Analytical method for animal health

REFERENCE: ANSES/SOP/ANA-I1.MOA.3500 - Version 03

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Morphological identification of *Tropilaelaps* spp. (adult form) (WOAH method)

Sophia-Antipolis Laboratory

National Reference Laboratory – Bee Health

European Union Reference Laboratory – Bee Health

History of the method

A method can be updated in order to take changes into account.

A change is considered major when it involves the analytic process, the scope or critical points of the analysis method, the application of which may modify the performance characteristics of the method and/or the results. A major change requires major adaptations and either total or partial revalidation.

A change is considered minor if it provides useful or practical clarifications, reformulates the text to make it clearer or more accurate, or corrects minor errors. A minor change in the method does not alter its performance characteristics and does not require revalidation.

The table below summarises the version history of this method and provides qualifications for the changes.

Version	Nature of changes (Major / Minor)	Date	Main changes
V02	Minor	01/04/2020	1. Reformatting of the method. 2. Updating of references. 3. Revisions of the protocol taking into account EURL practical feedback and results of the comparative laboratory testing TROPMORPH19: - Precisions in the protocol: sampling procedures, methods for measuring the size ratio. - Removal of the optional identification criterion : "Presence of a tritosternum" - Addition of the possibility to perform a confirmatory molecular biology analysis in case of inconclusive result. - Precisions in the "analytical results" section for "inconclusive" cases - Addition of sections on opinions and on performance characteristics of the method.
V03	Minor	15/09/2023	- « OIE » now « WOAH » (World Organisation for Animal Health) - New Anses logo - Updates in the introduction (regulatory context) - Precisions in §7 (sample conservation) - Precision concerning the orientation of the ventrianal plate (§ 8.2 / Tab.2 and Annex / Fig. 7)

Foreword

This method was developed by:

ANSES - Sophia-Antipolis Laboratory

French National Reference Laboratory for Bee Health

European Reference Laboratory for Bee Health

WOAH Reference Laboratory for Tropilaelaps infestation

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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.

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Introduction

Mites of the genus *Tropilaelaps* are ectoparasites of bee brood, native to Asia. 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 a few days.

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.

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)[1] and internationally (WOAH, 2018). In order to limit the risk of introducing this exotic pest into the EU, EU regulation 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.

In case of suspicion, rapid and reliable diagnosis is crucial to implement sanitary measures and avoid dissemination of the mite. This method, based on the WOAH Manual (WOAH, 2018), describes a protocol for the morphological identification of adult *Tropilaelaps* spp. It provides a result in a short time with a low cost technique and is therefore indicated for first intention diagnosis.

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

[1] Commission implementing regulation (EU) 2018/1882 of 3 December 2018 on the application of certain disease prevention and control rules to categories of listed diseases and establishing a list of species and groups of species posing a considerable risk for the spread of those listed diseases. In this regulation, infestation with Tropilaelaps spp. is listed in the categories D '"listed disease for which measures are needed to prevent it from spreading on account of its entry into the Union or movements between Member States ») and E ("listed disease for which there is a need for surveillance within the Union »).

[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.

Warnings and safety precautions

The user of this method should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations.

All actions taken in accordance with this method must be performed by employees who have attended relevant training.

1. Purpose and scope

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 bumblebees.

The identification method described here is based on the method described in WOAH manual (2018). It consists in the visual examination of individuals with recording of morphological characteristics and, if necessary, comparison of the sample to be identified with a reference sample or detailed photographs.

The visual examination described in this protocol is not sufficient to provide adequate differentiation among the four species of *Tropilaelaps* as they are morphologically very similar (Anderson and Morgan, 2007; Tangjingjai *et al*., 2003).

Molecular methods can be used as a second line to identify the *Tropilaelaps* species. In addition, in case of inconclusive morphological results, they should be systematically performed to confirm the diagnosis.

2. Reference documents

[1] ***WOAH, 2018.*** **Tropilaelaps *infestation of honey bees (*Tropilaelaps *spp.)*. Chapter 3.2.6. In: Manual of standards for diagnostic tests and vaccines for terrestrial animals, Paris.**

3. Terms, abbreviations and definitions

EU: European Union

WOAH: World Organisation for Animal Health

PCR: Polymerase Chain Reaction

4. Principle of the method

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 3.2.6 of the WOAH Terrestrial Manual (WOAH, 2018) and in various scientific publications (Anderson and Morgan, 2007; Delfinado and Baker, 1961; Fernandez and Coineau, 2007).

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

5. Reagents

Warning: Trade names or supplier names may be mentioned in the description of the products required to implement this method. This information is provided for users of the method and does not mean that ANSES recommends the exclusive use of these products. Similar products may be used if it has been demonstrated that they achieve the same results.

- Ethanol diluted to approximately 70% (not denatured)
- Lactic acid
- Mounting medium (*e.g.*: Hoyer's medium[3]) and clear nail polish for the long term conservation of the microscopic slides

Note: Not denatured ethanol shall be used in order to limit the risk of inhibition of the PCR (if performed later).

6. Equipment and materials

Warning: Trade names or supplier names may be mentioned in the description of the equipment and materials required to implement this method. This information is provided for users of the method and does not mean that ANSES recommends the exclusive use of these materials. Similar materials may be used if it has been demonstrated that they achieve the same results.

- 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
- Stereomicroscope
- Compound microscope (1000x)
- Heating plate

[3] Hoyer's medium is used as a reference in the field of acarology.

7. Samples

7.1 Acceptance conditions for samples

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 least at -70°C during, at least for 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%, in order to kill them.

7.2 Sample storage before analysis

The specimens are stored in ethanol 70% in capped tubes at room temperature.

7.3 Storage of samples or residual materials after analysis

The specimens are stored in ethanol 70% in capped tubes at room temperature.

8. Procedure

8.1 Protocol

1. Lay-out of the work area

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

2. Sampling for analysis

- Place all the specimens in a dish.
- Count the number of specimens present in the sample and record the result. If the number is greater than 100, stop at 100 and note: ">100".
- Carry out the analysis sampling on the basis of the number of specimens counted according to the protocol presented in the Table 1. The specimens are taken at random using fine-tipped tweezers or needle holders (fishing line equipped). Place them in a dish.

Table 1 – Analysis sampling strategy

Total number of specimens present in the sample	1-30	31-100	> 100
Number of specimens to analyse	all	30 + 30 % of the remaining specimens (total number of specimens – 30)*	51

** Round up to the next whole number.*

Example: if the total number of specimens in the sample is 58, analyse 30 + 30/100 x (58-30) = 38.4, i.e. *39 specimens to examine.*

Note: The sampling strategy is inspired by the referential of the French Association for the Study of epidemiology of animal diseases (Toma *et al*., 2010), and based on the data allowing the detection of an expected prevalence of 5% of positive cases in a population, with a risk of error of 5% (for a population of 1 to 180 units).

3. 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 8.2).

Note: Evaluate the ratio of mite body length to width by measuring the specimen in dorsal view:

- Use the same magnification to measure length and width.
- Measure where the body is widest / longest.
- For length, do not include mouthparts and antennae.
- For width, do not include legs.

If at least one of the criteria 1 to 3 is missing, compliance with the other criteria is not achieved.

4. 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 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).

5. 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 8.2).

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

- Reference microscopic slides could be observed for comparison if available.

6. Conclusion of the analysis

- If different specimens are identified during the examination, sort them by category (sub-samples), and analyse them separately according to the criteria detailed in paragraph 8.2 and annexe 1.
- If the result is inconclusive (non-assessable criteria, or if the physical condition of the specimen makes morphological identification difficult or even impossible), molecular identification will be necessary.
- In case of a positive result, the official sanitary authorities must be informed with no delay.

7. 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, directly from lactic acid (to prevent the formation of air bubbles).

Place a cover glass over the slide.

The drying time depends on the medium used (2-4 weeks at room temperature with Hoyer's medium, depending on room conditions). Drying can be accelerated by using a heating plate (heating to around 50°C for 1 to 2 weeks).

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, to prevent the formation of air bubbles and the shrinkage of the mounting medium.

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

8.2 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).

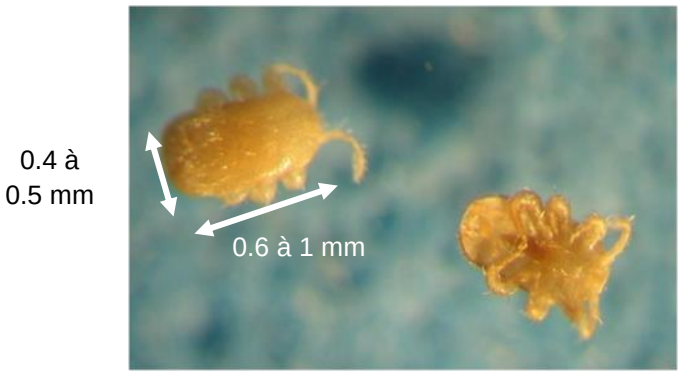

Figure 1- *Tropilaelaps* spp., as seen through a stereomicroscope.
Source: Anses, Sophia Antipolis.

Table 2 - 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 AND 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[4] between coxa[5] III and IV (Figures 6, 7, and 8). → Order Parasitiforms		**400X**
5. Presence of elongated peritremes[6] (Figures 6, 7, and 8). → Suborder Mesostigmata		**200X**
6. Epigynial plate, posteriorly rounded or sharp. Triangular-shaped ventrianal plate (Figures 2 and 7). Note: The point of the "triangle" is cranially oriented. → Family 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**

[4] The stigmata are tracheal openings in Arthropods.
[5] The coxa is the first leg segment of Arthropods which connects the leg and the body.
[6] The peritremes are tubular structures running on from stigmata. They could have a role in the respiration.
[7] Reticulated means that it has broken eggshell or fishscale pattern.

9. Results

9.1 Reporting of results

Table 3 – Results and conclusions

Analysis results	Conclusion
Positive: All the morphological characteristics of the adult mite *Tropilaelaps* spp. are confirmed (criteria 1 to 9).	Positive
Negative: 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
Inconclusive: Impossibility to rule on the positive or negative character of the sample: it was not possible to rule on the presence/absence of certain morphological identification criteria (e.g. damaged specimen). → Molecular identification systematically realised.	Inconclusive

9.2 Guidelines for expressing opinions

Opinions, taking into consideration the results of the different analysis performed (morphology and/or molecular diagnosis), can be expressed according to the decisions rules described in Table 4.

Table 4 - Decision rules for giving opinions

		PCR result			
		Analysis not performed	Positive	Negative	Inhibited
Morphological examination result	Positive	(1)	(1)	(3)	(1)
	Negative	(2)	(1)[i]	(2)	(2)
	Inconclusive		(1)	(2)	(4)
	Analysis not realised		(1)	(2)	(4)

[i] Note: Molecular identification of *Tropilaelaps* can be accompanied, in case of a positive result, by sequencing to identify the species of *Tropilaelaps*. Therefore, it is considered that a positive PCR result (associated with an identification of the *Tropilaelaps* species by sequencing) takes precedence over the result of the morphological identification for the final analytical conclusion.

(1): « Positive identification of *Tropilaelaps* spp.. ». In the case where species identification is carried out by molecular sequencing, this information will be indicated in the commentary: "Species identified: *Tropilaelaps X*".

(2): « Negative identification of *Tropilaelaps* spp.. »

(3): «Suspected identification of *Tropilaelaps*. Further molecular analysis is required to ascertain the identification ».

(4) « Inconclusive result of *Tropilaelaps* spp. (PCR analysis inhibited). »

10. Performance characteristics of the method

Table 5 – Performance characteristics of the method (final result).

Sensitivity	100%
Specificity	100%
Intra-laboratory precision	100%
Inter-laboratory precision	100%

Notes:

- Given the difficulty in obtaining negative and positive specimens, the performance characteristics were evaluated on a limited number of acarines. The data were consolidated through a literature review.
- The inter-laboratory precision (reproducibility) was evaluated in 2019 through the organisation of a comparative laboratory testing (CLT) involving five EU National Reference Laboratories, accredited for the method, and the EU Reference Laboratory for Plant Health (Anses, Montpellier laboratory).
- The detailed results of the morphological criteria assessment coming from the 2019 CLT showed that, the reproducibility mean is >95% for most of the features. However, in case of negative species, the reproducibility of some criteria was a little bit lower at about 80%, depending on the features and the species examined.

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ANNEX - Figures

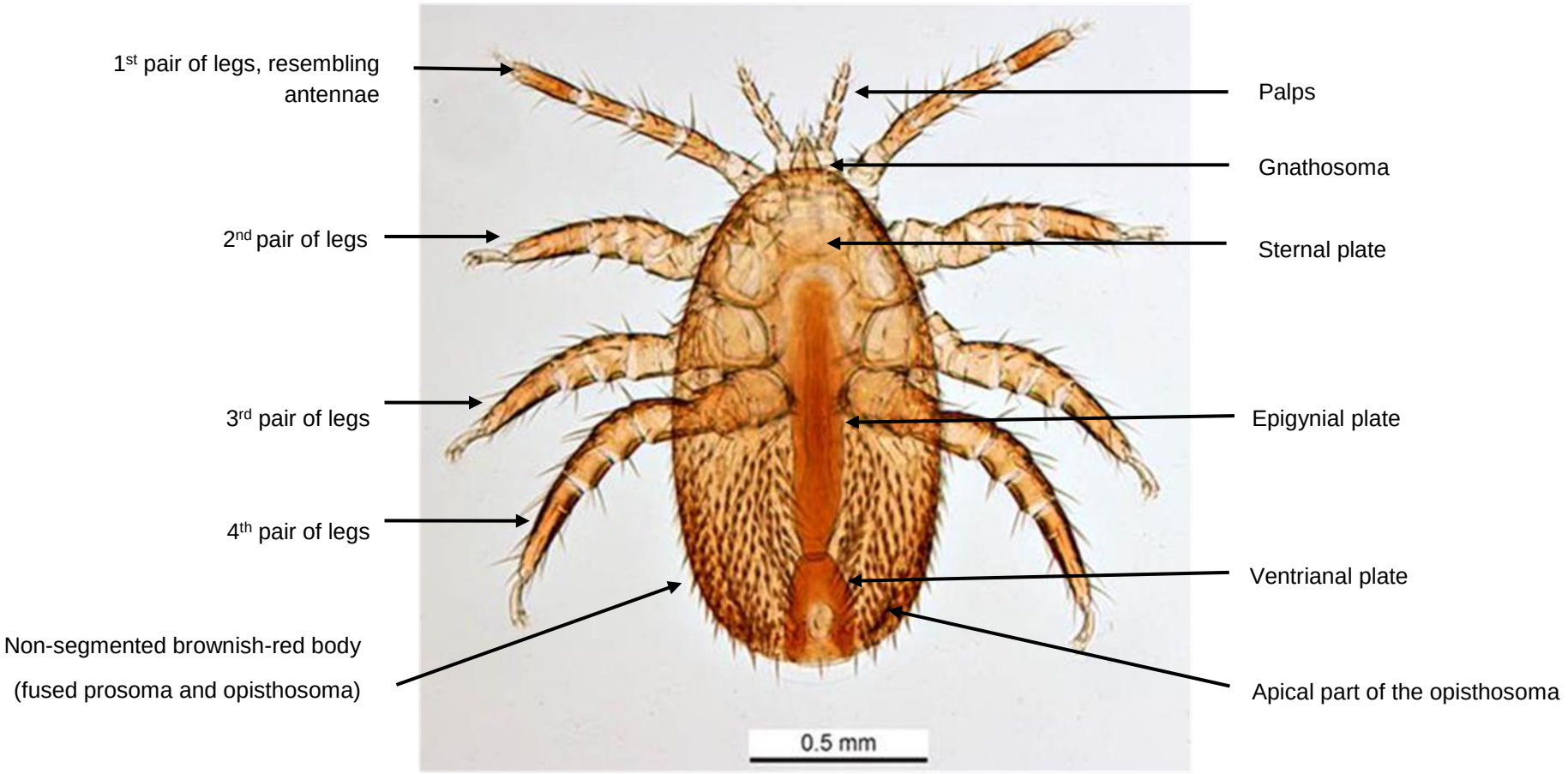


Figure 2 - *Tropilaelaps mercedesae*, female (ventral view).

Source: Ken Walker, Victoria Museum, Australia.

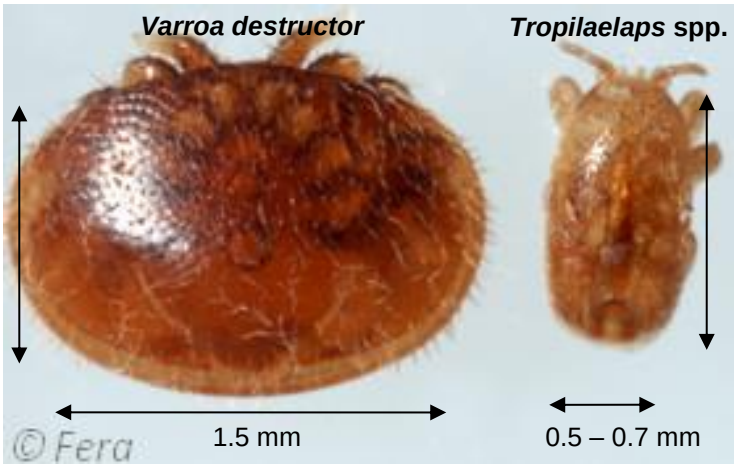


Figure 3 - *Varroa destructor* and *Tropilaelaps* spp. (dorsal view).

Source: Fera, Crown copyright.

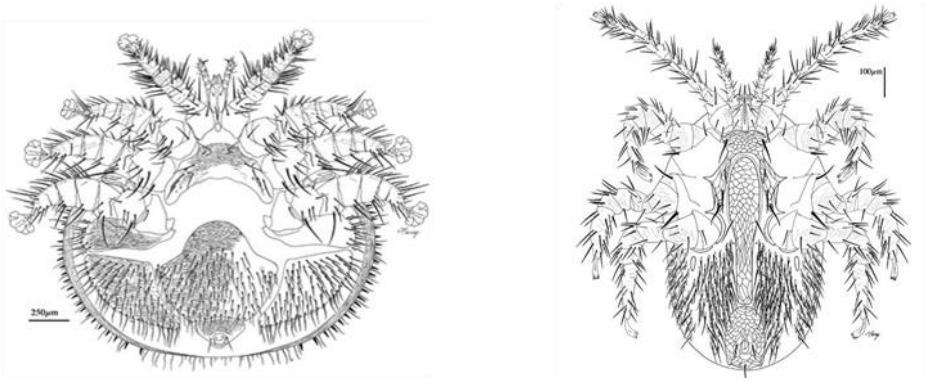


Figure 4 - *Varroa destructor* and *Tropilaelaps* spp. (ventral view).

Source: Walter et al., 2006.

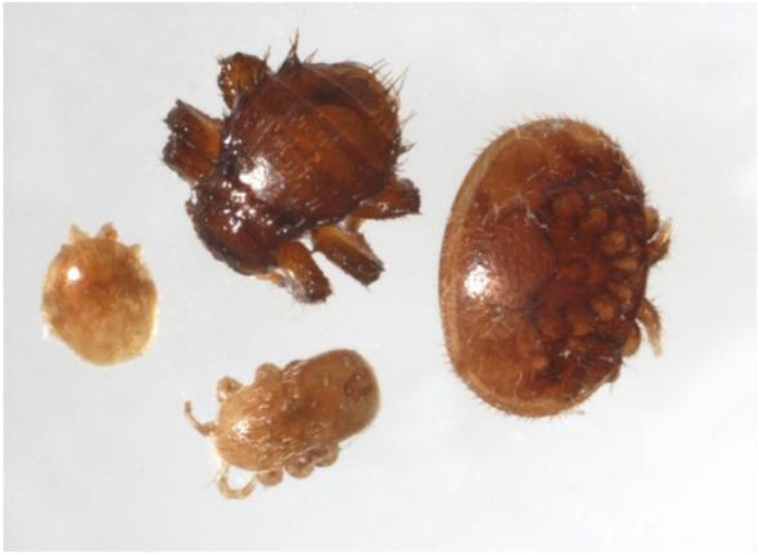
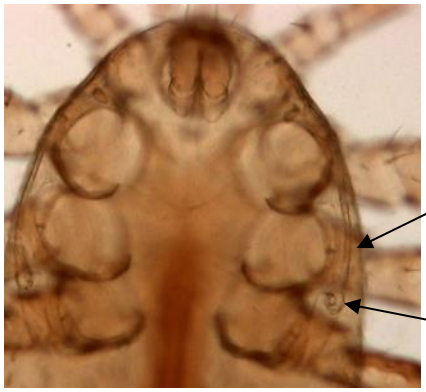
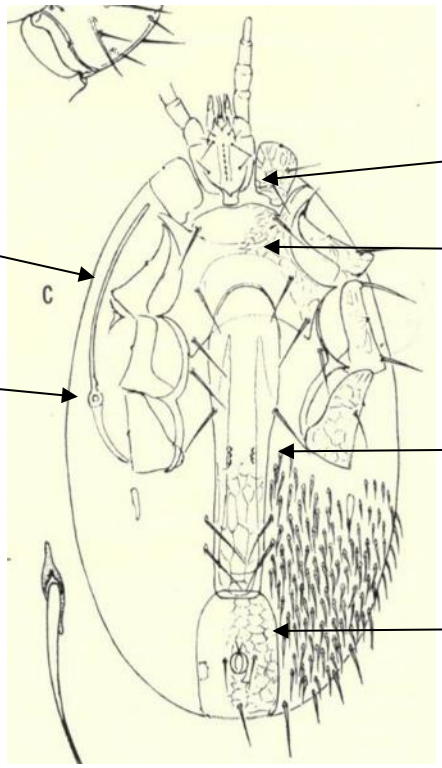


Figure 5 - *Braula coeca* (above), *Varroa destructor* (right), *Tropilaelaps* spp. (below centre) and *Melittiphis alvearius* (left) (dorsal view).

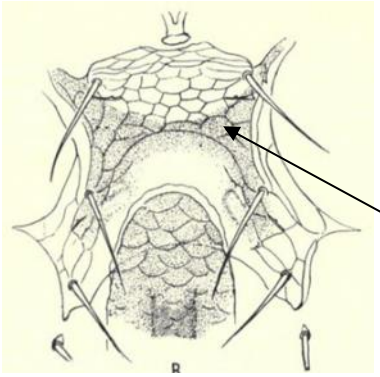
Source: FERA, Crown copyright.



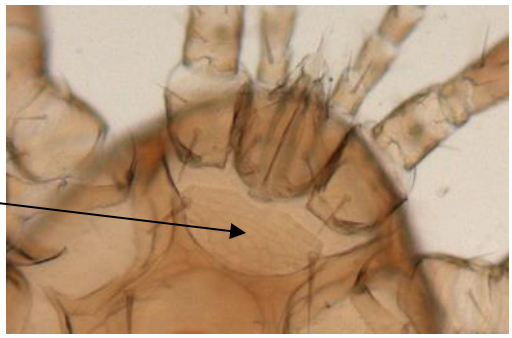
T. clareae – Microscope 100X



T. clareae, female (ventral view)



Sternal plate and epigynial plate (ventral view)



T. clareae – Microscope 200X

Gnathosoma (ventral view)

T. clareae – Microscope 200X

Figure 6 - *Tropilaelaps clareae*, anatomy.

Source: Delfinado and Baker, 1961.

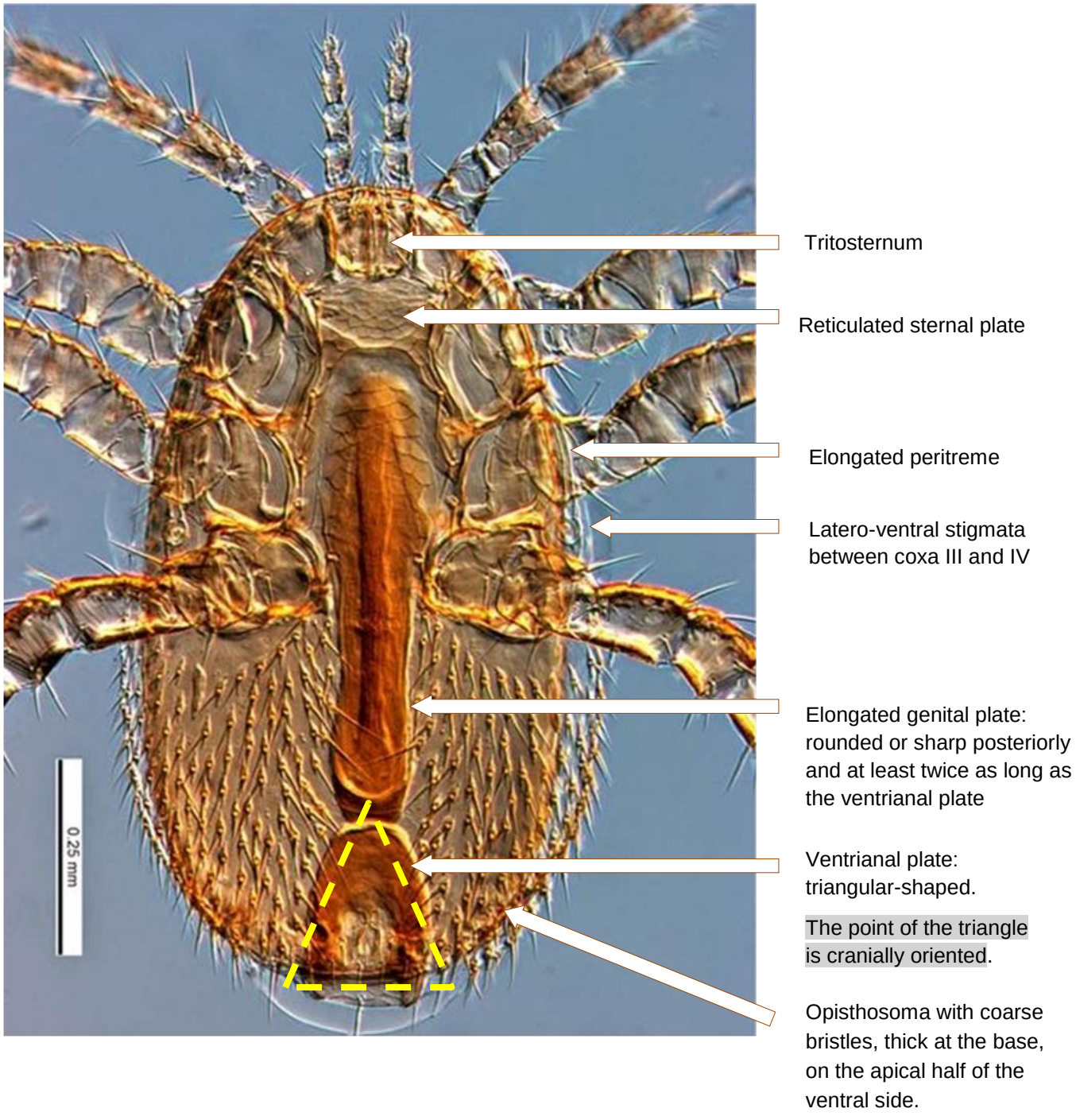


Figure 7 - *Tropilaelaps clareae*, female (ventral view).

Source: Ken Walker, Victoria Museum, Australia.

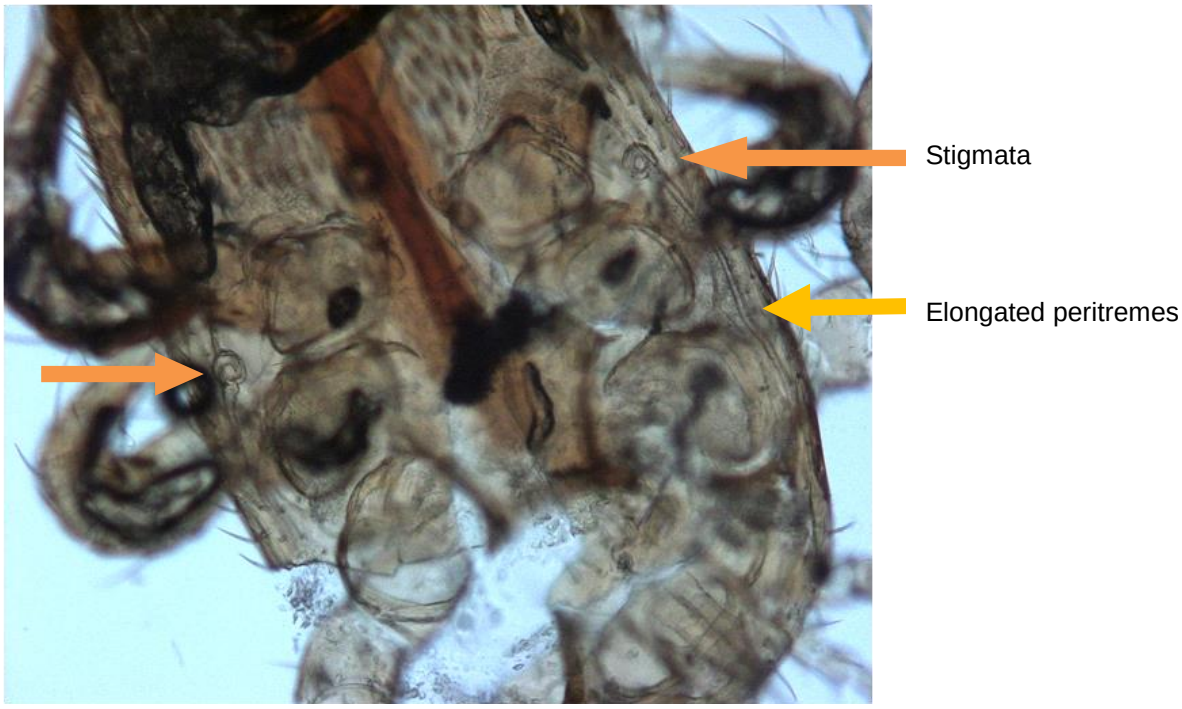
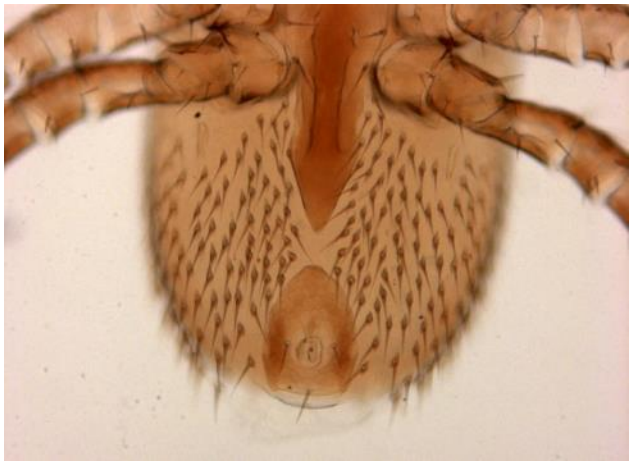
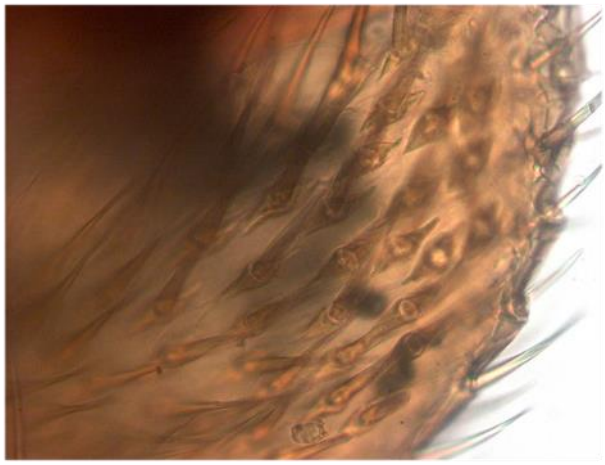


Figure 8 - *Tropilaelaps* spp. - Lateroventral view. Stigmata between coxas III and IV (200X).

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.

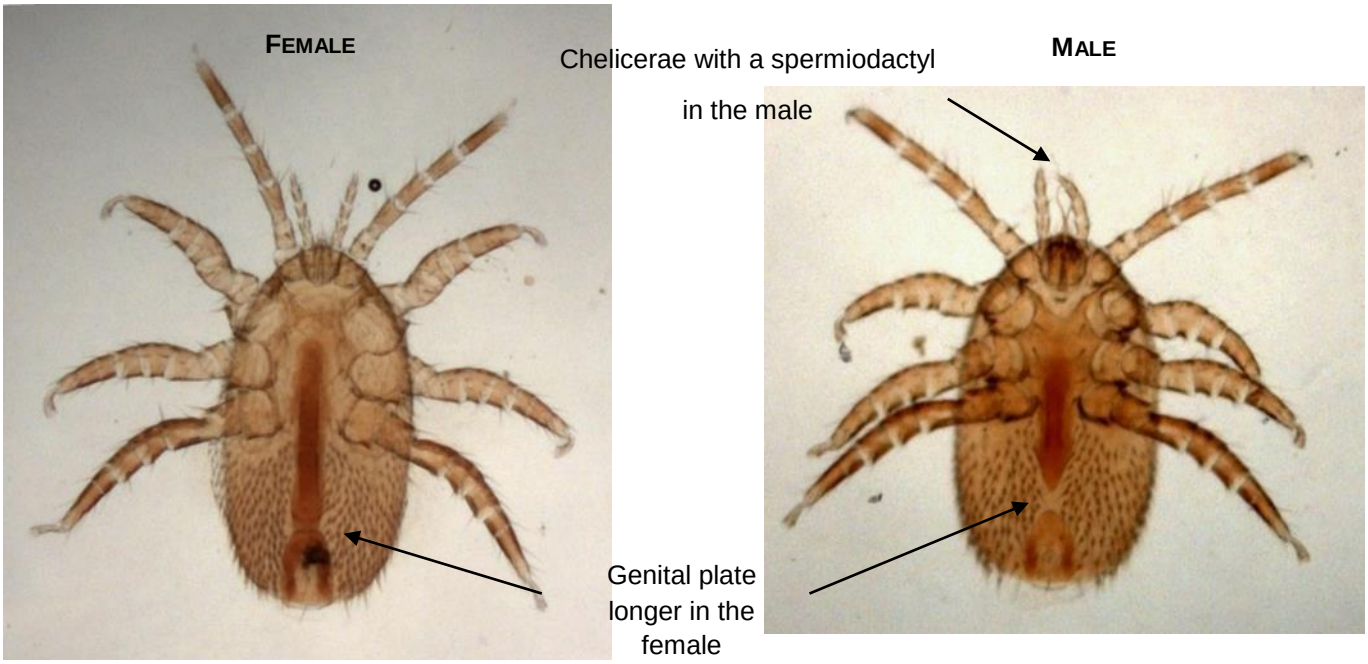


Figure 10 – *T. clareae*, male and female (ventral view).

Source: Anses, Sophia Antipolis.

Spermiodactyl not present in the female

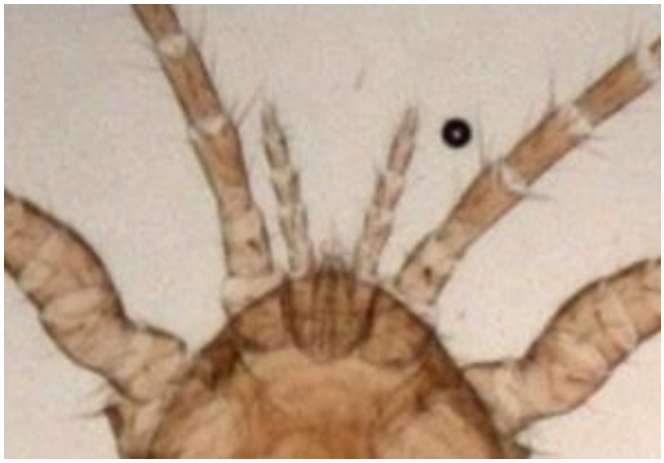


Figure 11 - *T. clareae,* female (anterior view).

Source: Anses, Sophia Antipolis.

Chelicerae with a spermiodactyl in the male

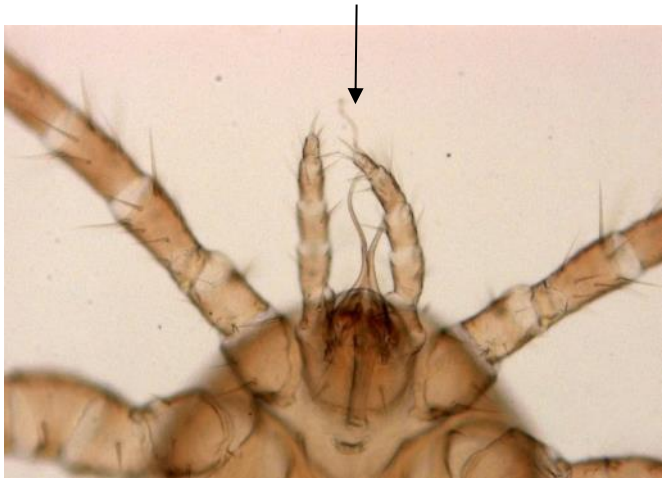


Figure 12 – *T. clareae*, male (anterior view).

Source: Anses, Sophia Antipolis.