offspring, either on the worker or drone broods. Checks in Java confirmed this behaviour (Anderson, 1994).

1.4 The uncovering of *Varroa destructor*

The peculiar behaviour observed in Java and New Guinea could not be explained from what was known about *Varroa* mites on *A. mellifera* in other regions. Hence, in the mid to late 1990's, studies were directed at finding out whether the inability of female mites to produce offspring on *A. mellifera* in New Guinea and Java was due to mite-resistant bees, an environmental component, or to the mites themselves.

To find out whether the *A. mellifera* were resistant, sister queen bees were raised in Western Australia and half moved to New Guinea and half to Germany (where *Varroa* mites were reproducing on the local *A. mellifera*). In New Guinea the *Varroa* mites failed to reproduce on the broods of the imported queens, but in Germany the *Varroa* mites did. This indicated that the mite behaviour in New Guinea was due to either an environmental factor or to the mites themselves.

An environmental factor was ruled out when *Varroa* mites in Java were first observed reproducing on *A. mellifera* during the mid-1990's, initially only in one location. The reproducing mites looked like the pre-existing non-reproducing ones except for their size: they were much larger. Closer observations showed that the smaller and larger mites were present in the same *A. mellifera* colonies, with the larger ones going on to reproduce and the smaller ones lacking the ability to reproduce. These observations indicated that the larger mites had been recently introduced into Java.

They also indicated that the reason for the smaller mites being unable to reproduce on *A. mellifera* was not environmental, but must have been due to a different type of mite (Anderson and Sukarsih, 1996). A comparison was then made of the DNA of the larger and smaller mites as well as the large mites in Germany and the small mites in New Guinea. It showed that the large and small mites were genetically different (Anderson and Fuchs, 1998).

To gain a better understanding of these genetic differences, a detailed study was carried in the late 1990's to find the extent of genetic variation among different populations of *V. jacobsoni*. Again, DNA methods were employed. Initially, only mites infesting *A. cerana* were investigated as that bee is the primary host of *Varroa* mites and, therefore, the mites on it would be expected to show the most variation.

In all, 18 genetically different mites were found. A computer-assisted cladistic analysis assigned them to one of 2 distinct species, while the identity of another four mites was unresolved. One of the two resolved species, redefined as *V. jacobsoni*, comprised several relatively small genotypes that infested *A. cerana* in the Malaysian-Indonesian region. Included was a Java genotype, specimens of which were used to describe *V. jacobsoni* nearly a century earlier. The other species, which comprised much larger mites, was new to science and was named *V. destructor*. It embraced genotypes that infested *A. cerana* in Japan and on mainland Asia. Of the 4 unresolved mites, one came from Sri Lanka and the other three from islands in the Philippines (Anderson, 2000; Anderson and Trueman, 2000).

Next, the identity of *Varroa* mites infesting *A. mellifera* in 32 countries was investigated. All were found to be *V. destructor*, and not *V. jacobsoni* as had been assumed. However, only 2 of the 6 *V. destructor* genotypes found on *A. cerana* were found infesting the *A. mellifera*. The most common was a Korea genotype of *V. destructor*, so-called because its primary host is *A. cerana* in Korea. It was found on *A. mellifera* in Europe, the Middle East, the Americas, Africa and Asia. The second genotype found on *A. mellifera*, the so-called Japan genotype of *V. destructor*, was only found in Japan, the Americas and Thailand (Anderson and Trueman, 2000). Recent research has also found very little genetic variation amongst the Korea type mites on *A. mellifera*, suggesting that they have all originated from a single female parent mite (Solignac et al., 2005).

1.5 Towards a new control for *Varroa destructor*

Since the discovery of *V. destructor*, a further 5 genotypes of *V. jacobsoni* or *V. destructor* have been found on *A. cerana*, bringing the number of known genotypes to 23 (Koeniger et al., 2002; Zhou et al., 2004). Each of these genotypes has been co-speciating on a particular genotype of *A. cerana* for a long time in complete isolation from *A. mellifera* (Anderson, unpublished data). It is only in recent times that those genotypes have come into contact with *A. mellifera*, following the introduction of that bee into Asia by humans. However, only 2, the Korea and Japan genotypes of *V. destructor*, are capable of reproducing on *A. mellifera* (Anderson and Trueman, 2000). The other genotypes are harmless to *A. mellifera* as they all lack the ability to reproduce on that bee (Anderson, 1994; Fuchs et al., 2000; Anderson, 2004). Finding the reason why those mites cannot reproduce on *A. mellifera* could present a cure for the varroa mite problem worldwide.

In theory, if one *Varroa* genotype can reproduce on *A. mellifera* then all *Varroa* genotypes should be able to, as they are all closely related. Indeed, all *Varroa* genotypes will attempt to reproduce on *A. mellifera* if given the opportunity (that is, when *A. mellifera* is introduced into areas of Asia where those genotypes live naturally on *A. cerana*). The reason why more than 90% of the genotypes cannot reproduce on *A. mellifera* lies in the way those genotypes have evolved on their own particular genotype of *A. cerana*.

As mentioned, all female *Varroa* mites only reproduce on capped drone brood of *A. cerana*. To reproduce on the drone brood they also all go through the same suite of behaviours.

After emerging from the drone cells with the emerging drones, young female mites move straight onto adult drone or worker bees where they hide, feed and mature in intersegmental spaces while awaiting the opportunity to re-enter drone brood cells to reproduce. Hence, a female *Varroa* mite spends the majority of her life hidden inside capped drone brood cells or in intersegmental spaces on adult bees. Outside of these two areas the mite becomes susceptible to attack by worker bees. Hence, the female will only leave an intersegmental space when she detects a brood pheromone that tells her she is above a susceptible drone brood cell (one that contains a drone prepupae at the precapping stage). At this point she extracts herself from the intersegmental space and drops off the adult bee into the cell below and immediately moves to the bottom of the cell and completely buries herself upside-down in the edge of the bowl of brood food, with just her breathing tubes (peritremes) protruding through the surface of the food. Worker bees then cap the cell. The drone larva inside the cell proceeds to consume the remaining food (eating outwards from the centre of the bowl) and, as it consumes the last of the food at the edge of the food bowl, releases the female mite. The mite then moves onto the body of the larva and shortly afterwards begins to lay eggs. The first egg is unfertilised and becomes a male while the others (usually up to 5) develop from fertilized eggs and become females. Occasionally, female mites will drop into a worker cell by mistake. In these cases they proceed through the same pre-egg-laying behaviour as females on drone larvae, but they do not go on to produce eggs or offspring.

All *Varroa* mites also show this same suite of behaviours when they find themselves inside an *A. mellifera* colony and are ready to reproduce. In other words, they have recognised behavioural cues (signals) on *A. mellifera* as being the same or similar to those they recognise on their native *A. cerana*. However, after entering brood cells at the precapping stage, only the Korea and Japan genotypes of *V. destructor* recognise the “begin egg-laying cue” on *A. mellifera*. German researchers have shown that when reproducing female Korean *V. destructor* are removed from *A. mellifera* cells and transferred into freshly capped cells they begin a new reproductive cycle by laying a male egg. This shows that a host (bee) cue (signal) present in freshly capped brood cells triggers both the start of mite reproduction and the subsequent sequence of sexes (Garrido and Rosenkranz, 2003).

The signal (or signals) that triggers varroa mite reproduction will almost certainly be a chemical that interacts with a mite receptor. In isolated populations of *A. cerana* it is very likely that, through evolutionary time, both the signal and the receptor have changed slightly through the action of mutation and natural selection. For example, a beneficial mutation in an isolated population of *A. cerana* could produce a signal that is more difficult for mites to recognise. Natural selection would then favour the survival of offspring of the few females that were better able to recognise the changed signal. Hence, over time, both the signal and receptor would change unpredictably to various degrees in the different *A. cerana* populations. Evidence that this has occurred can now be seen in parts of Asia when exotic honeybees (*A. mellifera*) come into contact with *A. cerana* carry particular genotypes of *Varroa* mite. In these instances, the different mite genotypes enter the *A. mellifera* colonies and invade susceptible brood cells, favouring drone over worker brood. After entering the cells they even enter the brood food in preparation for reproduction. Hence, up to this point, the mites have recognised the chemical signals that have guided them into the colony and brood cells of *A. mellifera* as being the same or similar to those inside colonies of their native *A. cerana*. However, only the Korea and Japan genotypes of *V. destructor* then recognise the signal on *A. mellifera* that tells them to commence egg laying. Furthermore, the Japan genotype does not appear to recognise the signal as well as the Korea genotype. This indicates that the egg-laying signal must vary or be released in different quantities or at slightly different times in different populations of *A. cerana*.

Identifying this signal could present a new way of controlling the Korea and Japan haplotypes of *V. destructor* on *A. mellifera*. Once the signal has been found, then various approaches can be pursued to produce varroa-resistant bees. The simplest would be to search for *A. mellifera* populations that produce a signal profile that is outside the receptive range of the Korea and Japan genotypes of *V. destructor*. Such bees could be easily propagated in isolation to produce varroa-resistant bee lines. Another approaches might be to feed bees a chemical analogue of the signal to confuse mites.

Yet another approach might be to employ genetic engineering techniques to produce bee-lines with signal profiles that mites cannot recognise. This latter approach has the possibility of completely eliminating the varroa mite as a pest of *A. mellifera*.

1.6 The general strategy and aim of the current project

The signal that triggers egg-laying in female *V. destructor* is produced by the bee and is present somewhere inside the capped cell during the first 70 hours after the cell is capped, as it is during this time that the first egg is laid. German researchers have determined that the signal exists in *A. mellifera* larval instar 5 (L5) but not in white-eyed pupae and could either be a nutritional or volatile chemical (Garrido and Rosenkranz, 2003).

In the studies here, the search for the signal is being carried out in *A. cerana* on which female mites reproduce on drone but not on worker brood (note: the mites cannot reproduce on worker brood even though they attempt to). Hence, on *A. cerana*, the signal will be present in drone L5 but not worker L5. Furthermore, once the signal is found in *A. cerana* drone L5 it should be relatively straight forward to find its equivalent in *A. mellifera* worker and drone L5. To narrow the field-of-search for the signal a model of the varroa mite reproduction system will be developed. This model will pinpoint the precise time when egg laying is first switched-on, thereby allowing for a very directed search for the signal. The model will be developed by tracing changes that occur in certain internal body tissues of female mites (such as nerve, fat and muscle tissue) after they enter susceptible bee cells up to the point where they commence egg-laying. However, before work can commence on developing the model, a procedure is needed to allow those changes to be traced.

The aim of the current project was to find a procedure that could differentiate the different internal body tissues of female *Varroa* mites. Following this project, it is anticipated that the procedure will be used in a second-stage project (of 3-years' duration) to develop a model of the varroa reproduction system. With that model in place, a third-stage project (also of 3-years' duration) will be needed find the signal that triggers mite reproduction and produce varroa-resistant bees.

2.0 Stage 1 – A Procedure for Differentiating Internal Body Tissues of Female *Varroa* Mites

2.1 Introduction

Very little is known about the *Varroa* mite reproductive system. Current knowledge is limited to the number and gender of offspring produced within a 70-hour time frame after adult female mites enter susceptible bee brood cells. More precise information than this is needed if the signal that switches-on mite egg-laying is to be found. Hence, in the second stage of this project it is proposed to construct a model of the *Varroa* mite reproductive system that will pinpoint the time when egg-laying is first initiated, so that a directed search can then be undertaken for the signal. Without such a step-wise approach it will be almost impossible to find the signal.

The aim of the current project was to find a procedure that will assist with the model building. This procedure must allow for monitoring of changes in the tissue of several different organs of female *Varroa* mites from the time they enter susceptible bee brood cells until they commence egg laying.

A light microscopy method was identified here that met that requirement. It involves collecting live mites from the field, embedding them in paraffin wax, obtaining ultra-thin sections from them and staining the sections with a specific stain.

2.2 Methodology

Collection and identification of mites

Ten specimens of each of the following mites were collected in Asia:

- (a) Reproducing female *V. destructor* from capped worker cells of a single *A. mellifera* colony on Luzon Island in the Philippines;
- (b) Non-reproducing female *V. jacobsoni* from worker cells of a single *A. mellifera* colony in Papua Province (formerly called Irian Jaya), Indonesia; and
- (c) Reproducing female *V. jacobsoni* from drone cells of a single *A. cerana* colony in Papua Province, Indonesia.

These mites were collected in 70% alcohol, transported under quarantine permit to the laboratory in Canberra and stored at -20°C until needed.

To confirm the identity of mites in the samples, DNA sequences were obtained from 2 mites in each sample, using methods described by Anderson and Trueman (2000).

Histology

In preliminary studies, one mite from sample (a) above was transferred to 70% then 90%, 95% and 3 changes of 100% alcohol, two changes of chloroform and 4 changes of hot paraffin wax, before being embedded into fresh hot paraffin wax. When cool, the paraffin block was sectioned using a feather S35 microtome blade in a Leitz 1512 microtome. The serial transverse 4µm ultra-thin sections were spread onto water, collected on glass microscope slides and dried in a hot air oven at 60°C for 1 hour before being de-waxed, stained and covered with glass cover slips. The cover slipped slides were then dried at room temperature overnight before being examined with a light microscope. Initially, several different stains were tested to see how well each differentiated mite tissue.

A Haematoxylin-Eosin stain (Gill's no. III Haematoxylin and 1% alcoholic Eosin) was found to best differentiate muscle, fat and nervous tissue and was therefore used in all further work.

Next, one entire mite from each of the 3 samples described above was embedded and serially transversely sectioned in a continuous fashion along its entire length. The stained sections were then examined with a light microscope.

2.3 Results

The tissue-sectioning technique used in combination with Haematoxylin-Eosin staining clearly differentiated nerve, fat body, ovary and muscle body tissues of both *V. jacobsoni* and *V. destructor* (see Appendix 1).

On average, 260 serial transverse sections were obtained from each of the 3 mites. Each section was digitally photographed and the photographs transferred on to DVD's for further use. The sections obtained from a female *V. destructor* are now being used in a collaborative study with Dr Paul Cooper from the Australian National University to construct a visual 3-D mite model, showing the shape and layout of its various body organs and tissues. Note that this model is different from the one that will be developed to pinpoint the time when *Varroa* mite reproduction is first initiated, but will nevertheless help during the development of that model.

3. Concluding Remarks

A light microscopy tissue-sectioning technique utilizing the Haematoxylin-Eosin stain was found to be superior to other procedures for differentiating the different internal body tissues of *Varroa* mites. The stain haematoxylin is not a dye, but develops colouring properties on oxidation to haematin. Eosin is an acid dye, which requires an acidic environment to work. For these reasons the haematoxylin stains the nuclei of cells whilst the cytoplasm is coloured by the eosin.

This technique will prove a useful tool during the proposed stage-two work of this project, which is to develop a model of the *Varroa* mite reproduction system. When developed, that model will be capable of pinpointing the time when reproduction is first initiated in female *Varroa* mites after they enter susceptible bee cells. This, in turn, will allow for a directed search for signals responsible for initiating mite reproduction and then to the production of varroa-resistant honeybees.

The development of varroa-resistant honeybees will open new markets for Australian-bred queen bees and safeguard Australian honey producers against losses in the event of a varroa mite incursion.

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5. Appendix 1

Transverse cross-sections of 3 different regions of a reproducing female *V. destructor* mite (indicated by the red lines) stained with Haematoxylin-Eosin to clearly show muscle (M), nerve (N), ovary (O) and fat body (FB) tissue. Note that the dorsal surface of the mite is the bottom edge of each cross-section.

