Analytical method for animal health

REFERENCE: ANSES/SOP/ANA-I1.MOA.3500 - Version 04

October 2025

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)
V04	Minor	01/10/2025	- Editorial improvements - Epidemiological and bibliographical updates - Minor modifications to two morphological criteria: description of the ventrianal plate shape, criterion concerning "elongated peritremes" removed (see details in §2). - Updates in §9: “Performance characteristics of the method”

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

Address: Les Templiers - 105 route des Chappes - CS 20111 - 06902 Sophia-Antipolis Cedex

Contact: eurl.bee@anses.fr

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, National Veterinary School of 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.

Contents

Introduction

Mites of the genus *Tropilaelaps* are ectoparasites of honey bees, native to Asia. They feed on the larvae and pupae, causing malformations, mortality and ultimately colony decline. Their life cycle is completed in approximately one week. Adult mites can be transported on adult bees (phoresy), but seem unable to feed on them due to their inability to pierce the cuticle, and therefore survive only a few days outside brood cells.

Four species of the genus *Tropilaelaps* have been described: *T. clareae* (Delfinado & Baker, 1961), *T. mercedesae* (Anderson & Morgan, 2007), *T. koenigerum* (Delfinado-Baker & Baker, 1982) and *T. thaii* (Anderson & Morgan, 2007). These species originally parasitised giant honey bees such as *Apis dorsata, Apis laboriosa and Apis breviligula.* Following the introduction of the European honey bee (*A. mellifera)* into Asian infested areas, two species, *T. clareae* and *T. mercedesae*, successfully adapted to this new host.

The European Union (EU) is currently free of *Tropilaelaps* mites. However, their recent spread in Central Asia, Black Sea-Caucasus region, and Eastern Europe poses a significant risk of introduction. To protect pest-free regions, infestation of honey bees by *Tropilaelaps* spp. is listed by the World Organisation for Animal Health (WOAH) and regulated within the EU[1].

Clinical signs of *Tropilaelaps* spp. infestation are very similar to those caused by *Varroa destructor*, a parasitic mite that is globally endemic in Europe. Differential diagnosis between these two mites is therefore essential.

In suspected cases, rapid and reliable diagnosis is critical to implement sanitary measures and prevent dissemination. The present method, based on the WOAH Terrestrial Manual (WOAH, 2025), describes a protocol for the morphological identification of adult *Tropilaelaps* spp. It provides results quickly and at low cost, making it suitable for first-line diagnosis. When necessary, morphological identification can be confirmed by molecular methods such as PCR or sequencing.

[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 »).

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 or queen bees.

The present method is based on the procedure described in the WOAH manual (2018). It consists of the visual examination of individual mites, with recording of their morphological characteristics and, when necessary, comparison of the specimen with a reference sample or detailed photographs.

The visual examination outlined in this protocol is not sufficient to provide adequate differentiation among the four *Tropilaelaps* species, as they are morphologically very similar (Anderson and Morgan, 2007; Tangjingjai *et al*., 2003). In such cases, molecular methods should be used as a second line approach to determine the species. Moreover, when morphological results are inconclusive, molecular confirmation should be systematically performed to establish a definitive diagnosis.

2. Reference documents

The method was developed and validated by the Anses Sophia Antipolis laboratory, and is published in Chapter 3.2.5 of the WOAH Terrestrial Manual (WOAH, 2025):

[1] ***WOAH, 2025. Infestation of honey bees with* Tropilaelaps *spp.* Chapter 3.2.5. In: Manual of standards for diagnostic tests and vaccines for terrestrial animals, Paris.**

Note: Since the last revision of the chapter, minor modifications have been made to the morphological identification criteria to improve the method: the description of the ventrianal plate shape has been slightly revised for clarity, and the criterion concerning "elongated peritremes" - which was considered imprecise - has been removed, as it was not relevant for characterising Mesostigmata mites.

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 (particularly *V. destructor*).

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% (avoid –denatured ethanol)
- Lactic acid
- Mounting medium (preferably Hoyer's medium, the reference in acarology) and clear nail polish for the long term conservation of the microscopic slides

Note: Non-denatured ethanol must be used, as denaturants may inhibit PCR if molecular analysis is 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
- Fine-tipped brush
- 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 sealed vials
- Glass microscope slides (classic and concave) and cover slips
- Stereomicroscope
- Compound microscope
- Heating plate

7. Samples

7.1 Acceptance conditions for samples

The sampling instruction sheet provided to customers must specifies that any mite suspected of belonging to the genus *Tropilaelaps* must be killed before being submitted to the laboratory.

In case of doubt, packages must be opened in containment conditions.

If the specimens are found to be alive on arrival, samples should be first placed at least at -70°C for approximately one hour before opening fully. 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%.

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 under the stereomicroscope 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, needle holders (fishing line equipped), or with a fine-tipped brush a moistened with ethanol. 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 larger specimens.

- Place the selected specimens on the slide in lactic acid with the needle holders (fishing line equipped) (or with extra-fine tweezers or a fine-tipped brush).
- 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).

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 (e.g. due to non-assessable criteria, or damage specimens), molecular identification is required.
- In case of a positive result, the competent sanitary authorities must be notified without 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 specimens on microscope slides for long term storage*
  - Deposit a few drops of 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.
  - Drying the slide:

At room temperature: allow to dry for approximately 2–4 weeks, depending on climatic conditions.
Accelerated drying: place on a heating plate at ~50 °C for 1–2 weeks.

  - Sealed the slide with clear nail polish at the end of the drying process, to prevent the formation of air bubbles and the shrinkage of the mounting medium.

Note: For further information on storage and mounting of mites, see Dietemann et al. (2013).

8.2 Identification of the adult *Tropilaelaps* spp. mite

Tropilaelaps spp. are mites belonging to the class Arachnida, subclass Acari, super-order Parasitiformes, order Mesostigmata and family Laelapidae (Anderson & Roberts, 2013).

They must not be confused with the mite *V. destructor*, a globally distributed parasite of honey bees that also belongs to the order Mesostigmata, but to the family Varroidae[2].

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).

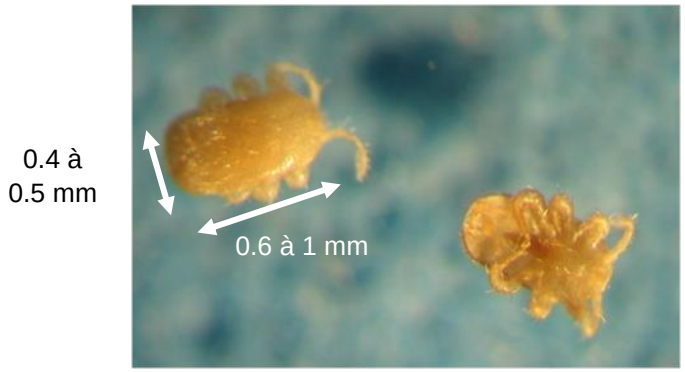

Figure 1- *Tropilaelaps* spp., as seen through a stereomicroscope.
Source: Anses, Sophia Antipolis.

[2] A recent publication (Oh et al., 2024), based on phylogenetic analyses, has proposed to reclassify *Varroa* within the family Laelapidae, subfamily Varroinae. To date, however, this new classification has not been adopted by all international taxonomic references (e.g. NCBI, GBIF).

Morphological criteria used to identify *Tropilaelaps* are summarised in Table 2.

Table 2 - Criteria for identifying *Tropilaelaps* spp.
(Anderson and Roberts, 2013; Delfinado and Baker, 1961; Smiley R.L., 1991)

	Stereomicroscope	Compound microscope
1. *Tropilaelaps* has four pairs of legs AND the first pair is vertically aligned, resembling antennae (Figure 2).	**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).	**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).		**400X**
5. Epigynial plate, posteriorly rounded or sharp. Ventrianal plate horseshoe-shaped. (Figures 2 and 7). *Note: The opening of the "horseshoe" is oriented caudally.*		**100X**
6. Elongated epigynial plate, at least twice as long as the ventrianal plate (Figures 2 and 7).		**100X / 200X**
7. Reticulated sternal plate[5] (Figure 7).		**400X**
8. 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. They are anteriorly extended by peritremes. Peritremes are tubular chitinous structures involved in mite respiration.
[4] The coxa is the first leg segment of Arthropods, which connects the leg and the body.
[5] Reticulated means that it has broken eggshell or fishscale pattern.

9. Results

9.1 Reporting of results

Table 3 presents how to report analytical results.

Table 3 – Results and conclusions

Analysis results	Conclusion
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 realised). - Or at least one out of the six other criteria (n°4 to 9) not confirmed.	**Negative**
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 carried out.	**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)*	(2)	(2)
	Inconclusive		(1)	(2)	(4)
	Analysis not realised		(1)	(2)	(4)

(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).

* *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.*

9. Performance characteristics of the method

The performance of the method was evaluated using *T. mercedesae* and *T. clareae* specimens collected from various geographical origins, as well as non-target mite species (Table 5).

Given the difficulty of obtaining negative and positive specimens, the data were also consolidated through a literature review.

To assess the reproducibility of the method across laboratories, a comparative laboratory testing (CLT) was organised in 2019, involving five EU National Reference Laboratories accredited for the method, and the EU Reference Laboratory for Plant Health (Anses, Montpellier laboratory). The CLT demonstrated 100% sensitivity, specificity, and reproducibility of the method (i.e. final analytical result). A more detailed analysis of the assessment of morphological criteria showed mean reproducibility rate values >95% for most of the features. However, for non-target species, the reproducibility of certain criteria was slightly lower, approaching 80%, depending on features and species examined.

Table 5 – Performance characteristics of the method

Caracteristic	Parameter	Value	Main information on the methods used for characterisation
Sensitivity (SE) & inclusivity (IN)	SE: Percentage of positive results found among the expected positive results. IN : Ability of the method to detect the target analyte from a wide range of strains, isolates, populations, etc.	**100%**	Test population: *T. clareae* - Philippines (n=3) *T. mercedesae* - Georgia, Indonesia, Nepal, Sri Lanka, Thailand (n=36)
Spécificité (SP)	Percentage of negative results found among expected negatives.	**100%**	Test population: Non-target mite species: 18 specimens corresponding to samples received for analysis (and collected in the environment of the hive) or to species used in biological control, in particular species belonging to the Laelapidae family.

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ANNEX - Figures

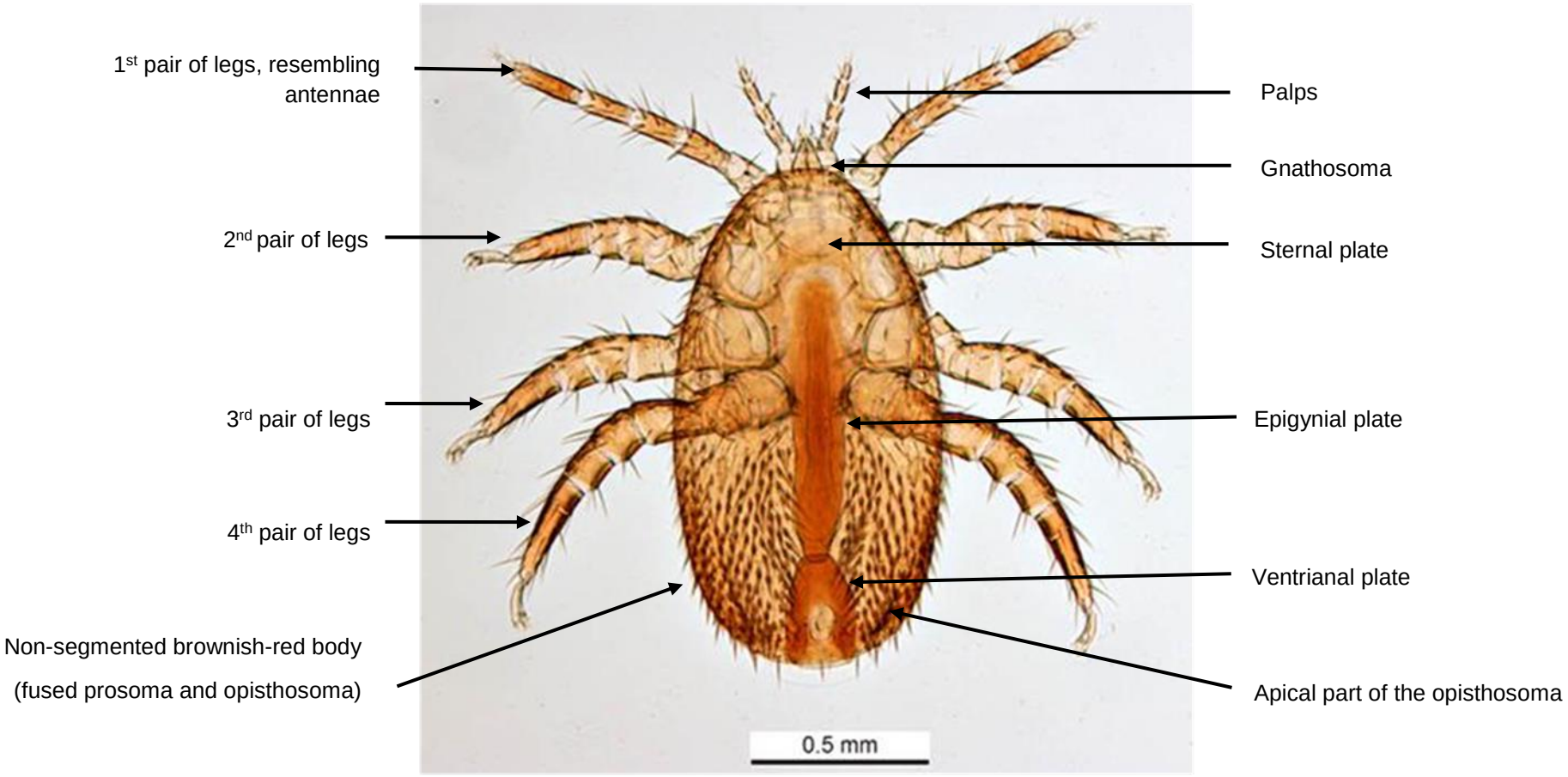


Figure 2 - *Tropilaelaps mercedesae*, female (ventral view).

Source: Ken Walker, Victoria Museum, Australia.

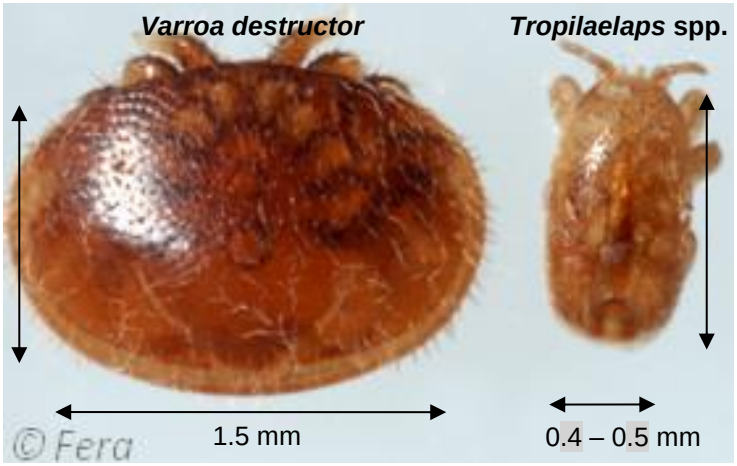


Figure 3 - *Varroa destructor* and *Tropilaelaps* spp. (dorsal view).

Source: Fera, Crown copyright.

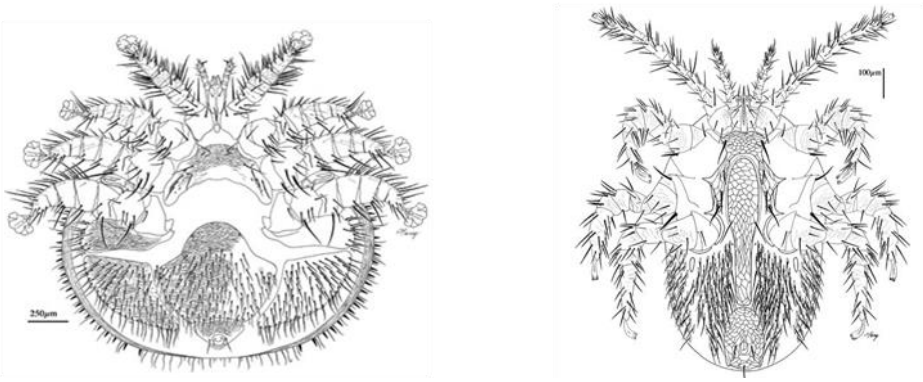


Figure 4 - *Varroa destructor* and *Tropilaelaps* spp. (ventral view).

Source: Walter et al., 2006.

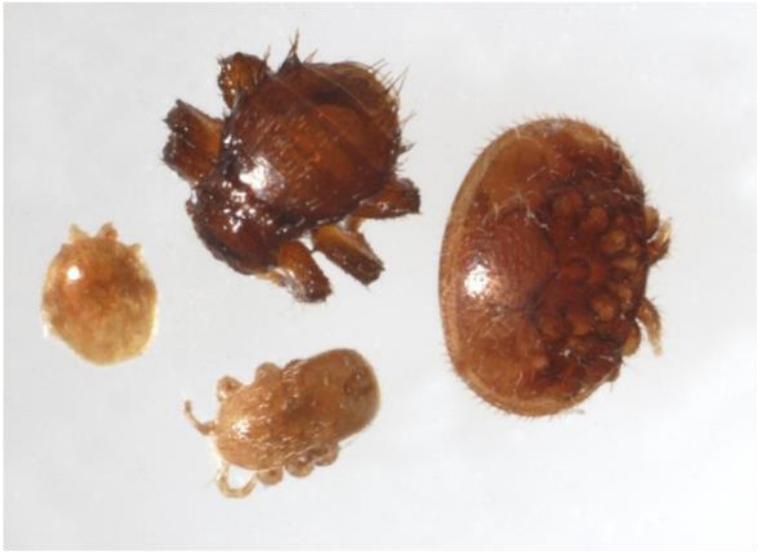
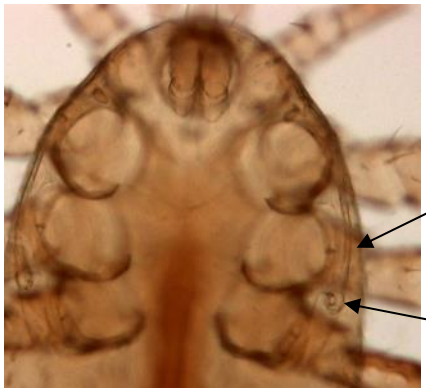
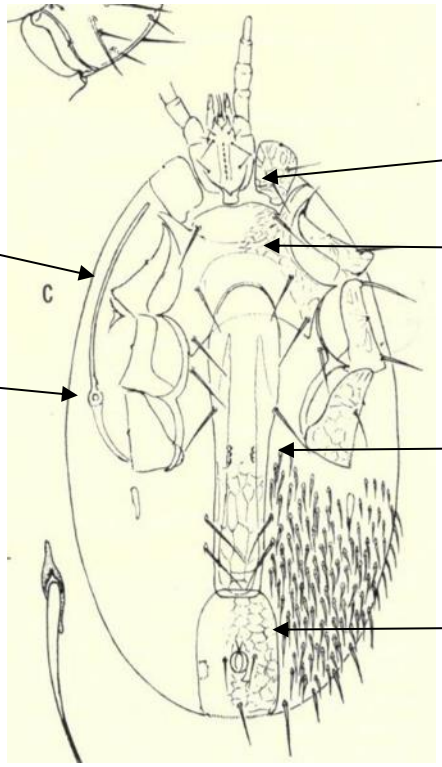


Figure 5 - *Braula coeca* (above), *Varroa destructor* (right), *Tropilaelaps* spp. (below centre) and *Melittiphis alvearius* (left) (dorsal view).

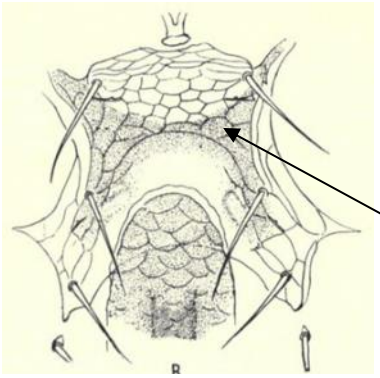
Source: FERA, Crown copyright.



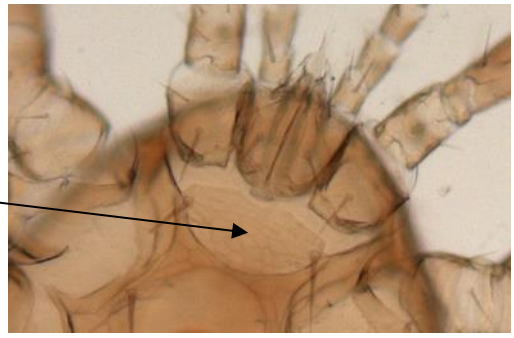
T. clareae – Microscope 100X



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.

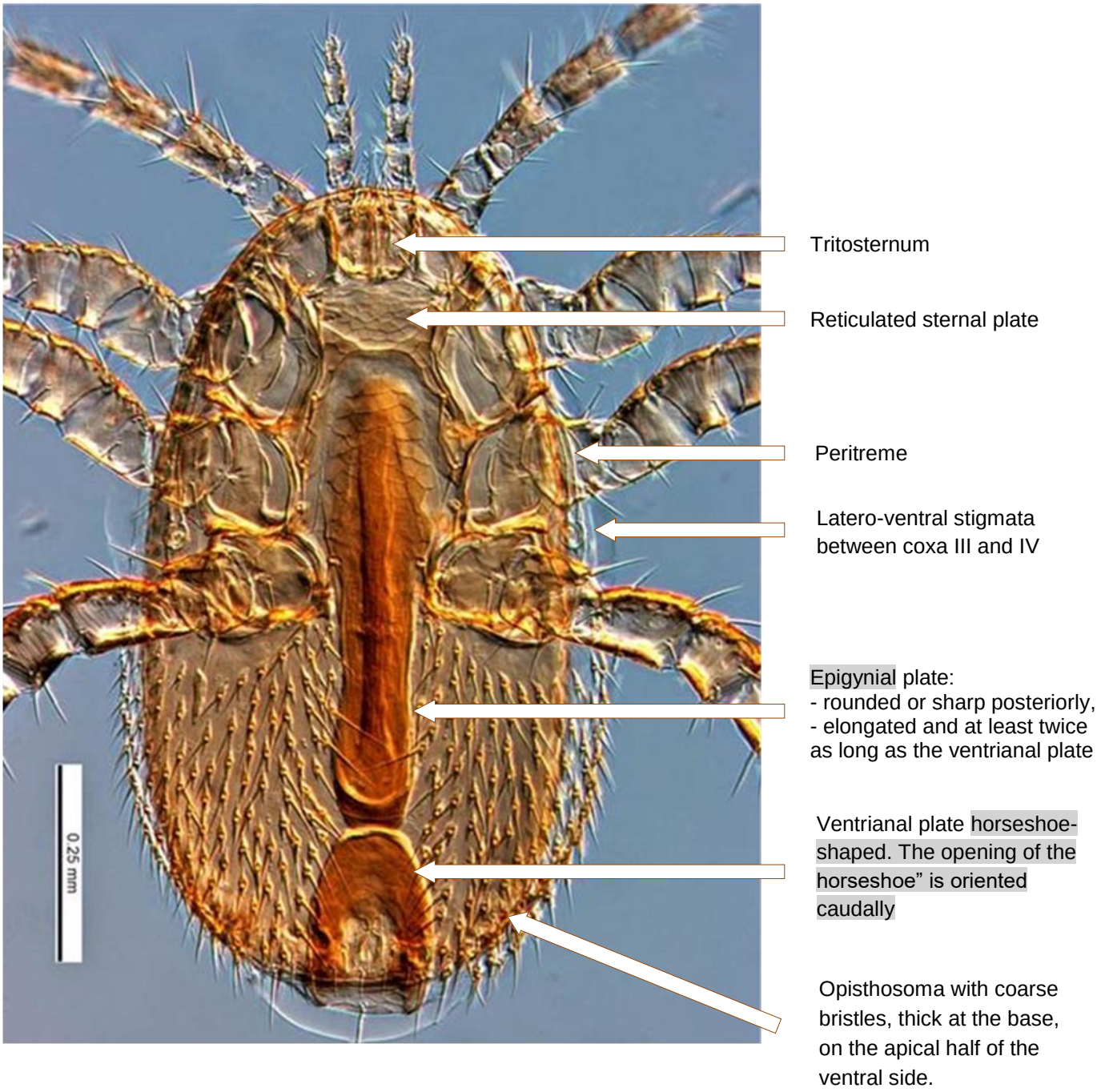


Figure 7 - *Tropilaelaps clareae*, female (ventral view).

Source: Ken Walker, Victoria Museum, Australia.

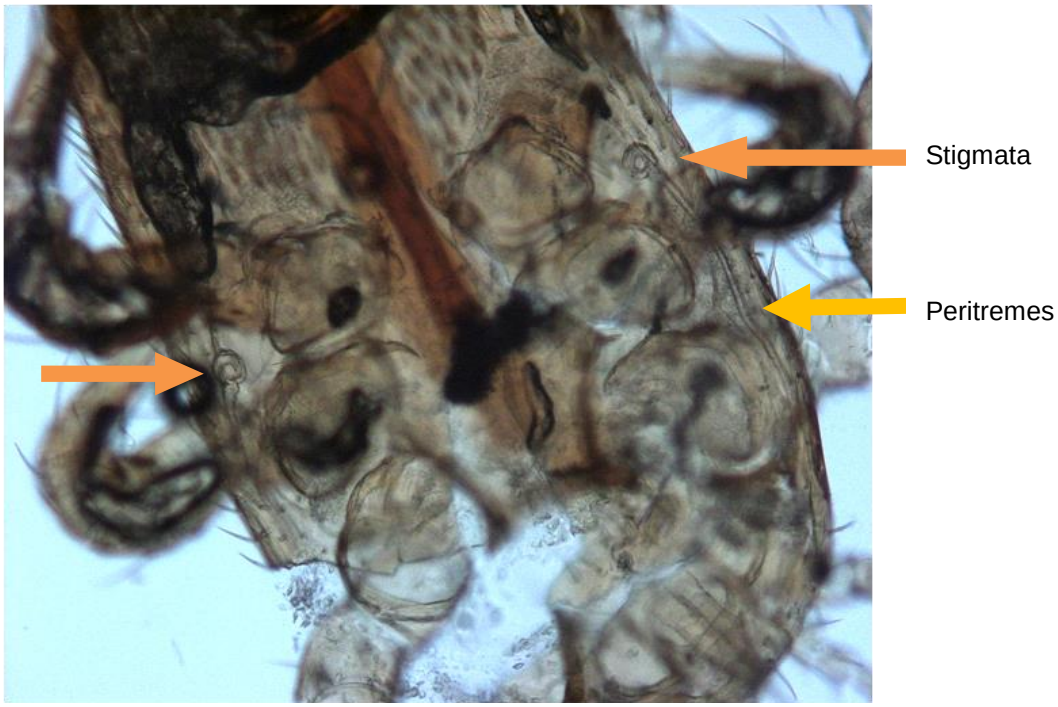
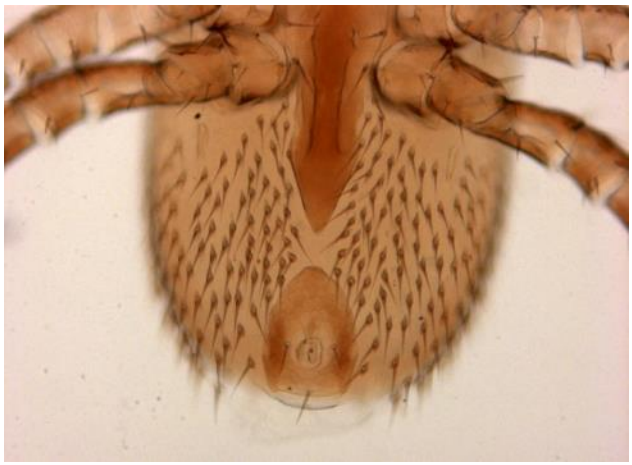
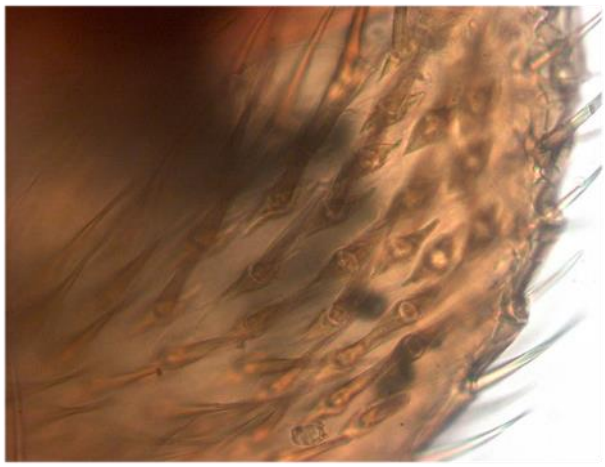


Figure 8 - *Tropilaelaps* spp. - Lateroventral view. Stigmata between coxas III and IV (200X).

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.

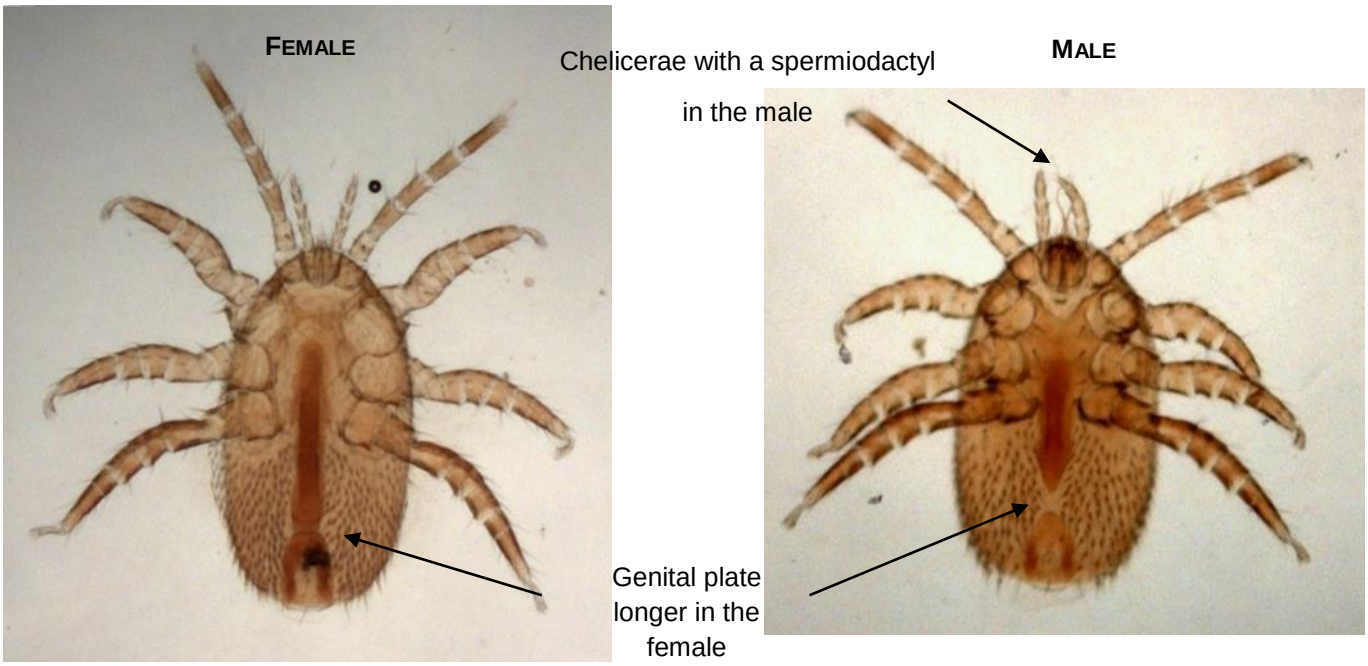


Figure 10 – *T. clareae*, male and female (ventral view).

Source: Anses, Sophia Antipolis.

Spermiodactyl absent in the female

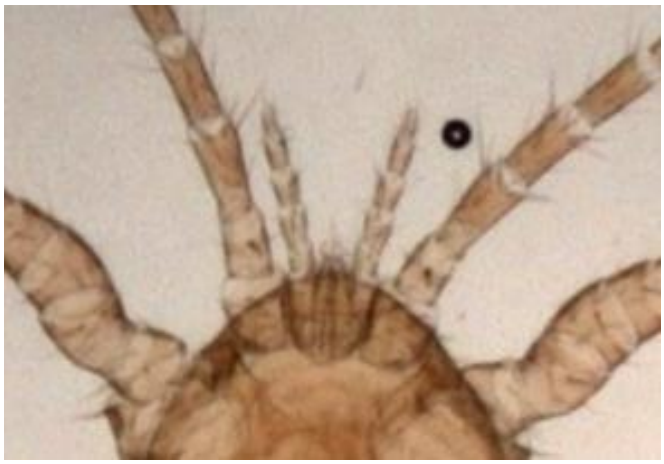


Figure 11 - *Tropilaelaps clareae,* female (anterior view).

Source: Anses, Sophia Antipolis.

Chelicerae with a spermiodactyl in the male

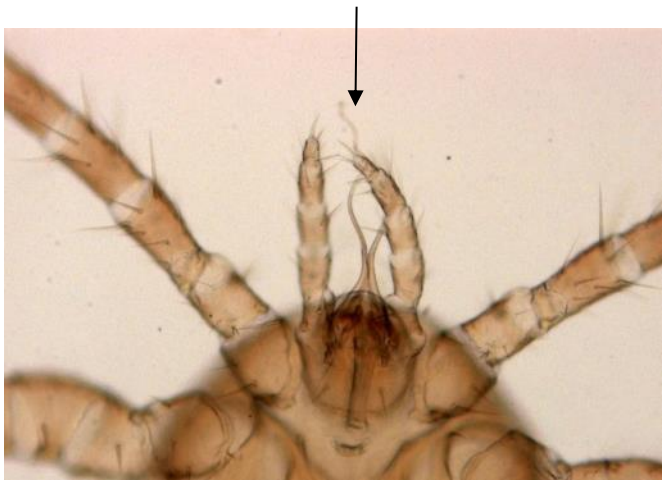


Figure 12 – *Tropilaelaps clareae*, male (anterior view).

Source: Anses, Sophia Antipolis.