Contents

Effective date: 21/09/15

Protocol established according to the ANA-I1.MOA.35_Rev01

This document describes a general protocol. Therefore neither reference nor names of equipment, reagents or consumables are given.

1. PURPOSE AND SCOPE

1.1 Background

Mites of the genus *Tropilaelaps* are ectoparasites of bee brood. They feed on the larvae and nymphs, and cause malformations, mortality and gradual colony decline. Their development cycle is approximately one week. Adult mites can be carried on the bodies of adult bees (phoresy), but are unable to feed (inability to pierce the cuticle) and, therefore, cannot survive for more than 74 hours.
Four species of the genus *Tropilaelaps* have been described: *Tropilaelaps clareae* (Delfinado and Baker, 1961), *T. mercedesae* (Anderson and Morgan, 2007), *T. koenigerum* (Delfinado-Baker and Baker, 1982) and *T. thaii* (Anderson and Morgan, 2007). Originally, these species were exclusively ectoparasites of the giant honey bee *Apis dorsata*. However, following the introduction of the European honey bee *A. mellifera* into regions infested with *Tropilaelaps*, two species, *T. clareae* and *T. mercedesae*, successfully adapted to parasitize this new host.
The following protocol describes the identification of adult mites of the genus *Tropilaelaps* by visual examination, in the event of a suspected infestation of colonies or during the routine examination of imported batches of bees, queen bees and bumble bees. The visual examination described in this protocol is not sufficient to provide adequate differentiation amongst the four species of *Tropilaelaps* as they are morphologically very similar (Anderson and Morgan, 2007; Tangjingjai *et al*., 2003).
Europe is currently free of this parasitic disease. Infestation of honey bee colonies by mites of the genus *Tropilaelaps* is regulated both within the European Union (EU) and internationally (OIE, 2008). In order to limit the risk of introducing this exotic pest into the EU, regulation (EU) no. 206/2010[1] requires that all imports of both honey bee and bumble bee queens from non-EU countries are subject to laboratory examination for the presence of this pest.
Clinical signs of colony and bee infestation by *Tropilaelaps* mites and *Varroa destructor* mites, which is endemic throughout Europe, are very similar. Therefore, it is essential to determine the causal mite by morphological examination.

Note on bumble bees (*Bombus* spp.):
According to existing literature, *Tropilaelaps* spp. mites have not yet been found in bumble bee colonies (*Bombus* spp.)[2]. However, *Varroa* has been found on foraging insects other than honey bees, including bumble bees (*Bombus pennsylvanicus),* despite its inability to reproduce in their colonies (Kevan *et al*., 1990). This suggests that phoretic transfer on bumble bees might also be considered to be a potential source for the introduction and spread of *Tropilaelaps* mites.

1.2 Biology of *Tropilaelaps* spp.

Tropilaelaps mites can be found on adult bees (phoretic phase) and/or in capped brood and are visible to the naked eye.

[1] Commission regulation (EU) No 206/2010 of 12 March 2010 laying down lists of third countries, territories or parts thereof authorised for the introduction into the European Union of certain animals and fresh meat and the veterinary certification requirements.
[2] Commission decision of 11 December 2003 regarding the health control and certification conditions governing the importation of Apidae (*Apis mellifera* and *Bombus* spp.) from certain non-EU countries and which amends Commission decision 2000/462/CE.

Gravid female *Tropilaelaps* mites invade the brood cell immediately before cell capping, and show a slight preference for drone (male) brood. Several females can infest a single brood cell. They begin egg laying 48 hours after infestation at a rate of one egg per day, each female laying up to four eggs. The eggs hatch after 12 hours and the larvae then feed on the haemolymph of the developing bee pupa. When the bee reaches adulthood and leaves its cell the parasite also emerges from the cell in search of a new host. The life cycle of the mite from egg to adulthood is approximately one week.
In contrast to the *Varroa* mite, *Tropilaelaps* is incapable of feeding directly on adult bees due to its inability to pierce the cuticle of the bee's body. However, when *A. mellifera* is infested by both parasites *Tropilaelaps* is the more serious problem, because the shorter life cycle means that the population is able to multiply at 25 times the rate of the *Varroa* mite.
The main pathway for the dissemination (or spreading) of this parasite is the phoretic movement of female mites on adult bees. Females have been found 18 times more frequently on adult bees than males (Fernandez and Coineau, 2007; OIE, 2008).

Clinical signs of infestation by mites of the genus *Tropilaelaps* (also encountered in cases of varroosis) are:

- **Bees**
- Weak, crawling
- Deformed and/or atrophied wings, legs, antennae
- Shrunken abdomens
- Phoretic mites present (female *Tropilaelaps*)
- Pupae with dark spots

- **Brood**
- Parasites present (on frames, adult bees, larvae and pupae)
- *Tropilaelaps* mites move much more quickly than *Varroa*
- Spotty brood
- Pierced cappings
- Dead brood present
- Brood with malformations: emerging bees/pupae with atrophied and/or deformed wings and legs, shrunken abdomens

If some or all of these symptoms are observed the material should be treated as suspect and the appropriate control measures adopted until the cause is determined. Information regarding any introductions to the apiary may also be useful in determining the risk of possible *Tropilaelaps* infestation.

Note: Due to the sanitary risk implied by this exotic parasite, the analysis must be done immediately after reception of the sample, in order to confirm or not the suspicion and to enable early implementation of official sanitary measures.

2. CONTENT

2.1 Principle

The scope of this method is to enable the identification of adult mites from suspect colonies or imported packages of bees or queen bees as belonging to the genus *Tropilaelaps*. The samples analysed may be of differing specimen types, e.g.: mites, other insects, etc.
The method is based on the visual examination of adult mites only and takes into account the morphological characteristics of the adult *Tropilaelaps* mite compared with those of other mite genera commonly found in bee colonies in Europe (particularly *V. destructor*). It is based on the diagnostic criteria referred to in chapter 2.2.6 of the OIE Terrestrial Manual (OIE, 2008) and in various scientific publications (Anderson and Morgan, 2007; Delfinado and Baker, 1961; Fernandez and Coineau, 2007).

2.2 Materials

- Fine-tipped tweezers
- Micro-dissecting needle holders equipped with minutien pins and with pins made of fishing line (with the extremity crushed in order to obtain a spoon-like shape)
- Dishes: glass Petri dishes, porcelain ceramic dishes, watch glass or similar
- Hermetic vials (hermetic seal)
- Microscope glass slides (classic and concave) and cover slips
- Lactic acid
- Mounting medium (e.g.: Hoyer's medium) and clear nail polish for the long term conservation of the microscopic slides
- Ethanol diluted to approximately 70% (not denatured)
- Stereomicroscope
- Compound microscope (1000x)
- Heating plate

2.3 Protocol

Note: Opening samples received by the laboratory.
The sampling instruction sheet sent to a customer specifies that any mite suspected of belonging to the genus *Tropilaelaps* must be killed before being submitted to the laboratory.
In case of doubt, the package must be opened in containment conditions.
If the specimens arrive alive to the laboratory, the sample must be placed in thermal confinement at around -80°C for a period of approximately one hour before being opened. This procedure immobilises the specimens in order to avoid their release into the environment.
Afterwards, the specimens are placed in a tube with ethanol 70%.

a) Lay-out of the work area

Clean the work area before the analysis and prepare the material required.

b) General observation of the specimens and sampling for analysis

- Place all the specimens in a dish
- Examine all the specimens with the stereomicroscope to verify the homogeneity of the sample.
- Take at least 10 specimens, or all of them if less than 10, using fine-tipped tweezers or needle holders (fishing line equipped).
- Place them in a dish.

Note: try to choose specimens that are not damaged**.**

c) Observation with the stereomicroscope

Examine the specimens in their entirety and appreciate some general characteristics for the identification (criteria n°1 to 3, detailed in paragraph 2.4).

d) Mounting specimens on glass microscope slides

The objective of this step is to clear the soft tissues in order to facilitate the observation of certain morphological characteristics.

- Deposit a few drops of lactic acid on a microscope slide.

  Note: use concave slides for big specimens.
- Place the selected specimens on the slide in lactic acid with the needle holders (fishing line equipped) (or with extra-fine tweezers).
- Using two holders (minutien pin equipped), position the specimens so as to have a ventral view.
- Place a cover glass over the microscope slide without crushing the mite and avoiding the formation of air bubbles.
- If possible: carefully press on the cover glass with a tweezer in order to spread open the legs which are curled up beneath the body.
- Place the slide on a heating plate (set to approximately 50 °C) and wait for the lactic acid to take effect (approximately 30 minutes).

  Note: the liquid should not boil on the slide (it would destroy the specimen).

e) Observation with the compound microscope

- Examine the slide(s) under the compound microscope at 100x, 200X, and then 400X magnification in order to fully observe the various diagnostic criteria (detailed in paragraph 2.4).
- Reference microscopic slides could be observed for comparison if available.

Note: depending of the thickness of the mite's body, you may need to vary field depth.

f) Results recording

Write down the results on a result sheet.

g) Sample conservation

- *Ethanol storage:*

Place the specimens in a hermetic vial with ethanol 70 %. They can be stored at room temperature.

Note: Denatured ethanol should not be used if molecular analyses are programmed.

- *Mounting on microscope slides for long term storage:*

Deposit a few drops of the mounting medium (e.g. Hoyer's medium) on a microscope slide.

Place and position the mites on the slide in the medium.

Place a cover glass over the slide.

The drying time depends on the medium used (two weeks at approximately 50°C for Hoyer's medium; for long term storage or for transporting, the edges of the cover slip should be sealed with water-resistant material such as clear nail polish).

Note: Detailed information concerning the storage and the mounting of mites is available in the Beebook chapter on *Varroa* mites (Dietemann *et al*., 2013).

2.4 Identification of the adult *Tropilaelaps* spp. mite

Tropilaelaps spp. belong to the class Arachnida, subclass Acari, order Parasitiformes, suborder Mesostigmata and family Laelapidae (Smiley R.L., 1991).

They should not be confused with the mite *V. destructor*, which is a member of the same family and a parasite that is well-established in Europe.
Tropilaelaps is visible to the naked eye. It is approximately between 0.6 mm and 1.0 mm long and between 0.4 to 0.5 mm wide. *Tropilaelaps* is smaller than *V. destructor* (Figures 1, 2, 3, 4, and 5).

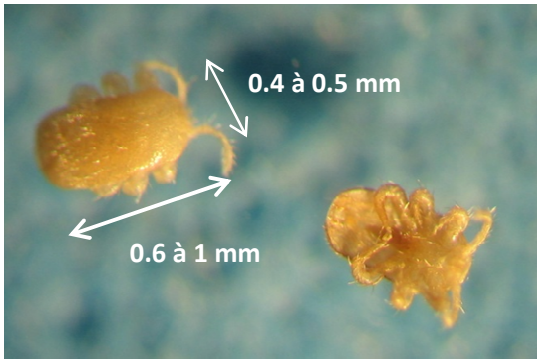


Figure 1 – *Tropilaelaps* spp., as seen through a stereomicroscope.
Source: Anses, Sophia Antipolis.

CRITERIA FOR RECOGNISING *TROPILAELAPS* SPP. (Anderson and Roberts, 2013; Delfinado and Baker, 1961; Smiley R.L., 1991, University of Michigan, 2013):	**Binocular microscope**	**Compound microscope**
1. Tropilaelaps has 4 pairs of legs. The first pair is vertically aligned, resembling antennae (Figure 2). → Class Arachnida	**X**	
2. The body is unsegmented, with a single visible region, due to the fusion of the prosoma (the equivalent of the cephalothorax) and the opisthosoma (or abdomen) into a single mass (Figure 2). → subclass Acari	**X**	
3. The body is longer than wide (as opposed to *V. destructor*) (Figures 3, 4, and 5). The ratio of length to width is greater than 1.3.	**X**	
4. It has a pair of latero-ventral stigmata[3] between coxa[4] III and IV (Figures 6, 7, and 8). → Order Parasitiforms		**400X**
5. Presence of elongated peritremes[5] (Figures 6, 7, and 8). Presence of a tritosternum[6] (Figures 6 and 7) (optional criterion, difficult to observe). → Suborder Mesostigmata		**200X**
6. Elongated epigynial plate, posteriorly rounded or sharp. Triangular-shaped ventrianal plate (Figures 2 and 7). → Laelapidae		**100X**
7. Elongated epigynial plate, at least twice as long as the ventrianal plate (Figures 2 and 7).		**100X / 200X**
8. Reticulated sternal plate[7] (Figure 7).		**400X**
9. Opisthosoma with coarse bristles, thick at the base, on the apical half of the ventral side (Figures 7 and 9).		**200X**
Comment: Criteria for distinguishing between males and females: the mobile digit of the male's chelicerae is filiform (spermiodactyls) (Figures 10, 11, and 12). The epigynial plate is shorter in the male than in the female (Figure 10).		**200X**

[3] The stigmata are tracheal openings in Arthropods.
[4] The coxa is the first leg segment of Arthropods which connects the leg and the body.
[5] The peritremes are tubular structures running on from stigmata. They could have a role in the respiration.
[6] The tritosternum is a bristle-like Y-shaped sensory organ located caudally to the gnathosoma (the gnathosoma is the body part of Acari that includes the mouthparts and oral aperture).
[7] Reticulated means that it has broken eggshell or fishscale pattern.

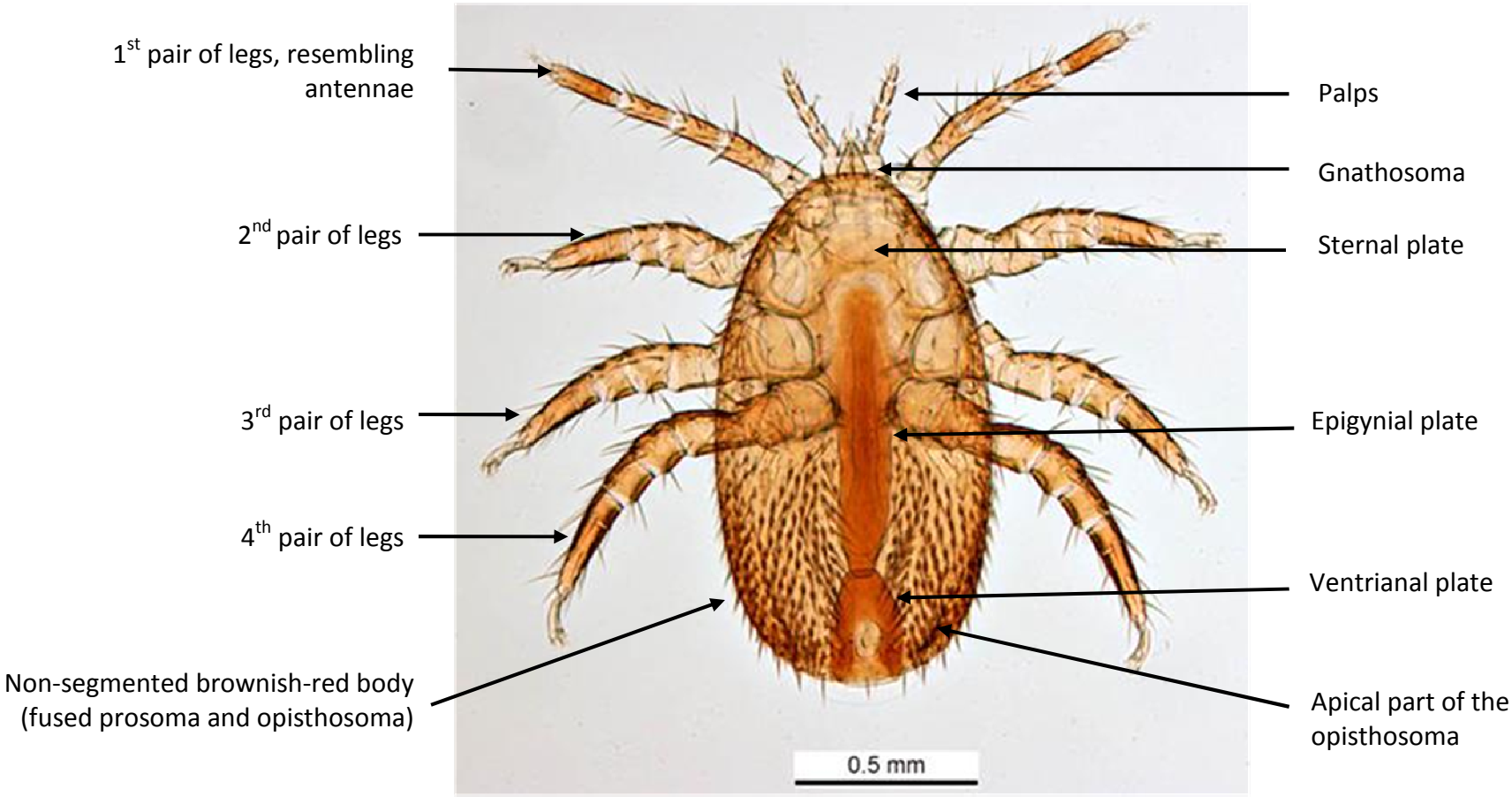


Figure 2 - *Tropilaelaps mercedesae*, female (ventral view).
Source: Ken Walker, Victoria Museum, Australia.

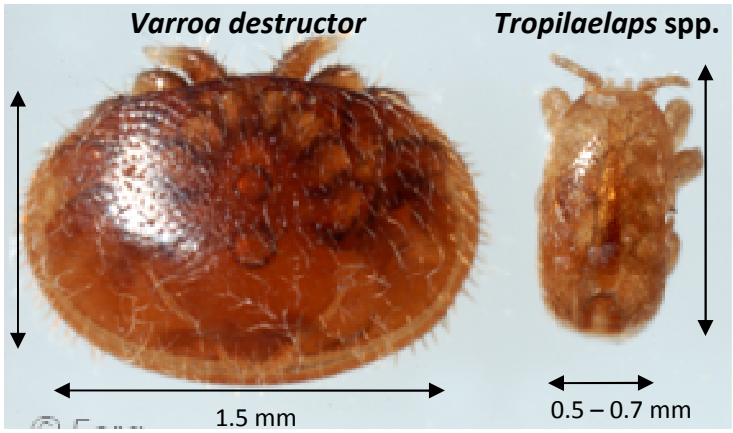


Figure 3 – *Varroa destructor* and *Tropilaelaps* spp. (dorsal view).
Source: Fera, Crown copyright.

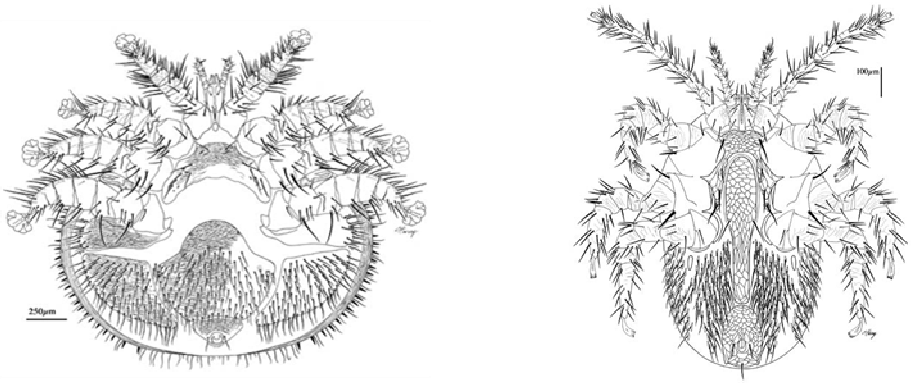


Figure 5 –*Varroa destructor* and *Tropilaelaps* spp. (ventral view).
Source: Walter et al., 2006.

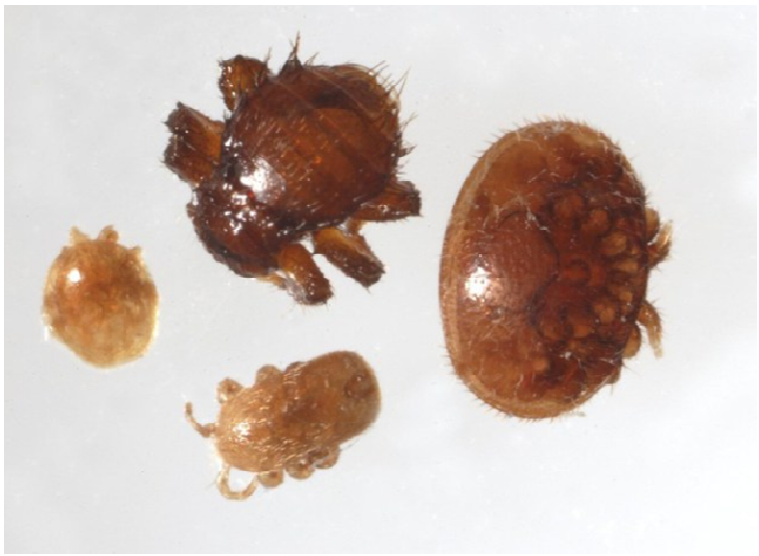
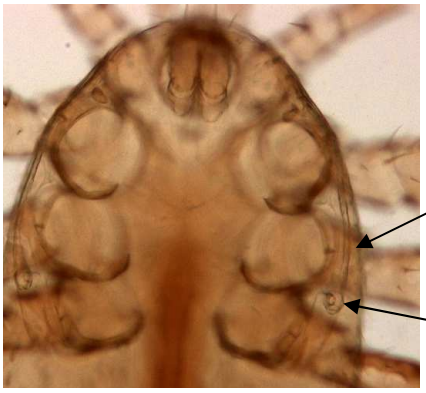
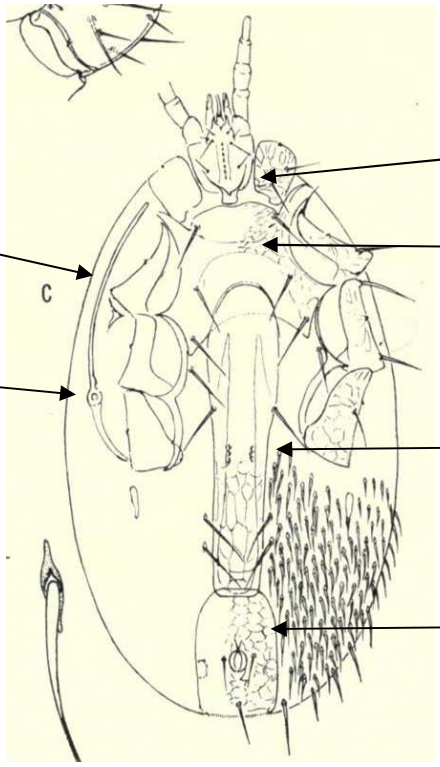


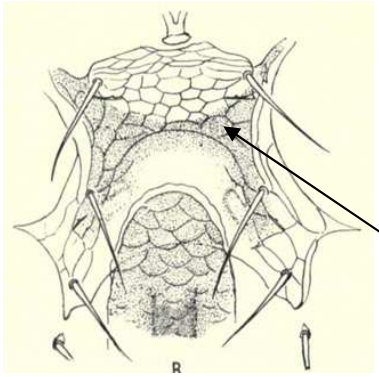
Figure 4 – *Braula coeca* (above), *Varroa destructor* (right), *Tropilaelaps* spp. (below centre) and *Melittiphis alvearius* (left) (dorsal view).
Source: FERA, Crown copyright.



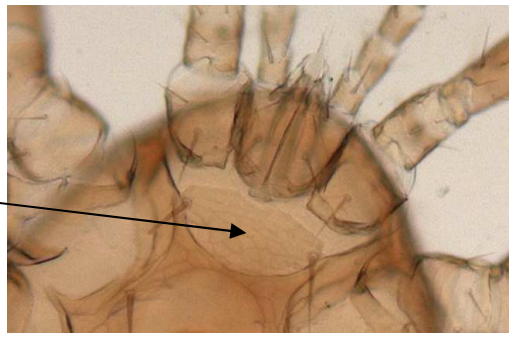
T. clareae – Microscope 100X
Source : Anses



T. clareae, female (ventral view)

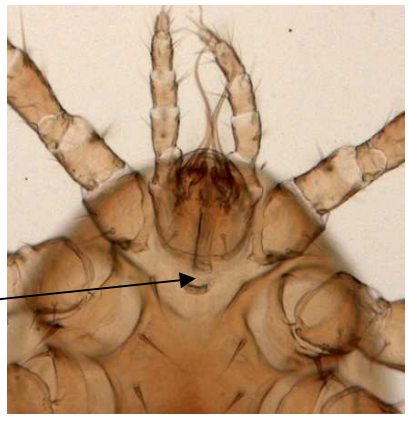


Sternal plate and epigynial plate (ventral view)



T. clareae – Microscope 200X
Source : Anses

Gnathosoma (ventral view)



T. clareae – Microscope 200X
Source : Anses

Figure 6 - *Tropilaelaps clareae*, anatomy.
Source: Delfinado and Baker, 1961.

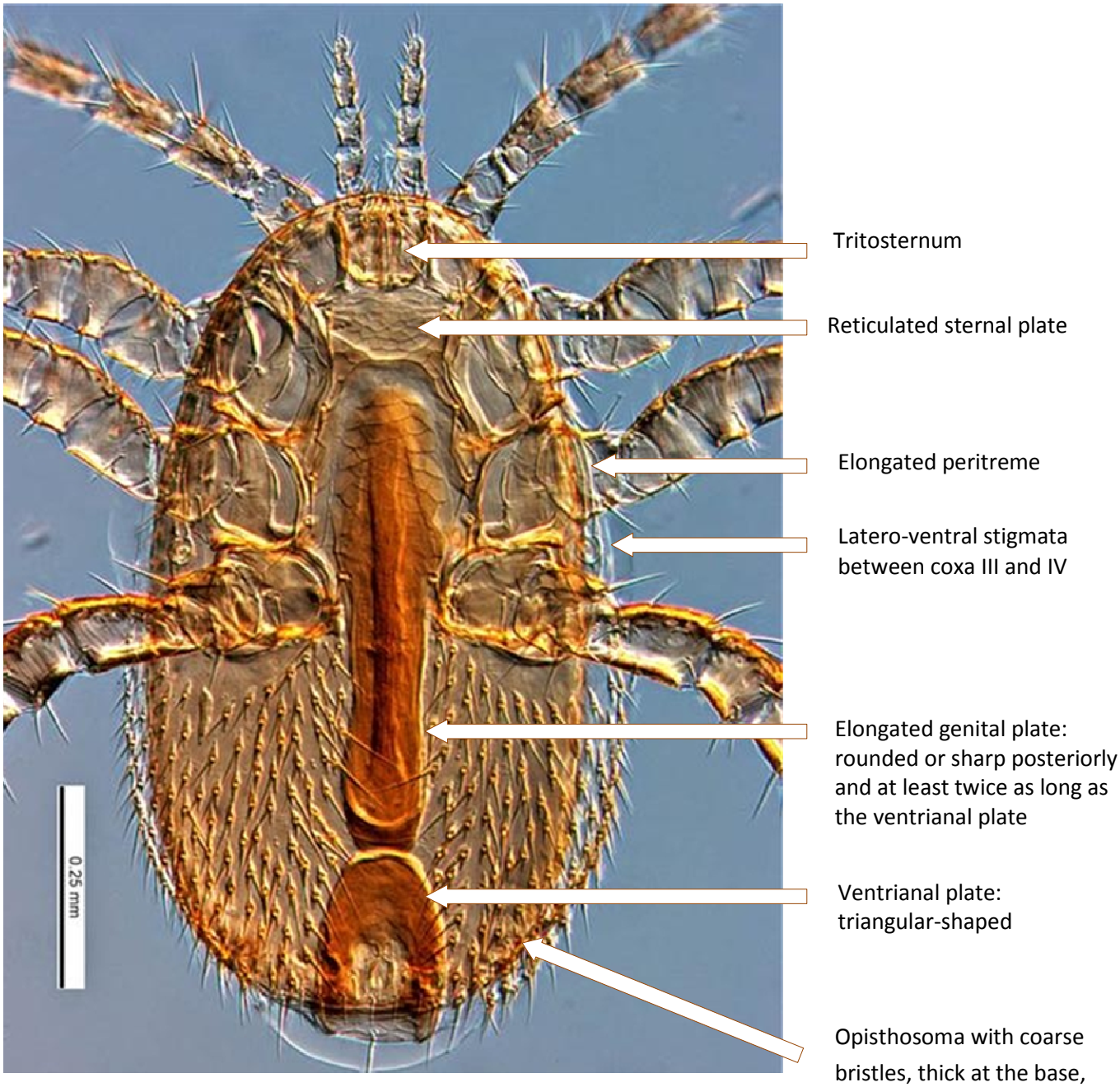
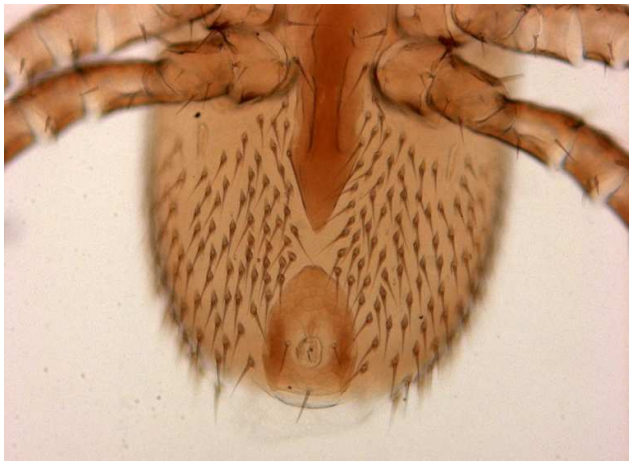
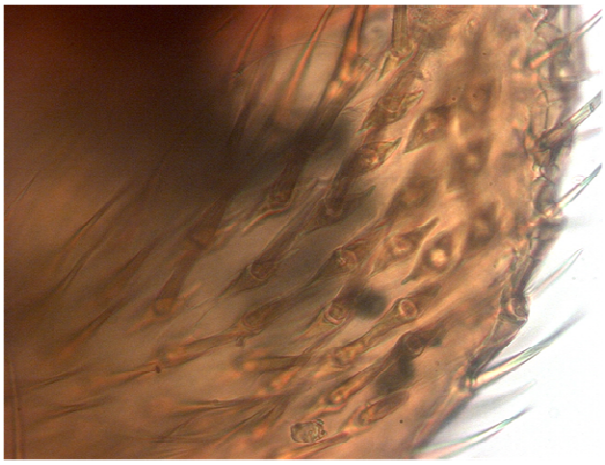


Figure 7 - *Tropilaelaps clareae*, female (ventral view).
Source: Ken Walker, Victoria Museum, Australia.

Figure 8 - *Tropilaelaps* spp. - Lateroventral view. Stigmata between coxas III and IV. Microscope 200 X
Source: Anses, Sophia Antipolis.



Microscope 100X



Microscope 400X

Figure 9 - *Tropilaelaps* sp. (ventral view). Opisthosoma, coarse apical bristles, thick at their base.
Source: Anses, Sophia Antipolis.

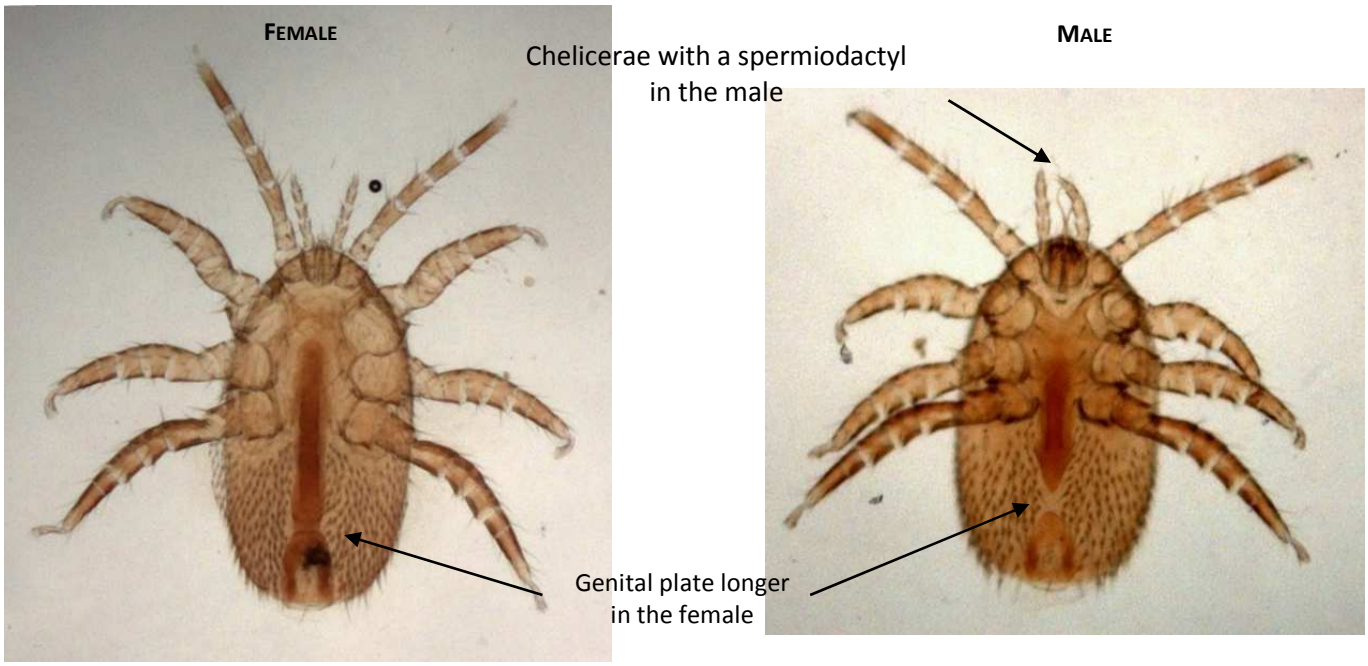


Figure 10 – *T. clareae*, male and female (ventral view).
Source: Anses, Sophia Antipolis.

Spermiodactyl not present in the female

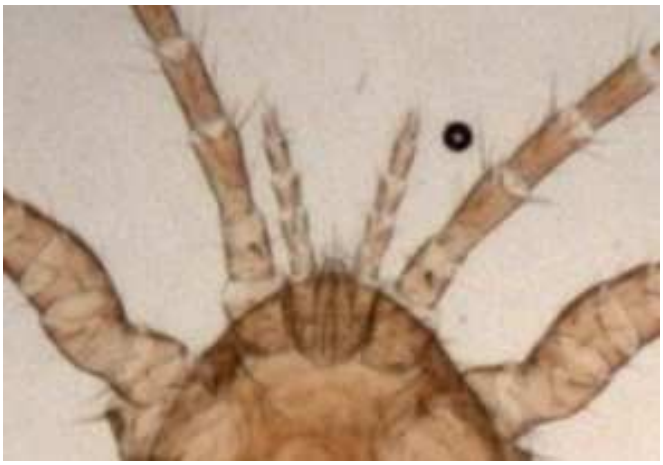


Figure 11 - *T. clareae,* female (anterior view).
Source: Anses, Sophia Antipolis.

Chelicerae with a spermiodactyl in the male

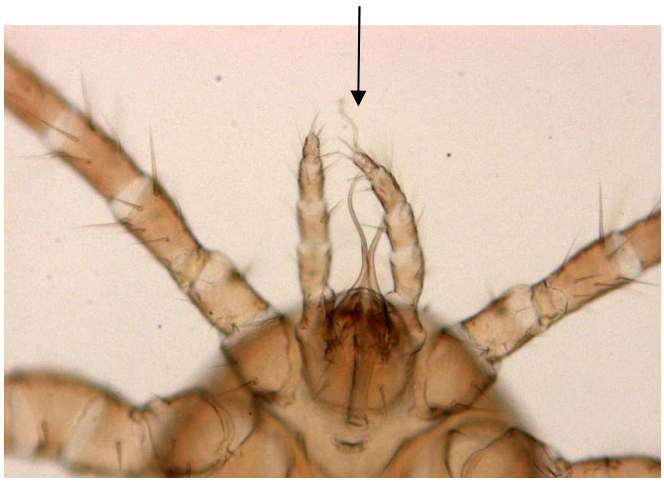


Figure 12 – *T. clareae*, male (anterior view).
Source: Anses, Sophia Antipolis.

3. ANALYTICAL RESULTS

Analysis results	**Conclusion** Analysis parameter: "*Tropilaelaps* spp. - adult form"
All the morphological characteristics of the adult mite *Tropilaelaps* spp. are confirmed (criteria 1 to 9).	Positive
Certain fundamental morphological characteristics of *Tropilaelaps* spp. are not present: • At least one out of the three criteria (n°1 to 3) not confirmed (in this case, the microscopic examination is not realized). • Or at least one out of the six other criteria (n°4 to 9) not confirmed.	Negative
Impossibility to confirm the presence or the absence of certain characteristics.	Inconclusive

Note:

- **In case of a positive result, the official sanitary authorities must be informed with no delay.**
- **In order to confirm the results and to identify the species of *Tropilaelaps*, molecular analysis (PCR) must be conducted.**

4. BIBLIOGRAPHY

Anderson, D.L., Morgan, M.J., 2007. Genetic and morphological variation of bee-parasitic *Tropilaelaps* mites (Acari: Laelapidae): new and re-defined species. Experimental Applied Acarology 43: 1-24.

Anderson, D.L., Roberts, J.M.K., 2013. Standard methods for Tropilaelaps mites research. In: Dietemann, V., Ellis, J.D., Neumann, P. (Eds), The COLOSS BEEBOOK, Volume II: Standard methods for *Apis mellifera* pest and pathogen research. Journal of Apicultural Research 52(4): http://dx.doi.org/10.3896/IBRA.1.52.4.21.

Delfinado, M.D., Baker, E.W., 1961. *Tropilaelaps*, a new genus of mite from the Philippines (Laelapidae, Acarina). Fieldiana Zoology 44: 53-56.

Delfinado-Baker, M., Baker, E.W., 1982. A new species of *Tropilaelaps* parasitic on honey bees. American Bee Journal 122: 416-417.

Dietemann, V., Nazzi, F., Martin, S.J., Anderson, D.L., Locke, B., Delaplane, K.S., Wauquiez, Q., Tannahill, C., Frey, E., Ziegelmann, B., Rosenkranz, P., Ellis, J.D., 2013. Standard methods for varroa research. In: Dietemann, V., Ellis, J.D., Neumann, P. (Eds). The COLOSS BEEBOOK, Volume II: Standard methods for *Apis mellifera* pest and pathogen research. Journal of Apicultural Research Apicultural Research 52(1): http://dx.doi.org/10.3896/IBRA.1.52.1.09.

Fernandez, N., Coineau, Y., 2007. La Tropilaelose. In : Atlantica (Ed), Maladies, parasites et autres ennemis de l'abeille mellifère, Biarritz, pp.255- 279.

Kevan, P.G., Laverty, T.M., Denmark, H.A., 1990. Association of *Varroa jacobsoni* with organisms other than honey bees and implications for its dispersal. Bee World 71(3): 119-121.

OIE, 2008. *Tropilaelaps* infestation of honey bees (*Tropilaelaps* spp.). Chapter 2.2.6. In: OIE (Ed), Manual of standards for diagnostic tests and vaccines for terrestrial animals, Paris, pp. 419-423.

Smiley R.L., 1991. Insect and mite pests in food, an illustrated key. In: J.R.Gorham (Ed), Food and Drug Administration, United States Department of Agriculture, p. 6.

Tangjingjai, W., Verakalasa, P., Sittipraneed, S., Klinbunga, S., Lekprayoon, C., 2003. Genetic differences between *Tropilaelaps clareae* and *Tropilaelaps koenigerum* in Thailand based on ITS and RAPD analyses. Apidologie 34(6): 513-523.

OConnor, B.. Potential threats to native and introduced pollinators: http://insects.ummz.lsa.umich.edu/beemites/index.html. University of Michigan, Museum of Zoology, North American Bee-Associated Mites. Last updated 16 July 2012. Web page accessed on 6 February 2013.

Walter, D.E.. Invasive Mite Identification: Tools for Quarantine and Plant Protection, Lucid v. 3.3: http://www.lucidcentral.org/keys/v3/mites/. Colorado State University, Ft. Collins, CO and USDA/APHIS/PPQ Center for Plant Health Science and Technology, Raleigh, NC. Last updated 24 July 2006. Web page accessed on 28/07/2014.

We appreciate the help of Philippe Reynault (Entomology and Invasive Plants unit of the Anses Plant Health Laboratory in Montpellier, France), Sarah Bonnet (INRA, USC Bartonella and ticks, Ecole Nationale Vétérinaire de Maisons-Alfort, France), and of Jon Pickup's team of the Virology and Zoology unit of SASA (Science and Advice for Scottish Agriculture division of the Scottish Government, Agriculture, Food and Rural Communities Directorate, Edingburgh, Scotland) with the writing of this instruction.