ANATOMY OF *TUNGA TRIMAMILLATA* PAMPIGLIONE *ET AL.*, 2002 (INSECTA, SIPHONAPTERA, TUNGIDAE) AND DEVELOPMENTAL PHASES OF THE GRAVID FEMALE

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Summary:

This paper deals with some internal anatomical features observed in histological sections and freshly dissected mounts of *Tunga trimamillata*, a Siphonaptera recently discovered in Andean regions of Ecuador from several mammals, including man. It was possible to study in males and also non-gravid and gravid females, the location and anatomy of several organs not previously described for this species: the testes, epididymis, *ganglia*, Malpighian tubules, eyes, rectal *ampulla* with one of its pads and structures which could be interpreted as midgut *diverticula*, whose presence has not been recorded in the Siphonaptera. The process of neosomy in the female during pregnancy is illustrated by photographs of the consecutive developmental phases, taken at the stereomicroscope. Furthermore, some details of the exoskeleton, *spermatheca* during different phases of pregnancy of the gravid female and the presence of a foreign body (parasite?) within the haemocoel have been displayed in specimens cleared with Hoyer's medium.

KEY WORDS : *Tunga trimamillata*, sand flea, anatomy, midgut *diverticula*, neosomic development.

Resumen : ANATOMÍA DE *TUNGA TRIMAMILLATA* PAMPIGLIONE *ET AL.*, 2002 (INSECTA, SIPHONAPTERA, TUNGIDAE) Y FASES DEL DESARROLLO EN LA HEMBRA DURANTE LA GRAVIDEZ

En este trabajo, gracias a las fotos de preparados histológicos y de disecciones, se dan a conocer algunas particularidades de la anatomía del Siphonaptera Tunga trimamillata, *que ha sido recientemente descubierto en varias localidades de los Andes ecuatorianos y representa una plaga de numerosas especies de mamíferos incluyendo el hombre. Además se evidencian, sea en el macho que en la hembra virgen y grávida, la posición y la anatomía de algunos órganos que no habían sido previamente descritos en esta especie: testículos, epidídimos, ganglios, tubos de Malpighi, ojo, ampolla rectal y algunas estructuras del mesenteron que podrían ser interpretadas como "divertículos". La presencia de estos últimos parece no había sido mencionada en las Pulgas. Se describen y se ilustran con fotografía al estereomicroscopio el desarrollo neosómico de la hembra durante la gravidez en las diferentes fases. Adicionalmente, después de haber clarificado los preparados con el líquido de Hoyer, se destacan algunas particularidades del exosqueleto, la espermateca de la hembra en las sucesivas fases del desarrollo y la presencia de un cuerpo extraño (¿parásito?) en el hemocele.*

PALABRAS CLAVE : Tunga trimamillata, *pulga penetrante, anatomía, divertículos del mesenteron, desarrollo neosómico.*

Résumé : ANATOMIE DE *TUNGA TRIMAMILLATA* PAMPIGLIONE *ET AL.*, 2002 (INSECTA, SIPHONAPTERA, TUGIDAE) ET PHASES DU DÉVELOPPEMENT DE LA FEMELLE GRAVIDE

Ce travail étudie, grâce à des images obtenues sur des préparations histologiques ou par dilacération, des détails anatomiques de Tunga trimamillata, *une puce-chique (Siphonaptera) récemment décrite de la région andine de l'Équateur, parasite de nombreuses espèces de mammifères y compris l'homme. Nous avons ainsi pu mettre en évidence, entre autres, tant chez le mâle que chez la femelle gravide ou non, la situation précise et la morphologie de plusieurs organes qui n'avaient pu encore être décrits pour cette espèce : les testicules, les épididymes, la chaîne nerveuse, les tubules de Malpighi, l'œil, l'ampoule rectale et certaines formations du mésenteron qui pourraient être considérées comme des "diverticules". La présence de ces derniers organites ne semble pas avoir été signalée chez les Siphonaptères. Le processus de néosomie chez la femelle pendant sa période de gorgement et de maturation des œufs a été illustré par des photos au microscope stéréoscopique, pendant les phases successives de développement. Certains détails concernant l'exosquelette, l'évolution morphologique de la spermathèque, de même que la présence d'un corps étranger (parasite ?) dans l'hémocèle ont été visualisés par l'utilisation du liquide de Hoyer.*

MOTS CLÉS : Tunga trimamillata, *puce chique, anatomie, diverticules du mésenteron, processus de néosomie.*

INTRODUCTION

Tunga trimamillata is a sand flea recently discovered in some Andean regions of Ecuador, parasitic of many species of mammals (cow, swine, goat, sheep) (Pampiglione *et al.*, 2002; 2004) humans included (Fioravanti *et al.*, 2003). Therefore, this sand flea is the tenth species of the genus *Tunga* (Insecta, Siphonaptera, Tungidae) described up to now and the 2nd observed in man and domestic animals, with evident importance, consequently, in human and veteri-

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nary medicine. The other eight species of the same genus are recorded only on wild animals and limited mostly to an unique or a few related species of hosts (anteaters, armadillos, rats and other small rodents). Having had the opportunity of obtaining several gravid and non-gravid females and males of this sand flea from Ecuador, we were able to study the anatomy of some internal organs which had not been described previously (Pampiglione *et al.*, 2003; 2004) due to a shortage of samples. This study involved histological sections and dissection of specimens.
Some external morphological details of the insect, essential for the diagnosis of this species, are also here displayed, as well as the external different aspects during the gravid female neosomic development.

MATERIALS AND METHODS

For this study, 20 males, 20 gravid females and 20 non-gravid females of *T. trimamillata* were critically examined. They were collected in Santa Isabel and Catacocha villages, located in the Andean provinces of Azuai and Loja (Ecuador), respectively. Males and non-gravid females were collected both with sticky traps placed on the ground and by human bait collections, while the gravid females were extracted from cattle. After fixation in 70 % ethanol or 10 % buffered formalin, the parasites underwent histological preparation, using 5-7 µm sections, stained with Masson Goldner's Trichromic, Picro-Mallory or Heidenhaim-Azan (the latter as suggested by Rothschild *et al.,* 1986). In a few cases the specimens were dissected and/or cleared in Hoyer's medium, sometimes supplemented with MIF (merthiolate, iodine, formalin), to highlight certain anatomical features. To study the sequence of the developmental phases of the females during pregnancy, a stereomicroscope was used to observe specimens extracted from the host, after removing tissue remnants and fixing the fleas in 10 % buffered formalin. For identification of the different organs and systems we used the above-mentioned manual (Rothschild *et al.*, 1986) for the internal structures of Siphonaptera and also papers by Jordan (1962) and Barnes & Radovsky (1969) on the morphology of *T. caecigena* and *T. monositus*, respectively.

RESULTS AND DISCUSSION

In previous studies (Pampiglione *et al.*, 2003, 2004) some prevalently external morphological details of *T. trimamillata* were presented. In this paper the histological structure of some of the biological systems and internal organs, important not only for the differential diagnosis of the parasite, but especially for understanding its anatomy, physiology and evolutionary development, are presented.
Since this study is principally illustrative, the figures have been grouped into five plates according to the following criteria in order to facilitate readability and discussion of the results:

MALE

Plate I displays the histological structures both in an overall view and in their more important specific structures presented at a higher magnification. Therefore, in Figures 1 and 2, the parasite is seen *in toto* in sagittal sections in a lateral position, and in Figure 3 in a dorso-ventral position. In Figure 1 we surprisingly found the midgut gorged with blood, which had not yet been digested, and the *proventriculus* open. The latter is also observed in greater detail in Figure 7, where the *acanthae* (proventricular spines) appear numerous, lightly tanned, with their points bifurcate and with well developed specialised cells of unknown function (according to Rothschild *et al.,* 1986) grouped in two globose formations in the "neck" of the midgut. Haematophagy, which has already been demonstrated in experiments with rats for the male of *T. penetrans* (Witt *et al.*, 2004) is now demonstrated in the male of *T. trimamillata*. Furthermore, in a sagittal section (Fig. 3), we managed to point out the distribution of the musculature in the entire body, including the limbs, as well as with specific sections, that of the *aedeagus* and a clasper (Figs 10, 11). We could see also the *testes*, containing developing sperm bundles, the fully developed epididymis containing mature sperm, with voluminous accessory glands attached (Figs 1, 2, 4, 8) as well as the brain (supraoesophageal and suboesophageal *ganglia*), thoracic and part of the abdominal *ganglia* of the nerve chord ventrally laying (Fig. 5); these are normally located. It's not the instance in two, taxonomically or biologically related fleas *Tunga monositus* Barnes & Radowsky, 1969 and *Phacopsylla inexpectata* (Smit, 1950) *teste* Beaucournu *et al.*, 1996. These organs have never previously been seen directly using Hoyer's medium. The same is true for the resilin pad (Fig. 9), the elastic substance used by the insect to jump (Rothschild *et al.*, 1975). The presence of partially tanned *taenidia* are apparent in the trachea of an abdominal spiracle shown in Figure 6. A group of fat cells is observed in Figure 12; these cells are distributed in several parts of the body and are considered to have a function somewhat analogous to that of the vertebrate liver and are the centre of intermediary metabolism (Rothschild *et al.*, 1986).

NON-GRAVID FEMALE

Plate II has the same arrangement of figures as in Plate I: the overall structures are therefore shown in sagittal

sections in a lateral position (Figs 1, 2) and in a dorso-ventral position (Fig. 3). The voluminous Malpighian tubules both longitudinally (Figs 2, 5) and transversally (Figs 3, 6) are displayed. The whole chord of *ganglia* including the abdominal ones, is visible in Figure 1; it runs along the entire length, under the intestinal tube. Details of two abdominal spiracles are shown in Figure 4. In this female stage we were able to display, in *T. trimamillata*, the whole *spermatheca*, structured with the *bulga*, its *area cribriformis*, the *hilla* and delimited by a transparent fragile wall (Fig. 7). In spite of numerous previous trials by dissection of non-gravid females of *Tunga trimamillata* and *T. penetrans*, we never obtained to isolate the entire *spermatheca* but only its thicker components (*area cribriformis* and *hilla*) (Pampiglione *et al.*, 2004, Fig. 5A, B), while the outline of its wall always resulted broken and vague. Actually, the *spermatheca* outline was never documented exactly in non-gravid Tungidae, with the exception of a para-sagittal section of *Tunga monositus* (Rothschild *et al.*, 1986, Fig. 182): the pictures reported by different authors were only showing partial details or were concerning gravid females (Karsten, 1865, Plate 8: Fig. 12; Pinto & Oliveira, 1942, Fig. 1; Hopkins & Rothschild, 1953, Plate 6: B and Plate 7: B, C, respectively; Johnson, 1957, Plate 113: 2; Barnes & Radovsky, 1969, Fig. 22). Some drawings have outlined the contour of the organ but they were always hypothetic and imperfect (Hopkins & Rothschild, 1953, Fig. 21D). For these reasons the present image (Fig. 7) represents the only real and complete picture of the *spermatheca* in a non-gravid female of a dissected Tungidae. The eye including its lens, optic nerve and pigmented layer is presented in Figure 8. The structure of the rectal *ampulla*, already seen in Figure 2, is shown with higher magnification in Figure 9 and the presence of a pad in its cavity (Fig. 9) is observed. In all our slides it was not possible to notice more than two rectal pads, as in *Tunga* and *Echidnophaga* (Rothschild *et al.*, 1986), while the number usually found in Siphonaptera is 6 (Grassé, 1951; Rothschild *et al.*, 1986). The structure of the *sensilium* and of the abdominal spiracles, again in a non-expanded female, are shown in Plate V (Figs 5, 11 and 4, 5, respectively). Of the latter, we particularly point out the importance of those of the 8th segment, which present a rake-shaped dilation formed by a series of delicate fibrils (about 20), incorporated into a sheath, which becomes transparent with Hoyer's medium (Plate V; Fig. 5). It appears as a pseudo tail, when the insect is seen in profile and characterises the non-gravid female of *T. trimamillata*, distinguishing it from other *Tunga* species (Hopkins & Rothschild, 1953; Pampiglione *et al.*, 2004). In the male, the 8th abdominal spiracle and its relative segment have a different shape, less differentiable from those of *T. penetrans*.

Gravid female

Plate III shows images of a gravid *T. trimamillata* female. A view of the overall structures of the parasite in a cross section of about three-quarters of the abdomen is shown in Figure 1. An oviduct, containing *ova*, is seen in Figure 2, while other eggs and ovarioles are visible here and there in Figures 1, 2, 3 and 6. The structure of the alimentary canal epithelium with *villi* both in the anterior and the distal parts of the midgut is shown in Figures 12 and 3. We were able to show also in Figures 4 and 6 the existence for *T. trimamillata* of some structures which could be tentatively interpreted as "midgut *diverticula* or pouches" of the dendritic type. Since, from the literature consulted, it does not appear that such *diverticula* have ever been described in Siphonaptera (Reinhardt, 1976) as in other Mecopteroids (Grassé, 1951), we suggest their existence in *T. trimamillata* with caution, awaiting further histological approaches incorporating serial sections and elective stains, or dissection of the insect, to confirm it. According to what we have verified up to now, they seem to develop only in pregnancy. In fact, we have never been able to observe them either in males or in non-gravid females, while in gravid females they are frequently present. Transverse sections of thoracic muscular fibres are reported in Figure 7, while Figure 8 shows some skeletal structures of the head, sunk between two of the three anterior protuberances, and also a section of the pharynx shaped like a small star. The structure of the *spermatheca* with its attached glands, both in histological sections and from dissection, is presented in Figures 9 and 10. An image of the *spermatheca* with its wall only slightly thickened, extracted by dissecting a gravid female at the beginning of expansion and treated with Hoyer and MIF is reported in Figure 11. From other dissections that we made in different phases of dilation it seems that the wall of the *spermatheca* already becomes thicker very early in pregnancy and that, therefore, the finding in Figure 11, consequently, represents the passage of the transparent virginal phase, illustrated in Figure 7 (Plate II), to the thickened and sclerotic phase of pregnancy (Figs 9, 10) (Pampiglione *et al.*, 2004, Fig. 5C, D). These changes in structure of *spermatheca* is only reported in genus *Tunga,* for this time and they could be in relation to the copulation stimulus and to necessity of a more consistent container for sperm. Some giant polyploid cells present in the hypodermal layer of the expanded cuticle during the process of neosomy, are illustrated in Figure 5. These cells were observed by Rothschild *et al.* (1986) and Rothschild (1988) in four other *Tunga* species and seemed to be associated with the massive necessity for protein synthesis during the rapid expansion of the cuticle. These cells are also present in the hypodermic layer of the anterior dila-

tion of the abdomen (Plate IV; Figs 7, 8) typical of the species (Plate V; Fig. 3).

Process of neosomy

Plate IV illustrates various developmental phases during pregnancy in females extracted intact from cattle skin and examined under a stereomicroscope. Development of neosomy with dilation of the abdomen, especially of the intersegmental membrane between the 2nd and 3rd abdominal *sclerites*, has been here documented in different phases of pregnancy, starting of a specimen not yet fertilized (Fig. 1) and continuing the gradual dilation process (Audy *et al.*, 1972; Rothschild, 1992) up to maturity with the appearance of characteristic anterior protuberances and the caudal cone (Figs 2-6). During development, as the size of the insect increases, the 1st abdominal segment is pushed forward, forming the three characteristic protuberances of *T. trimamillata*, which almost protect the head and the thorax as an airbag (Fig. 6; Plate V, Fig. 3); some histological details of these protuberances are presented in Figures 7 and 8. The enormous dilation of the 2nd abdominal segment pushes the successive segments more and more caudally. When the abdomen has reached its maximum expansion, the flea becomes spherical or cylindrical, showing a continuous wall on which the three protuberances project anteriorly and caudally, there is moreover a slightly protruding terminal cone (Fig. 6). This develops into a caudal disc, slightly funnel-shaped, with the genital pore and anus at its centre, surrounded by *tergites* and *sternites* of the 3rd to 8th abdominal segments, overlapping one another (Plate V, Fig. 6). Such formation probably permits telescopic and elastic movements, favouring the fast expulsion of the eggs, as observed in other *Tunga* species (Jordan, 1962; Barnes & Radovsky, 1969).

Taxonomic separation characters

Regarding taxonomic separation of *T. trimamillata* and *T. penetrans* (Pampiglione *et al.*, 2004), we have stressed the most distinctive characteristics, essential for such a diagnosis, in Plate V. Of special importance is the structure of the maxillary palp in the non-gravid female (Fig. 1): whereas in *T. penetrans* the second segment is generally longer than the others (Westwood, 1840; Hopkins & Rothschild, 1953) and numerous bristles are implanted on its entire surface, in *T. trimamillata* the first segment is always the longest and, in addition to bristles, bears very peculiar short, thick, slightly claw-like spines. Taxonomic separation between the two species in the non-gravid female is also evident in the particular structure of the above-mentioned 8th abdominal spiracle (Fig. 5) whereas, in the gravid female, the presence of three anterior protuberances (Fig. 3) and, in both males and females, the arrangement of the spines on the tibia and on the 1st tarsal segment of the 3rd pair of legs (Fig. 2): a row of 9-12 spines on the antero-medial surface of the tibia are present in *T. trimamillata*, absent in *T. penetrans*; 9-13 spines on the edge of the first tarsal segment are present in the former species, while in the latter species the number is always lower. The structure of the spiracles of the 5th, 6th, 7th and 8th abdominal segments of the non-gravid female is illustrated in Figure 4, while the *sensilium*, which is shown detached, observed from the posterior end so as to render the mechanoreceptors (8 in number) visible on both sides, is reported in Figure 11. In Figure 6 the caudal extremity of the gravid female, seen from the posterior end and cleared with Hoyer, can be observed; in it, the *sternites* of the 3rd to the 8th abdominal segments surrounding the caudal disc are clearly seen.

Some elements of the exoskeleton (Fig. 8), which we were unable to show in our previous studies (Pampiglione *et al.*, 2003; 2004), have, by using Hoyer's medium, now been identified in both males and non-gravid females for both species. They are the three intersegmental membranes (Fig. 7) which project bilaterally from the thorax at the level of *pronotum*, *mesonotum* and *metanotum*, like three triangular transparent sails, and for this reason are often not recognizable. They are often curled under, sloping from the largest to the shortest. There are no records that they have been described in other species of chigoes. We have identified them not only in *T. trimamillata* but also in *T. penetrans*, both in males and in females. It is possible that the method of clearing specimens currently used (10 % NaOH solution) makes these membranes so transparent that it is impossible to see them. In contrast using Hoyer's medium they are frequently recognised. Similarly, the *metepimeron* (Fig. 7) in *T. trimamillata* was rarely visible when NaOH solution was used. In contrast, it was clearly and frequently recognisable when Hoyer was applied, or when SEM examinations were made. The presence of the *metepimeron* having this form, which is not specific to *T. trimamillata*, had already been reported from the first observations on *T. penetrans* in the 1800s (Karsten, 1865; Blanchard, 1890) and had been interpreted incorrectly by some past authors as a wing (Dugès, 1836). The 8th spiracle and the *sensilium* in the male are depicted in Figure 12. The presence of a foreign body in the haemocoel (Figs 9, 10), probably the sclerotised and destroyed larval form of a parasite or an extraneous body incorporated by the flea larva, has been observed in only one male specimen of *T. trimamillata* and is therefore considered a rare exception.

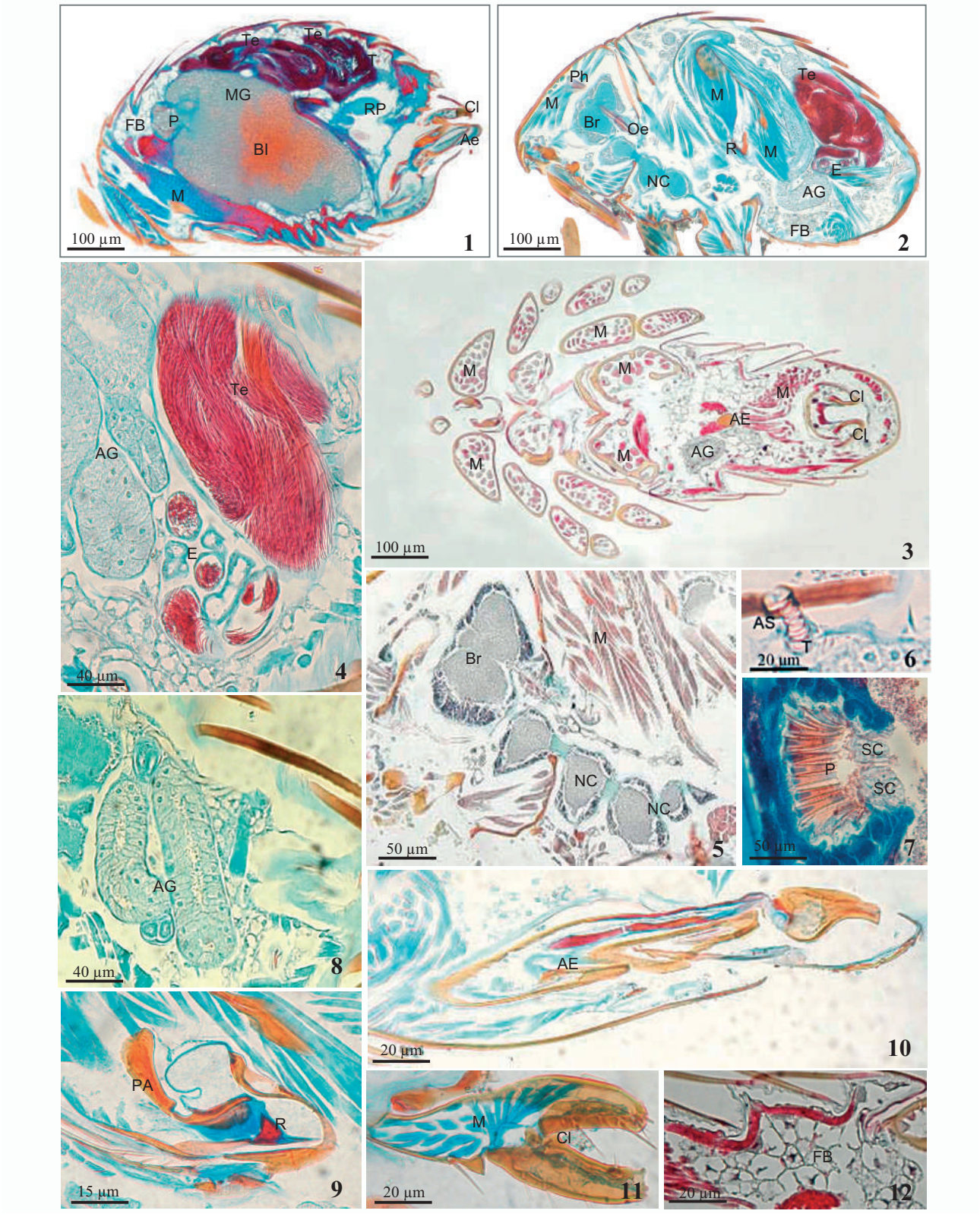

Plate I. – Anatomical details of a male of *T. trimamillata*: Ae = *aedeagus*; AG = accessory glands; AS = abdominal spiracle; BI = blood ingested; Br = brain; Cl = claspers; E = epididymis; FB = fat body; M = muscles; MG = midgut; NC = nerve chord; Oe = oesophagus; P = *proventriculus*; PA = pleural arch; Ph = pharynx; R = resilin pad; RP = rectal pad; SC = specialised cells; T = trachea; Te = testes. (Stains: Heidenhaim Azan = Figs 1, 2, 3, 7, 8, 9, 10, 11; Masson Goldner = Figs 4, 5, 6, 12).

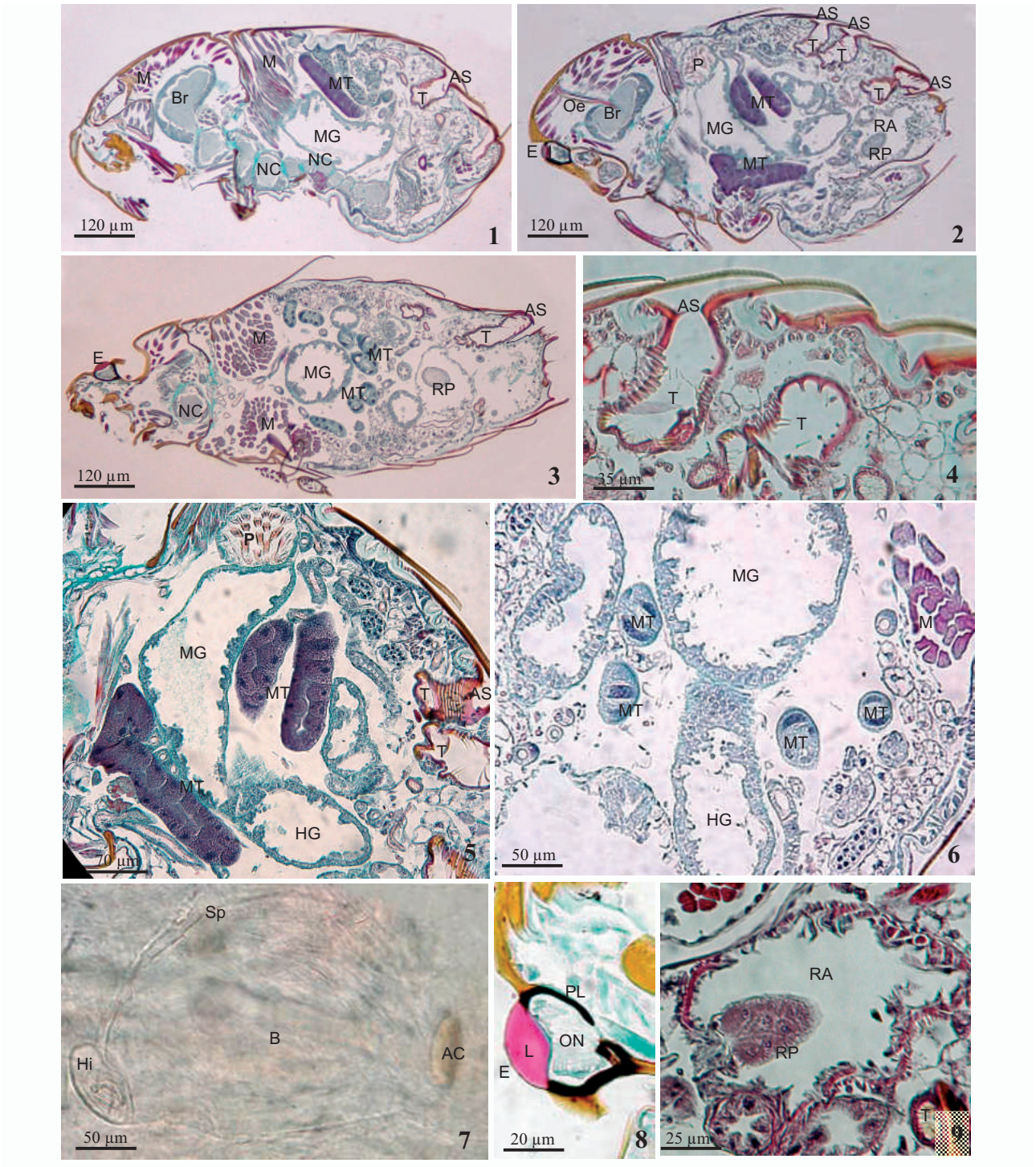

Plate II. – Anatomical details of a non-gravid female of *T. trimamillata*: AC = *area cribriformis*; AS = abdominal spiracles; B = *bulga*; Br = brain; E = eye; HG = hindgut; Hi = *billa*; L = lens; M = muscles; MG = midgut; MT = Malpighian tuabules; NC = nerve chord; Oe = oesophagus; ON = optic nerve; P = *proventriculus*; PL = pigmented layer; RA = rectal *ampulla*; RP = rectal pad; Sp = *spermatheca*; T = trachea. (Stains: Hoyer medium = Fig. 7; Picro Mallory = Figs 1, 2, 3, 5, 6; Masson Goldner = Figs 4, 8, 9).

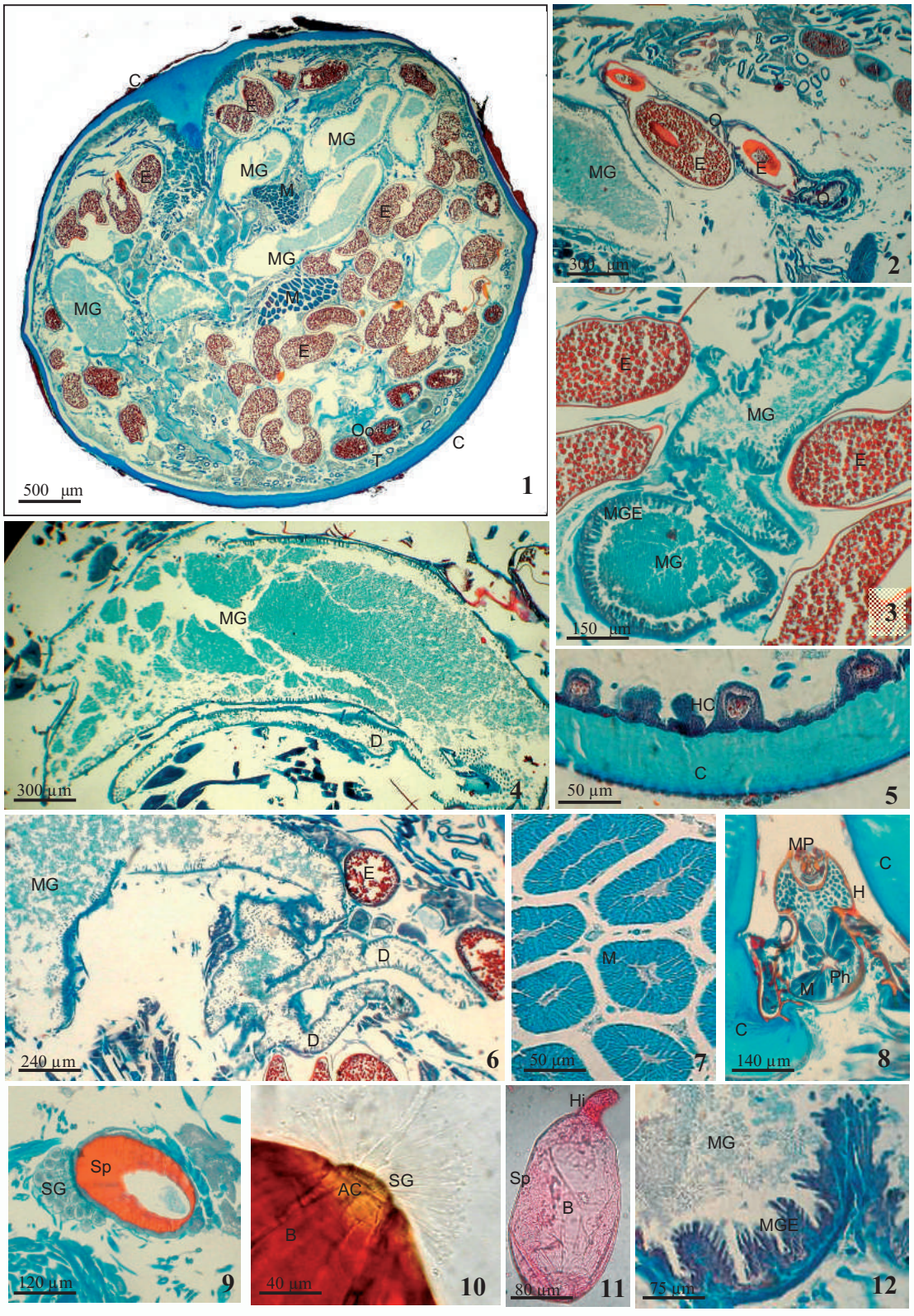

Plate III. – Anatomical details of a gravid female of *T. trimamillata*: AC = *area cribriformis*; B = *bulga*; C = cuticula; D = *diverticulum*; E = eggs; H = head; HC = hypodermal cells; M = muscles; MG = midgut; MGE = midgut eptihelium; MP = mouth parts; O = oviduct; Oo = oocites; OP = Oral Parts; Ph = pharynx; Sp = *spermatheca*; SG = spermathecal glands. (Stains: Heidenhaim Azan = Figs 1, 2, 3, 4, 5, 6, 7, 8, 12; Hoyer medium = Fig. 10; Hoyer + MIF = Fig. 11).

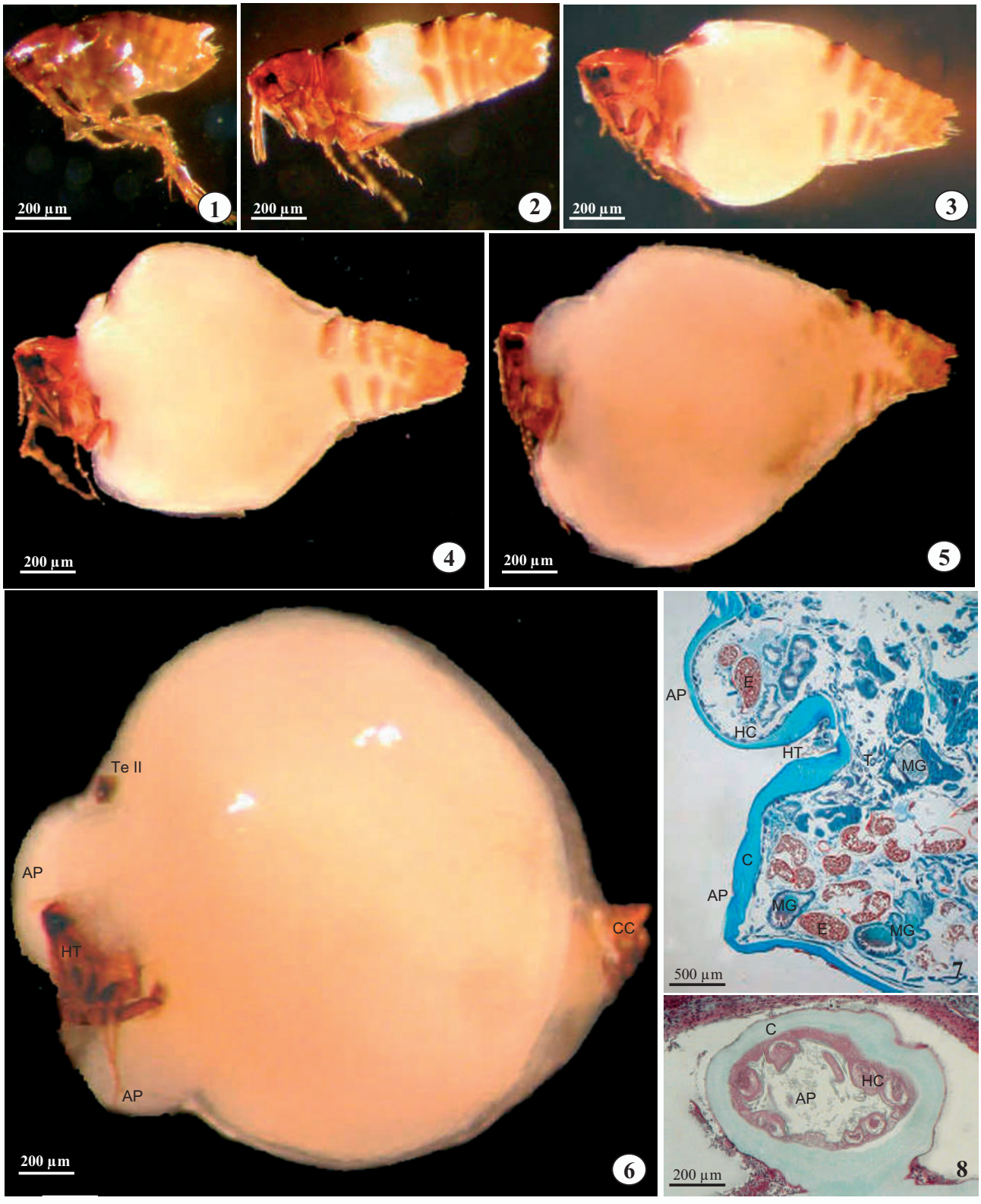

Plate IV. – Neosomic development of *T. trimamillata* from a non-gravid to a 3/4 grown gravid female (Figs 1-6); AP = anterior protuberances; C = cuticula; CC = caudal cone; E = eggs; HC = hypodermal cells in an anterior protuberance; HT = head and thorax; MG = midgut; T = tracheae; Te II = *tergum* of II abdominal segment. (Stains: Heidenhaim Azan = Fig. 7; Masson Goldner = Fig. 8; without stain = Figs 1, 2, 3, 4, 5).

Plate V. – Some other morphological details of *T. trimamillata* (evidenced by Hoyer medium, with the exception of Figure 3). Figures 7, 9 and 10 are concerning a male, Figures 3 and 6 a gravid female, the others refer to non-gravid females): Ab = abdomen; AP = anterior protuberances; AS V-IX = abdominal spiracles of V-IX abdominal segments; Cl = claspers; CD = caudal disc; FB = foreign body; H = head; HT = head and thorax; IM = intersegmental membranes; Ma = *maxilla*; ME = *metepimeron*; MP = maxillary palp; Se = *sensilium*; Ti = tibia of 3rd pair of legs; Te III-VIII = terga of abdominal segments III-VIII; TS = first tarsal segment of 3rd pair of legs. (Stains: Hoyer medium: Figs 1, 2, 4, 6, 7, 9, 10, 11, 12; Hoyer + MIF: Figs 5 and 8; without stain: Fig. 3).

CONCLUSIONS

This study provides a series of competent photographs, both of the internal and the external structures of *T. trimamillata*. In this manner we have been able to demonstrate the location and the histological structure of several internal organs in this species for the first time and we were sometimes able to capture some of their important functions.
Thus we were able to identify:
a) in the male: testes, epididymis, accessory glands, *proventriculus, ganglia*, resilin pad, rectal *ampulla*; and haematophagy was demonstrated;
b) in the non-gravid female: the whole profile of the *spermatheca*, Malpighian tubules, the structure of the eyes and a pad in the rectal *ampulla*;
c) in the gravid female: oviduct, some ovarioles chains, fertilised and non-fertilised *ova*, some possible midgut *diverticula*, the accessory glands of *spermatheca*, the giant polyploid cells of the hypodermic layer and the structure of the intestinal epithelial layer;
d) the whole process of neosomy during the female pregnancy with the formation of the three anterior abdominal protuberances and the caudal cone;
e) the principal anatomical characters of taxonomic separation differentiating the species.
Since we think that the anatomy-morphology of an insect is "a *sine qua non* to interpret and understand the many mysteries in the other subdisciplines of entomology" (Gupta, 1994), we have begun, in addition, a SEM examination of *T. trimamillata*, to complement what has been reported here, which will furnish a more complete picture of both males and gravid and non-gravid females using different techniques.

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