THESIS

STUDIES ON THE EXTERNAL MORPHOLOGY OF *BOOPHILUS MICROPLUS* MIDGUT BY SCANNING ELECTRON MICROSCOPE

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GRADUATE SCHOOL, KASETSART UNIVERSITY
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THESIS

STUDIES ON THE EXTERNAL MORPHOLOGY OF *BOOPHILUS MICROPLUS* MIDGUT BY SCANNING ELECTRON MICROSCOPE

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A Thesis Submitted in Partial Fulfillment of
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The morphology of the midgut of *Boophilus microplus* was studied using the scanning electron microscope (SEM). The alimentary tract of *B. microplus* was divided into 3 parts; foregut, midgut and hind gut. The foregut contains the pharynx and the esophagus, the midgut consists of the ventricular caeca and the stomach, while hind gut, the last part of the alimentary tract, consists of the rectal sac and the rectum. The midgut is the most important part of the alimentary tract, because several proteins identifiable here lead to the commercial vaccine against *B. microplus.* However, there is limited data concerning the morphology of *B. microplus*, especially the midgut. Therefore, we used the SEM in combination with the LM technique to clarify the morphology of the *B. microplus* midgut. This study analyzed the midgut of a sample of 30 engorged adult females of *B. microplus* of 4-6 mm in length. The midgut consists of 7 pairs of ventricular caeca and can be divided into two parts, the anterior and posterior part. The anterior part consists of 4 short pairs (a.l.1-a.l.4) and 1 long pair of ventricular caeca (a.l.5) while the posterior part consists of 2 long pairs of ventricular caeca (p.l.1-p.l.2). The stomach (STO) is situated between the anterior and the posterior part. A rectum forms from the ventral part of the stomach and enlarges becoming a rectal sac (RS). Even though the midgut is formed of 7 pairs of ventricular caeca and the stomach, it is possible to categorize them into 3 groups according to the cells composing each part. Group 1 consists of STO, RS, a.l.1 and p.l.1. Group 2 consists of a.l.2, a.l.3 and a.l.5 and group 3 consists of a.l.4 and p.l.2. This study shows that each ventricular caeca of the midgut of *B. microplus* can be identified distinctly either observing them by SEM or analyzing the components of the composing cells by LM technique.

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Student's signature Thesis Advisor's signature

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Hathairat Chanphao

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LIST OF ABBREVIATIONS

a.l.	=	anterolateral
AN	=	anus
CH	=	chelicera
CL	=	claw
cm	=	centimeter
CPD	=	critical point dryer
CX	=	coxa
DE	=	early stage of digestive cells
DHL	=	digestive cells with hemoglobin and leukocyte
DHV	=	digestive cells with hemoglobin without leukocyte
DM	=	mature stage of digestive cells
DS	=	spent digestive cells
EM	=	pulvillus
FE	=	festoon
FG	=	foregut
GN	=	gnathosoma
GO	=	genital opening
H	=	hypostome
H&E	=	haematoxylin and eosin
HG	=	hindgut
LM	=	Light microscope
µm	=	micrometer
mm	=	millimeter
min	=	minute
NaH_2PO_4	=	sodium phosphate, monobasic
Na_2HPO_4	=	sodium phosphate, dibasic
NAOH	=	sodium hydroxide
PBS	=	phosphate buffer solution
p.l.	=	posterolateral
PP	=	palpus

LIST OF ABBREVIATIONS (Continued)

RS	=	rectal sac
SD	=	standard deviation
SEM	=	Scanning electron microscope
S_1E	=	early stage of secretory cells type 1
S_2E	=	early stage of secretory cells type 2
S_1M	=	mature stage of secretory cells type 1
S_2M	=	mature stage of secretory cells type 2
ST	=	stem cell
STI	=	stigma
STO	=	stomach
TA	=	tarsus

STUDIES ON THE EXTERNAL MORPHOLOGY OF *BOOPHILUS MICROPLUS* MIDGUT BY SCANNING ELECTRON MICROSCOPE

INTRODUCTION

The economic importance of ticks is widely acknowledged and is related to their feeding habits. When feeding, almost all species of ticks transmit diseases such as protozoa, viruses, rickettsias and spirochetes, to man and other animals (Rey, 1973). Among the species belonging to the family Ixodidae, the tick *Boophilus microplus*, known as tropical cattle ticks, which is of the great veterinary importance, because it transmits the disease known as cattle babesiosis or cattle fever. This disease causes severe economical losses to the cattle industry in many countries (Arthur, 1996). This ectoparasite produces weakness, reduced milk and meat production (Boue, 1998). Global economic losses caused by *B. microplus* ticks have been estimated at US$ 7 per animal per year (Patarroyo, 2002). In Cuba alone, babesiosis and anaplasmosis have caused more than 100,000 deaths over the last decade (Boue, 1998). Brazil has the fifth largest cattle herd in the world and economic losses around US$ 800 million have been estimated due to direct and indirect effects from B. microplus infestations (Patarroyo, 2002).

Numerous studies are currently underway aiming to analyze the feeding processes of the cattle tick *B. microplus* (Canestrini) (Tatchell, 1968). Lara et al (2003) studied the pathway of haem detoxification in the midgut of the cattle tick B. microplus. Other studies focused on the internal anatomy of Argas ticks (Roshdy, 1961, 1962, 1963, 1966), internal organs, especially ovaries of *B. microplus* (Saito, 2005) and digestive cells of midgut of *B. microplus* (Tatchell, 1968). Finally, another study analyzed the feeding processes and digestion of *B. microplus* (Lara et al, 2003). Numerous studies are aiming to find an efficient control strategy that would minimize the damages caused by these parasites such as anti-cattle-tick vaccine (Tellam *et al*, 1992; Willadsen, 1997). The midgut of the ticks can be used as an immunogenic. Recently, vaccines containing the recombinant *B. microplus* midgut antigen Bm86 have been developed (Fuente, 1999). Antigens from partially engorged females of

B. microplus have been used with variable success to immunize animals against tick infestation (Akhtar, 1999). Although the midgut has an important role for the commercial vaccine, it is also one of the main parts synthesizing proteins that protect the tissues and organs of the ticks. Even though there were many attempts to establish cattle ticks vaccines from the midgut of *B. microplus*, there is limited data concerning its morphology.

The present work was aimed to collect new data on the morphology of the female midgut of *B. microplus* in order to provide information that would contribute to the future control of this parasite. This study shows that each ventricular caeca of the midgut of *B. microplus* can be identified distinctly either observing them by SEM or analyzing the components of the composing cells by LM technique.

LITERATURE REVIEW

1. Background information

Background information about *Boophilus microplus* can be retrieved from Table 1.

Table 1 Background information about *Boophilus microplus*

Genus	Rhipicepharus (Horak, 2002)
Species	Microplus
Location	Australia, West Indies, Mexico, Central
	America, South America, Asia, South Africa
Life Cycle	One host tick, eggs hatch on the ground
Mating Habit	Females lay up to 4400 eggs
Feeding Habit	Causes irritation and loss of condition
	Loss of blood in severe infestation can lead to death
Hosts	Primary: cattle
	Also found on: sheep, goats, horses and deer
Tick Borne Diseases	Cattle: *Babesia bigemina* (redwater fever),
	Anaplasma marginale, (gall fever), *Babesia berbera*

Source: Soulsby, 1982 and Baker, 1999

2. Morphology

Boophilus microplus is a member of the Ixodidae family (hard ticks). Hard ticks have a dorsal shield (scutum) and their mouthparts (capitulum) protrude forward when they are seen from the top. Boophilus ticks have a hexagonal basis capitulum. The spiracular plate is rounded or oval and the palps are very short, compressed, and

ridged dorsally and laterally. Males have adanal shields and accessory shields. The anal groove is absent or indistinct in females, and faint in males. There are no festoons or ornamentation. *B. microplus* adults have a short, straight capitulum. The legs are pale cream and there is a wide space between first pair of legs and the snout. The body is oval to rectangular and the shield is oval and wider at the front. The snout is short and straight. The nymphs of this species have an orange–brown scutum. The body is oval and wider at front. The body color is brown to blue–gray, with white at the front and sides. *B. microplus* larvae have a short, straight capitulum and a brown to cream body. Larvae have 6 legs (Baker, 1999).

3. Biology

Boophilus microplus has been eradicated from the United States. It is still found in Mexico and Africa as well as Australia, Central and South America, Madagascar, and Taiwan. The common occurrence of parthenogenesis in this species aids its survival when harsh conditions restrict the size of a population. Cattle are the primary hosts, but sheep, goats, horses, and other animals may be infested (Gerald, 2005).

4. Importance

Boophilus microplus is a hard tick that can be found on many hosts including cattle, buffalo, horses, donkeys, goats, sheep, deer, pigs, and dogs. It can transmit babesiosis (*Babesia bigemina* and *Babesia bovis* infections) and anaplasmosis (infection by *Anaplasma marginale*). Heavy tick burdens on infested animals can decrease production and hidden damage (Baker, 1999).

5. Life Cycle of Tick *Boophilus microplus*

After hatching, the *Boophilus microplus* larvae find their vertebrate host. During the following a few months, the tick larva feeds on small amounts of blood, and after maturation, over a period slightly longer than 1 day, the adult female ingests blood equivalent to approximately 100 times its own body mass. Being a single-host

tick, the engorged female drops from the bovine host and die approximately 1 month later. Most of the digestion takes place over a few days following the meal, in parallel with the development of a large number of eggs (Gough and Kemp, 1995) (Figure 1).

5.1. Non-parasitic part of the life cycle

This begins when the fully engorged female tick, the stage most easily seen on infested cattle, falls to the ground and finds a suitable place to lay eggs. The pre-egg laying period is dependent on environmental temperature and relative humidity, and can be as short as one to two days or as long as 40 days.

The duration of egg laying is also temperature-controlled and can range from two to 44 days. Each female tick may lay up to 3,500 eggs. During the wet season when both temperature and humidity are optimal, eggs hatch in approximately 18 to 21 days.

The six-legged larvae that hatch from eggs are known as seed ticks. These are extremely active in response to moving objects. The close proximity of an animal is sufficient to activate them to climb to the tips of grass, where they can attach more easily to a passing host. During the evening, seed ticks seek protection in the vegetation. The longevity of seed ticks is influenced by temperature and humidity. They are extremely vulnerable to very low ambient temperatures and low humidity. In northern Australia the maximum longevity is two to four months depending on the season.

The non-parasitic part of the life cycle ends when seed ticks find suitable hosts. These may not necessarily be cattle. The cattle tick has been known to infest horses, sheep, dogs, buffalo, deer, pigs and hares, though cattle are the preferred host. (Radunz, 2003)

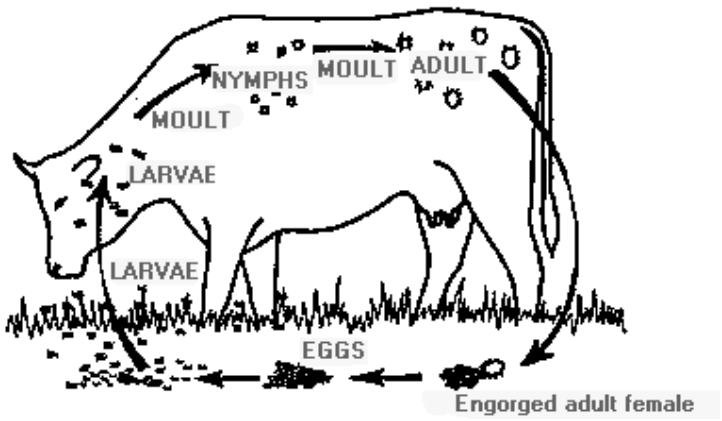


Figure 1 Life cycle of *Boophilus microplus*

Source: Hibberd (2007).

6. Alimentary System

The entrance to the alimentary canal, the tubular buccal canal, is formed dorsally by the chelicerae and ventrally by the hypostome (Figure 2). These two parts of the gnathosoma form the anchorage of the tick to the host skin during feeding. The chelicerae are paired, sheathed, rigid, sclerotized tubes with two segments (other acarians have three segments) ending in cutting digits with recurved teeth with which the tick penetrates the host skin. The unpaired hypostome is an extended, toothed anterior process of the basis capituli with retrograde denticles on the ventral surface. In ixodid ticks with the exception of some *Ixodes* spp., the feeding channel is further sealed during host attachment by attachment cement, a salivary gland product which solidifies almost immediately, making the tick alimentary canal continuous with the lesion in the host skin. The cement seals off and firmly embeds the mouth parts. If some tick species are removed manually from the host, the cement often remains attached to the mouth parts.

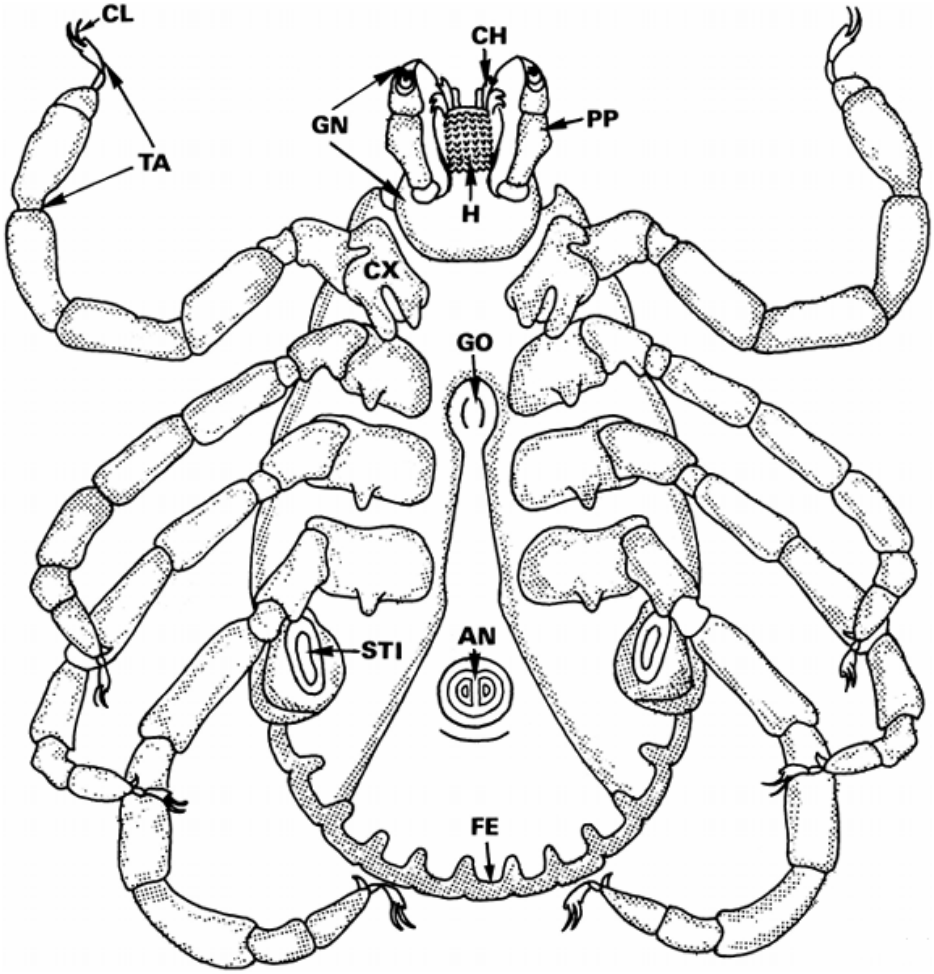


Figure 2 Diagrammatic representation of an ixodid tick from its ventral side
AN, anus; CH, chelicera; CL, claw; CX, coxa; EM, pulvillus; FE, festoon; GN, gnathosoma (capitulum); GO, genital opening; H, hypostome; PP, pedipalpus; STI, stigma; TA, tarsus.

Source: Mehlhorn (2004)

The buccal canal is a common duct for the intake of host tissues and for the outflow of saliva. It passes into the pharynx, a powerful suction organ with several sets of constrictor and dilator muscles, which, in conjunction with the pharyngeal valve, moves host tissues into the esophagus. The esophagus, a narrow tube adjoining the pharynx, passes through the synganglion or "brain" (as in other acarians) before

leading to the midgut or ventriculus. This consists of a large central chamber from which several pairs of blind-ending diverticula or ceca lead off, providing additional surface area on which digestive processes can take place. Some are branched or form numerous loops. The midgut fills most of the body cavity of the tick. It is well equipped with muscle fibers situated externally and arranged both longitudinally and transversely, and is capable of peristaltic and other movements. When the leg of a tick is cut off, as in investigations for hemolymph stages of *Babesia* spp. or *Theileria* spp., the hemolymph is frequently mixed with gut contents because ceca protruding into the leg are also damaged (Baker, 1999).

The midgut has a fairly uniform structure throughout, the wall consisting of a single epithelial cell layer resting on a thin basal lamina, with muscle fibers on the hemocoelic side. According to Balashov (1968) and Sonenshine (1993), three types of cells are present in the epithelium: reserve or stem (undifferentiated) cells, secretory cells and digestive cells. Agdebe and Kemp (1985), described intermediate digestive cells (digestive cell series) and two different secretory cells in *Boophilus microplus*. There are indications that each cell can differentiate successively to serve both secretory and digestive functions, particularly in argasid ticks. In ixodid ticks, it appears more likely that each cell differentiates irreversibly to take either a secretory or a digestive role. The apical or luminal surface of epithelial cells is covered with microvilli, while the distal plasma membrane is folded until the time when the tick begins ingesting blood. Because of projecting epithelial cells, the midgut lumen is small.

A short intestine (sometimes called the small intestine) joins the midgut to the rectal sac. It is a tube narrowing towards the rectal sac which it enters anteroventrally. In the rectal sac, the fecal discharge accumulates, together with the products of the malpighian tubules, to be expelled through the anus.

In ixodid ticks, the salivary gland plays a major role during feeding, and it is also important for the development of a variety of pathogens, many of which conclude their development there before being transmitted to the host. The role of the salivary

gland in argasid ticks is different, the excretion of fluid into the host during feeding being minimal. In ixodid ticks, salivary excretion into the host animal body is responsible, to a large extent, for preventing the excess dilution of the tick body fluids by eliminating the major part of the blood meal's liquid content. It has been the subject of extensive studies, both by light and electron microscopy.

The salivary gland is a paired organ with a similar appearance in both sexes. It consists of grapelike clusters of acini extending from the level of the peritremes along the sides to the gnathosoma, where the paired main ducts open into the salivarium, which opens dorsally into the buccal canal.

7. Feeding Habits

The larvae, nymphs and adults of one-host ticks all feed and molt on the same host. Ticks of the genus *Boophilus* are such examples where larvae attach to bovines and engorged females drop off the host 3–4 weeks later. Except for the engorged female, all stages are very small. The genus *Margaropus* also includes one-host ticks. Acaricide resistance in ticks is particularly prevalent among one-host ticks (*Boophilus*) where selection pressure is directed against all stages and heritably resistant mutant individuals have a greater chance of survival and of becoming the progenitors of resistant populations. In the two-host ticks larvae feed and molt without leaving the host, the replete nymph dropping and molting on the ground. The adults then seek a second host to complete development. *Rhipicephalus evertsi*, with two subspecies, is a two-host tick found throughout sub-Sahelian Africa. Some species of *Hyalomma* are also two-host ticks and among them are ticks that can use two or three hosts in different generations. The majority of hard ticks require three hosts to complete development, each stage becoming replete on a host and then dropping to the ground to molt. Larvae, nymphs and adults each seek a different host, the engorged female dropping from the third host to lay eggs on the ground. In two- and three-host ticks, the different stages may prefer completely different host species. In many three-host ticks, larvae and nymphs prefer small rodents and lagomorphs as

hosts while adults attack larger mammals. In some cases, the larvae and nymphs of a species are unable to survive on the host species of the adults.

The argasid ticks have different feeding habits from hard ticks, with usually much shorter feeding periods and up to seven nymphal feedings (*Ornithodoros coriaceus*) as well as up to seven meals in the adult stage (*Argas persicus*), with a different host each time. Ticks with these feeding habits can be termed multihost ticks. *Otobius* spp. are exceptions, showing a modified one-host pattern of feeding.

In ticks, reproduction and feeding are often closely related. However, there are many cases of larval and adult ticks not requiring a blood meal for further development. In many prostriate species, the male does not feed.

As a rule, the period of feeding is short in argasids and long in ixodids. In most species of *Argas* and *Ornithodoros,* adults and nymphs do not require more than 15–60 min to engorge, the range being approximately 2 min to 2 h. Larvae of argasid ticks require longer feeding times than the corresponding nymphs and adults. *Argas persicus* and *A. reflexus* larvae require 5–10 days and *A. boueti* 16–25 days. During engorgement, soft ticks ingest quantities of blood corresponding to 3 or 4 times their original body weight. In hard ticks, this quantity can be 50–200 times the weight of the unfed female. They remain attached to the host and engorge in 5–12 days, unless they do not mate, in which case they may remain for several weeks. Larvae and nymphs of *Rhipicephalus appendiculatus* increase their body weight to the same degree as the female, i.e., about 100 times. Male ixodid ticks feed for 3–5 days, during which time their weight more or less doubles and after which they will ingest further blood only if the nutrients are exhausted while they are searching for or waiting for a female. They may remain on the host, sometimes seeking a fresh host, for several weeks or months (Baker, 1999).

MATERIALS AND METHODS

Materials

1. Equipments for Tick Midgut Preparation

1.1 paraffin petridish
1.2 brade and knife
1.3 needles with curve tip and straight tip
1.4 forceps
1.5 alcohol lamp
1.6 stereoscope
1.7 small test tube with cover
1.8 ice pack
1.9 beaker 25 ml
1.10 dropler
1.11 empty petridish

2. Equipments for Paraffin Technique

2.1 small cassette
2.2 embedding ring
2.3 mold
2.4 slide box
2.5 paraplast
2.6 staining jar
2.7 slide tray
2.8 slide warmers
2.9 racks
2.10 oven incubator
2.11 tissue floatation bath
2.12 automatic tissue processor

2.13 tissue embedding center
2.14 hood
2.15 rotary microtome
2.16 clean glass slides
2.17 cover slips
2.18 light microscope

3. Equipments for SEM

3.1 hood
3.2 stub
3.3 adhesive tabs
3.4 critical point dryer (CPD)
3.5 ion sputter
3.6 Scanning Electron Microscope

4. Chemical Compound

4.1 buffered saline solution pH 7.2-7.4
4.2 glutaraldehyde
4.3 paraformaldehyde
4.4 osmium tetroxide 1%
4.5 acetone 50%, 70%, 80%, 90%, 95% and100%
4.6 alcohol 70%, 80%, 95% and 100%
4.7 melt paraffin
4.8 harris's hematoxylin
4.9 eosin dyes
4.10 fixative agent

Methods

1. Sample Preparation

Sixty adult female ticks at the 5-day-fed stage with the length of 4-6 mm. were used in this study. The ticks were dissected in a petridish containing 0.1 M phosphate buffer pH 7.4. Dorsal part of scutum was carefully cut open. All the internal organs except the midgut were removed. After cleaning with PBS, half of the samples were measured and then paraffin embedded for serial section. The other half subjected to SEM study.

2. Preparation of Paraffin-Embedded Sections for Light Microscopic Study

2.1 Sectioning

Each prepared tick was fixed in Bouin's fluid fixative for 24 hours and transferred to 70% alcohol solution. Afterwards the ticks are transferred and processed in the automatic tissue processor and the tissue embedding device. 5 μm thick tissue sections were prepared and cut from paraffin-embedded blocks on a microtome and mounted with warm water (40-50ºC) onto adhesive microscope slides. Sections are allowed to dry overnight at 40ºC. (Adhesion of the section to the slide is essential to prevent tissue loss during subsequent incubations and washes) (Drury and Wallington (1967) and Hubbard (1994). They were deparafinized with xylene and dehydrated with graded ethanol (Table 2) before haematoxylin and eosin staining.

2.2 Haematoxylin and Eosin Staining Method

2.2.1 Stain in hematoxylin (after deparafinization) in a jar, for 7 min.

2.2.2 Wash well in running tap water for 2-3 minutes. The section may be examined microscopically at this stage to confirm a sufficient degree of staining. If insufficient, return to the stain.

2.2.3 Remove excess stain by decolorizing (differentiating) in 0.5-1 per cent hydrochloric acid in 70 per cent alcohol for a few seconds. The blue staining of the hematoxylin is changed to red by the action of the acid.

2.2.4 Regain the blue color and stop decolorization by washing in alkaline, running tap water for at least 5 minutes. The stain should again be checked microscopically until proficiency in naked-eye control of decolorization has been gained by experience with stain and tissues.

2.2.5 Stain in 1 per cent aqueous eosin for 1-3 minutes.

2.2.6 Wash off surplus stain in water.

2.2.7 Examine microscopically. Cytoplasm and muscle fibers should be deep pink, while collagen should be a lighter pink. Red blood cells and eosinophil granules should be a bright orange-red.

2.2.8 Dehydrate in alcohol and clear in xylene as outlined in the general scheme, bearing in mind that aqueous eosin is removed from tissues by water and low grade alcohol, less readily by absolute alcohol. The degree of staining of eosin is thus easily controllable and a slight over-staining when sections are examined prior to dehydration will be remedied during the passage through alcohol.

2.2.9 Mount in a synthetic resin medium.

3. SEM Preparation

Midgut ticks were flushed in buffer before being transferred into paraformaldehyde: glutaraldehyde (1:1) in PBS (Karnovsky, 1965) for 1-2 h at 4ºC. The tissues were rinsed twice in phosphate buffer at 15-20 min. intervals, transferred into post fixative (2% osmium tetroxide) for 2 h at 4ºC. The tissues were rinsed twice in phosphate buffer at 15-20 min intervals, dehydrated through graded acetones; 50%,

70%, 80%, 90%, 95% and 100% 2 times. Then they were transferred into a critical point drying apparatus (Hitachi Criticalpoint Dryer HCP-2, Japan) for 20 min. Dried tissues were mounted on stubs using adhesive tape and then coated with gold using a sputter coater (JEOL Fine Coat Ion Sputter JFC-1100, Japan) for 10 minutes (Gabriel, 1982).

Table 2 Deparafinization method

Xylene	2 to 3 changes, 5 minutes each
100% alcohol	2 changes, 3 minutes each
95% alcohol	2 changes, 3 minutes each
80% alcohol	3 minutes
70% alcohol	3 minutes

Source: Hubbard (1994)

RESULTS

The midgut of *Boophilus microplus* consists of 7 pairs of ventricular caeca, a.l.1, a.l.2, a.l.3, a.l.4, a.l.5, p.l.1, p.l.2, and a wide central tube which is the stomach (STO). These 7 pairs of the ventricular caeca can be classified based on their positions into anterior and posterior groups. The anterior group consists of 4 short pairs (a.l.1-a.l.4) and 1 long pair (a.l.5) of the ventricular caeca. All of them originate from the anterior end of the stomach. The anterior group arises over the salivary gland in the lateral corner of the anterior part of the tick body. The posterior group consists of 2 pairs of long ventricular caeca (p.l.1-p.l.2) which originate from the posterior end of the stomach. a.l.5, p.l.1 and p.l.2 are comparatively longer than a.l.1-a.l.4, and extend first towards the posterior part, then taking a retrograde curve towards the anterior part.

The stomach (STO) is situated between the anterior and the posterior parts of ventricular caeca. The rectum originates from the ventral part of the stomach with the terminal part enlarged to form rectal sac (RS). This rectal sac is extending into the ventral body cavity. The mean lengths of a.l.1, a.l.2, a.l.3, a.l.4, a.l.5, p.l.1, p.l.2 ventricular caeca and STO are 0.2333, 0.2810, 0.3583, 0.3633, 0.9300, 1.0133, 0.9867 and 0.1967 cm. respectively (Table 4). Their lengths are decreasing in the following order: p.l.1 (the longest), p.l.2, a.l.5, a.l.4, a.l.3, a.l.2, a.l.1, STO (the shortest) (Figure 3).

The shape of ventricular caeca of *Boophilus microplus* midgut varies and can be observed into 3 phases: nodular, elongated-nodular and straight. The nodular phase is characterized by 6-8 round shape nodules for each ventricular caeca of a.l.1-a.l.4, while a.l.5, p.l.1, p.l.2 contain each 12-15 round shape nodules. Each nodule does not connect directly to the next, being separated by a constriction interval (Figure 9). The elongated-nodular phase is characterized by nodules longer than those in nodular phase, while maintaining the constriction interval between nodules. For this phase, a.l.1-a.l.4 contain 3-4 elongated nodules in each ventricular caeca, while a.l.5, p.l.1, p.l.2 contain 5-8 elongated nodules each (Figure 10A). Finally, the

straight phase does not present any constrictions on the ventricular caeca (Figure 10B). The surface during nodular phase is folded and pleated, having a constricted part with deep folds on the surface. However, during the elongated-nodular phase there are shallow folds on the surface and usually less constrictions than during the nodular phase (Figure 11 and Figure 12).

Table 3 The length of each ventricular caeca studied from 30 female ticks *B. microplus* (Fresh specimen).

Sample No.	Length of ventricular caeca (cm)							
	a.l.1	a.l.2	a.l.3	a.l.4	a.l.5	p.l.1	p.l.2	STO
1	0.15	0.28	0.40	0.30	0.70	1.00	1.00	0.20
2	0.20	0.20	0.25	0.40	1.10	0.90	1.00	0.30
3	0.20	0.20	0.20	0.25	0.60	0.70	1.00	0.20
4	0.20	0.20	0.20	0.35	0.90	1.00	1.10	0.20
5	0.15	0.20	0.40	0.30	1.10	1.10	1.00	0.15
6	0.20	0.30	0.30	0.50	0.90	1.00	1.00	0.20
7	0.20	0.20	0.30	0.30	0.90	0.90	1.00	0.20
8	0.30	0.30	0.50	0.50	1.00	1.20	1.00	0.30
9	0.30	0.40	0.50	0.50	1.20	1.30	1.40	0.20
10	0.20	0.40	0.40	0.30	1.00	1.10	0.80	0.20
11	0.30	0.40	0.50	0.50	0.90	1.20	1.00	0.20
12	0.20	0.30	0.30	0.40	0.80	0.90	1.00	0.20
13	0.20	0.30	0.40	0.40	1.00	1.00	0.80	0.20
14	0.20	0.30	0.30	0.30	0.80	0.90	1.00	0.15
15	0.30	0.40	0.50	0.30	1.00	1.10	1.10	0.30
16	0.20	0.25	0.30	0.30	1.20	1.00	1.00	0.20
17	0.40	0.50	0.40	0.50	1.10	1.20	0.90	0.30
18	0.30	0.30	0.40	0.40	1.00	0.90	1.00	0.30
19	0.30	0.30	0.50	0.50	1.00	1.30	1.30	0.20
20	0.30	0.30	0.40	0.40	1.00	1.20	1.10	0.20

Table 3 (Continued)

Sample No.	Length of ventricular caeca (cm)							
	a.l.1	a.l.2	a.l.3	a.l.4	a.l.5	p.l.1	p.l.2	STO
21	0.20	0.30	0.30	0.30	0.80	0.80	0.90	0.15
22	0.30	0.30	0.60	0.40	0.90	1.00	1.20	0.20
23	0.20	0.20	0.20	0.20	1.00	1.10	1.00	0.15
24	0.30	0.30	0.50	0.50	0.70	0.90	1.00	0.10
25	0.20	0.20	0.30	0.30	1.00	0.90	0.80	0.15
26	0.20	0.30	0.30	0.30	1.00	1.00	0.90	0.15
27	0.20	0.20	0.30	0.30	0.90	1.00	0.90	0.20
28	0.20	0.20	0.30	0.30	0.90	1.00	0.90	0.15
29	0.20	0.20	0.30	0.30	0.80	1.00	0.80	0.15
30	0.20	0.20	0.20	0.30	0.70	0.80	0.70	0.20

a.l.1, a.l.2, a.l.3, a.l.4, a.l.5, p.l.1, p.l.2 =ventricular caeca 1-7, STO=stomach

Table 4 Mean and SD of the lengths of the midgut components

ventricular caeca	Length from origin to end (cm.)				
	N	Minimum	Maximum	Mean	Std. Deviation
a.l.1	30	0.15	0.40	0.2333	0.05921
a.l.2	30	0.20	0.50	0.2810	0.07924
a.l.3	30	0.20	0.60	0.3583	0.10834
a.l.4	30	0.20	0.50	0.3633	0.08996
a.l.5	30	0.60	1.20	0.9300	0.14657
p.l.1	30	0.70	1.30	1.0133	0.14559
p.l.2	30	0.70	1.40	0.9867	0.14559
STO	30	0.10	0.30	0.1967	0.04901

a.l.1, a.l.2, a.l.3, a.l.4, a.l.5, p.l.1, p.l.2 =ventricular caeca 1-7, STO=stomach

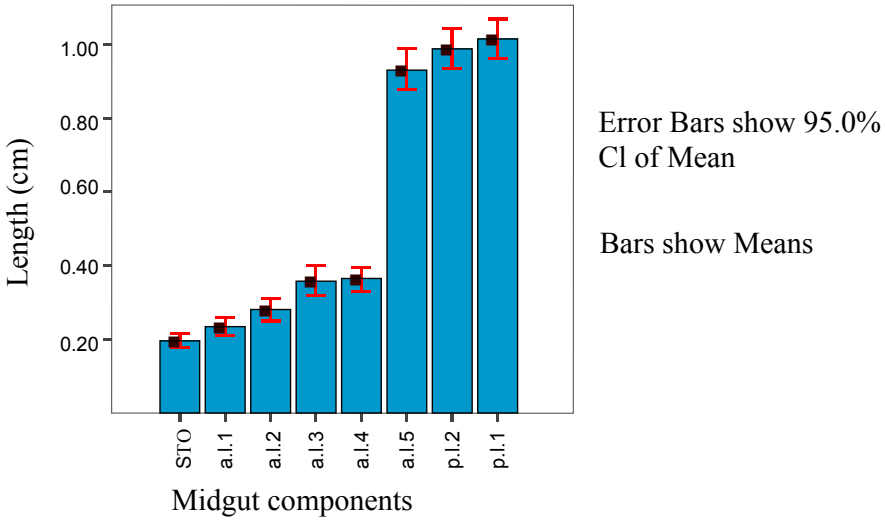


Figure 3 Histogram of midgut components (ventricular caeca) shows mean and SD of length (cm)

A **B**

Figure 4 (A) Female tick of *B. microplus* that has 4-6 mm in length (B) Midgut of tick *B. microplus* was measured after cleaning with PBS.

Figure 5 The midgut of *B. microplus* after being removed from the body cavity

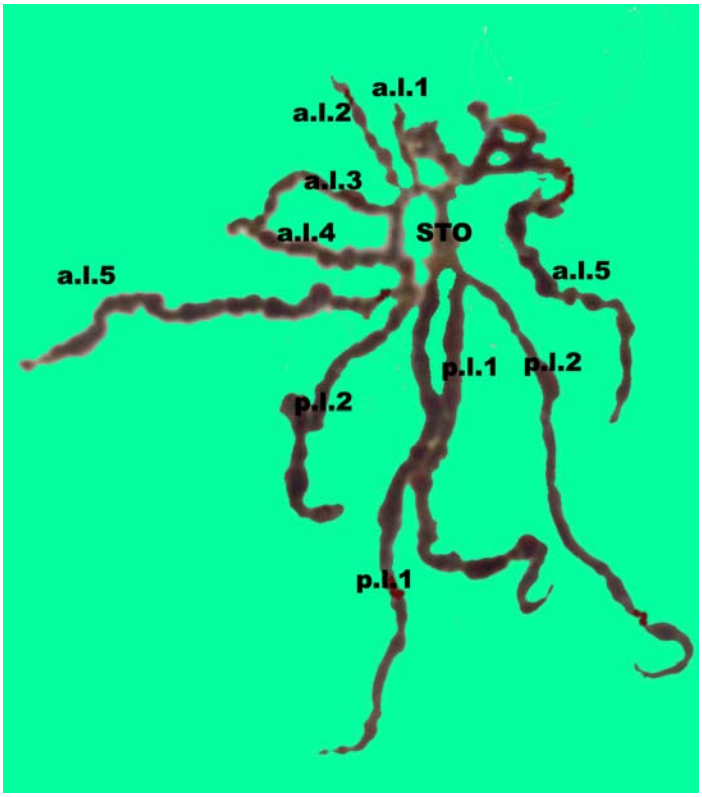
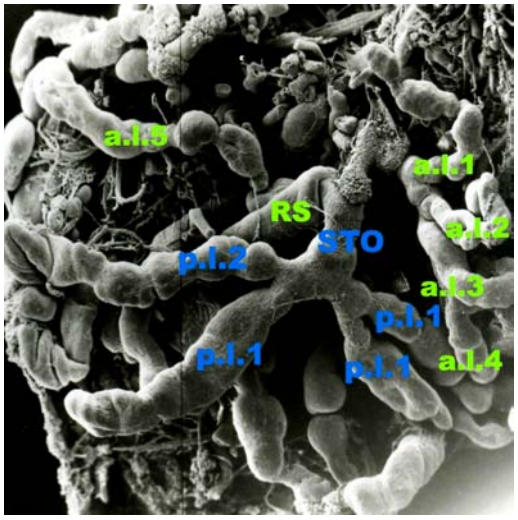
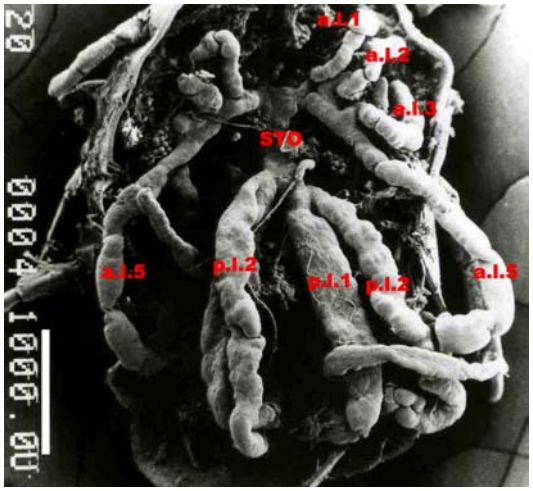


Figure 6 The midgut of *B. microplus* after being removed from the body cavity and cleaned of all of the respiratory system and the reproductive system



A



B

Figure 7 (A)The SEM of the whole midgut of tick *B. microplus* shows the position of all ventricular caeca; a.l.1, a.l.2, a.l.3, a.l.4, a.l.5 starting from the anterior part; STO is the stomach; p.l.1, p.l.2 start from the posterior part. (B) a.l.5, p.l.1 and p.l.2 are comparatively longer than a.l.1-a.l.4 and extend first towards the posterior part, then taking a retrograde curve towards the anterior part

Bar = 1000 µm

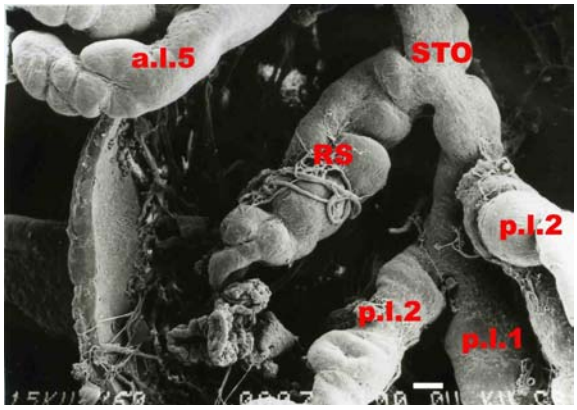


Figure 8 The SEM lateral view of the midgut; a rectum forms from the single ventricular caecum separating from the stomach (STO) and enlarges becoming a rectal sac (RS). This rectal sac is extending into the ventral body cavity.
Bar = 1000 µm

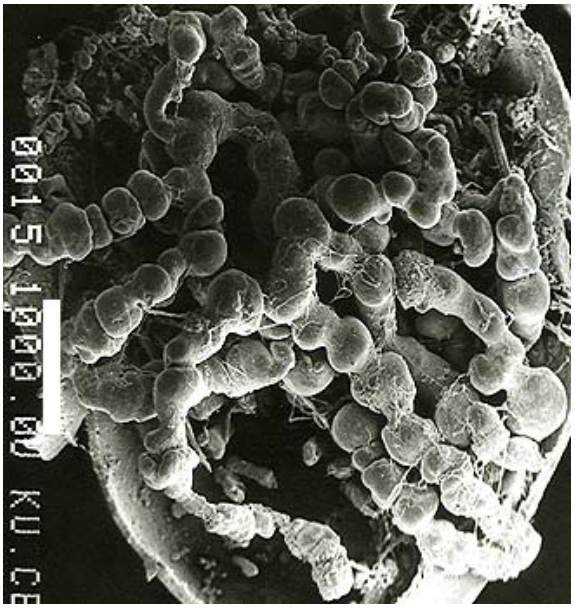
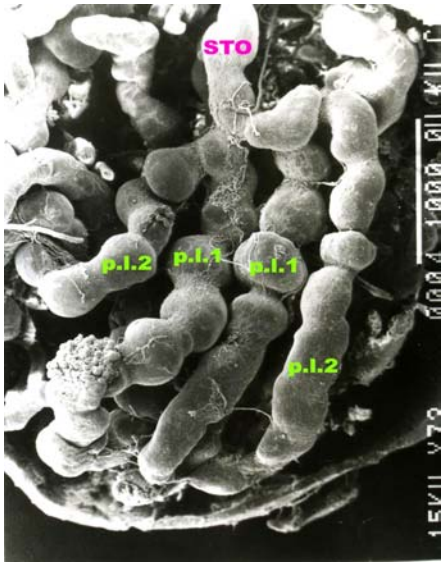
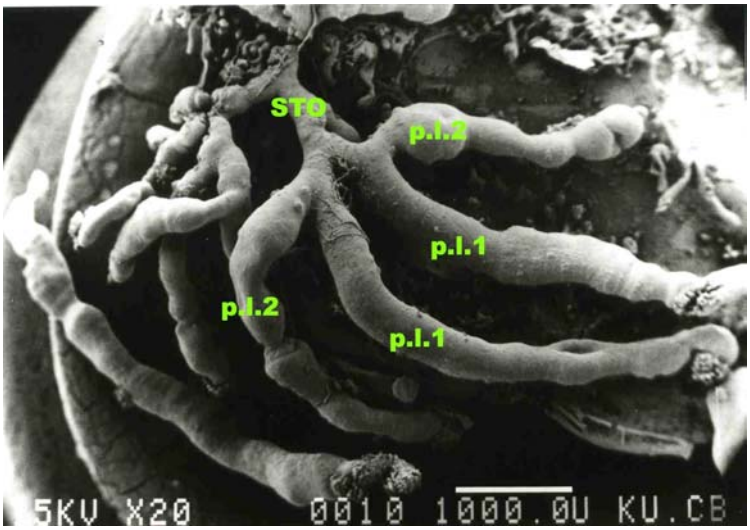


Figure 9 The SEM of the whole midgut of tick *B. microplus* shows the ventricular caeca nodular phase that contains 6-8 round shape nodules in each of a.l.1-a.l.4 ventricular caeca and 12-15 round shape nodules in each a.l.5, p.l.1, p.l.2 ventricular caeca



A



B

Figure 10 (A, B) The SEM view of the posterior part of the midgut consists of 2 pairs of long ventricular caeca (p.l.1-p.l.2) that divide from the posterior end of the stomach (STO). The elongated-nodular phase of ventricular caeca (A) is longer than the nodular phase, still having constriction intervals between nodes. The straight phase of ventricular caeca (B) does not have any constriction intervals.
Bar = 1000 μm

Figure 11 The surface of the nodular phase is deep fold and pleat.

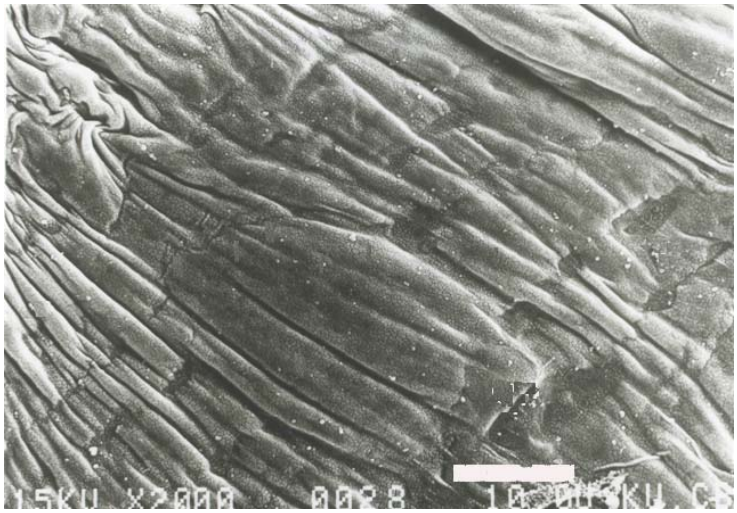


Figure 12 The elongate-nodular phase has shallow folds on surface and usually has fewer constrictions than the nodular phase.

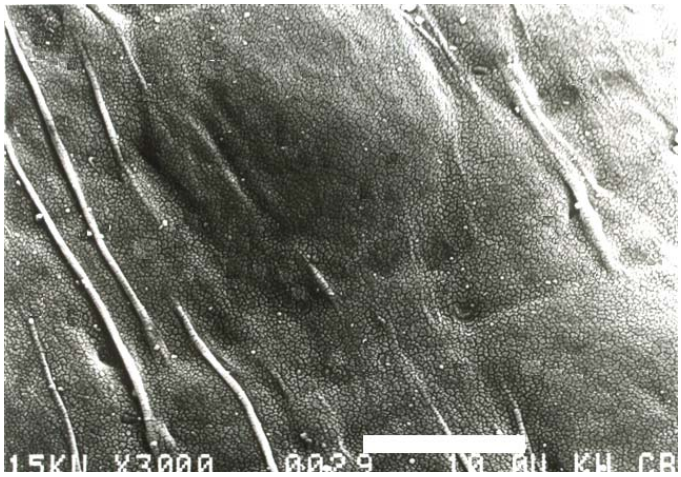


Figure 13 The surface of the straight phase is flat and smooth. This phase does not have any constrictions on the caeca because of a large amount of blood content in the midgut cavity

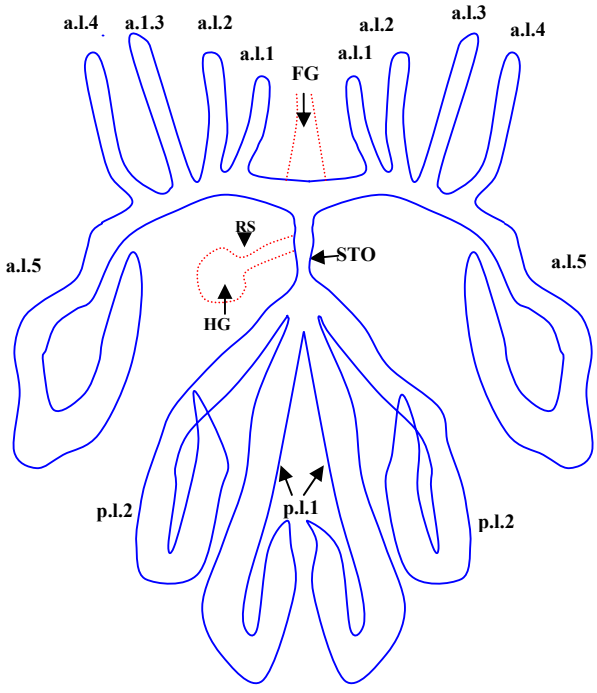


Figure 14 The midgut of *B. microplus*: a.l.1-a.l.5 are ventricular caeca on the anterior part, p.l.1 and p.l.2 are on the posterior part, STO is the stomach, RS is a single ventricular caecum with rectal sac, FG is the foregut and HG is the hindgut

The midgut wall consists of 3 types of cells: stem, digestive and secretory. Stem cells (ST), located on the basal part of the gut, have small dome shape and they are the generating cells of other types. The digestive cells are the dominant cell type in the epithelium of the midgut and they have a cuboidal shape. Their cytoplasm is filled with inclusion body of different types and sizes. Several stages can be observed during the development of digestive cells: the early stage (DE), the mature stage (DM) and spent stage (DS).

Cells in the early stage have a larger size than the stem cells and have a few food vacuoles, a slightly elongate shape with a round nucleus and a large deeply staining nucleolus. Mature digestive cells (DM) contain several food vacuoles presumably containing hemoglobin and other soluble blood constituent. DM cells can be divided into 2 subtypes depending on their stage of development. Subtype I describes mature digestive cells that have many vacuoles containing tick leukocytes and presumably soluble constituents of the diet (DHL). Subtype II (DHV) describes a mature digestive cell that develops later and has no leukocytes in the large vacuoles. Spent digestive cells (DS) contain haematin granules and some vacuoles. Some of them are budded into lumen from where they either break or are excreted as a whole granule. Others, which are still attached to the basal laminar, release their haematin when they break; their nuclei are irregularly polygonal and often masked by cell inclusions. Spent digestive cells were the last stage during the evolution of the digestive cell.

Secretory cells can also be observed into 2 subtypes, S_1 and S_2. S_1 can be observed in 2 stages: early secretory cell (S_1E) and mature secretory cell (S_1M). S_1E and S_1M have both a dark staining nucleus in the cytoplasm, while during the mature stage (S_1M) the density of eosinophilic granules in the cytoplasm is greater and granules appear to be released into the lumen. The second secretory cells subtype, S_2, can be observed in stages S_2E and S_2M. During S_2M, the secretory cells have a highly granular cytoplasm that is secreted as a colloidal mass when the apical end of the cell buds of into the lumen. During S_2E the cell is narrow and dark, having an immature form with an intensely staining nucleus (H&E). During S_2M stage, its shape becomes

columnar, often with a bulbous tip. The cells in each ventricular caeca have different morphologies that can be observed in a cross section through the midgut of *B. microplus* after staining with H&E.

Table 5 Cell types of midgut components

Midgut Component	Gut cells									
	S_1E	S_1M	S_2E	S_2M	DE	DM	DS	ST	DHL	DHV
STO			✓	✓	✓		✓	✓		
a.l.1	✓	✓		✓	✓	✓	✓	✓		✓
a.l.2	✓	✓			✓	✓	✓	✓		✓
a.l.3	✓	✓			✓	✓	✓	✓		✓
a.l.4	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
a.l.5	✓	✓			✓	✓	✓	✓		✓
p.l.1	✓	✓		✓	✓	✓	✓	✓	✓	✓
p.l.2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

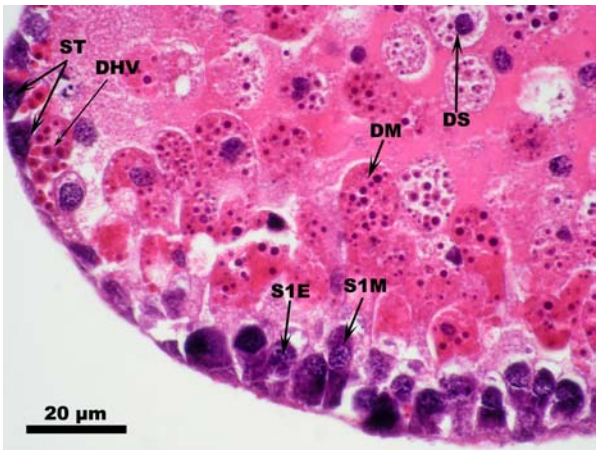


Figure 15 a.l.1 of Midgut of tick *B. microplus* longitudinal section, LM H&E DHV: digestive cell with hemoglobin without leukocyte, DS: spent digestive cell, S_1E, S_1M: early and mature stage of secretory cell, ST: stem cell.

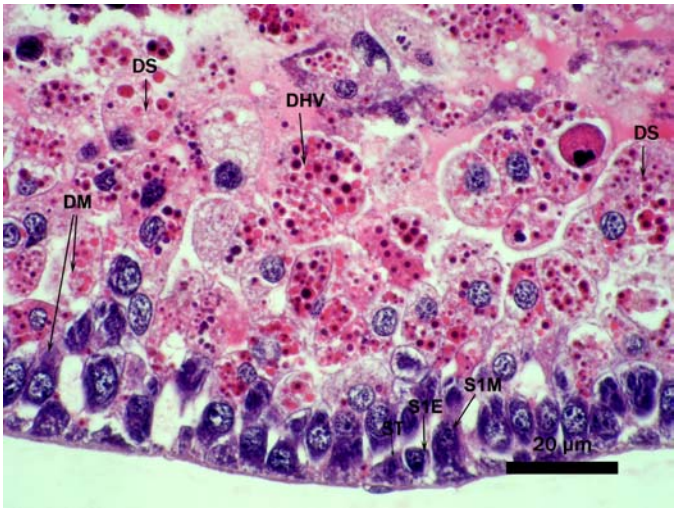


Figure 16 a.l.2 of tick Midgut of *B. microplus* longitudinal section, LM H&E DHV: digestive cells with hemoglobin without leukocyte, DM: mature stage of digestive cells, DS: spent digestive cells, S_1E, S_1M: early and mature stage of secretory cell, ST: stem cells.

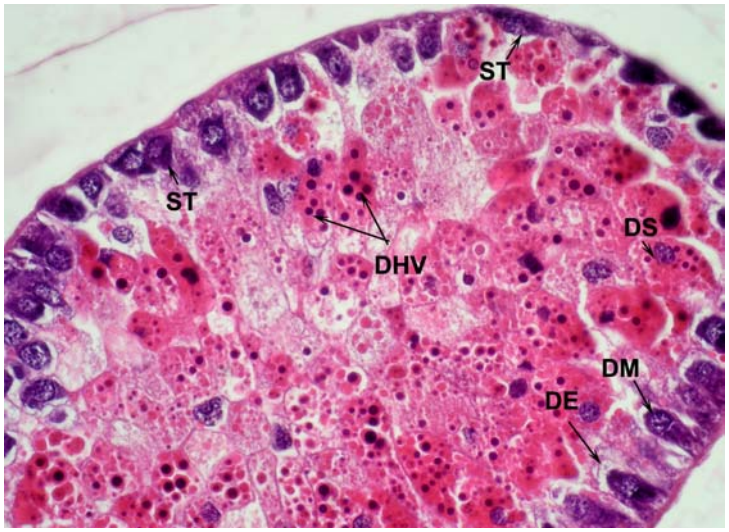


Figure 17 a.l.3 of Midgut of tick *B. microplus* longitudinal sections, LM H&E DE: early stage of digestive cells, DHV: digestive cells with hemoglobin without leukocyte, DM: mature stage of digestive cells, DS: spent digestive cells, ST: stem cells.

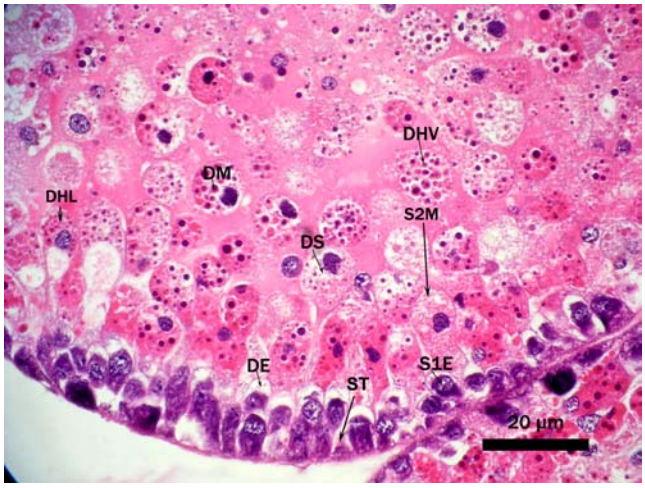


Figure 18 a.l.4 of Midgut of tick *B.microplus* longitudinal section, LM H&E
DE: early stage of digestive cells, DHL: digestive cells with hemoglobin and leukocyte, DHV: digestive cells with hemoglobin without leukocyte, DM: mature stage of digestive cells, DS: spent digestive cells, S_1E: early stage of the secretory cells, S_2M: mature stage of the secretory cells, ST: stem cells.

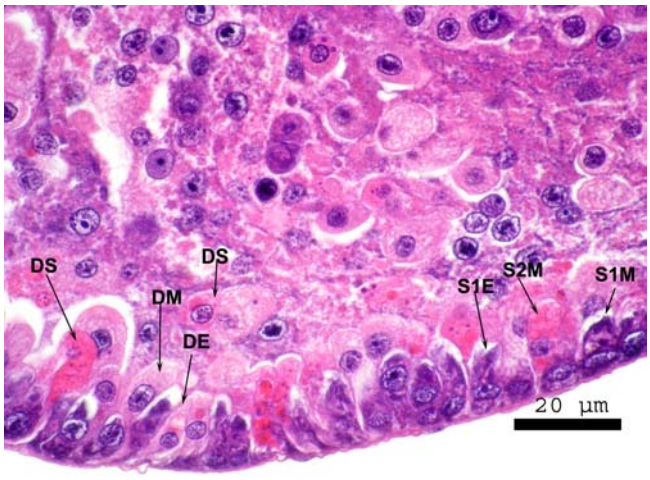


Figure 19 p.l.1 of Midgut of tick *B. microplus* longitudinal section, LM H&E
DE, DM: early and mature stage of digestive cells, DS: spent digestive cells, S_1E: early stage of the secretory cells, S_2M: mature stage of the secretory cells.

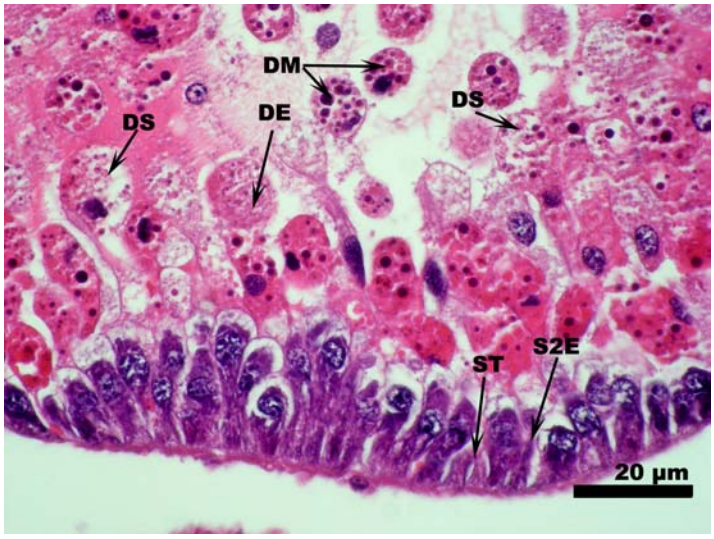


Figure 20 p.l.2 of Midgut of tick *B. microplus* longitudinal section, LM H&E DE, DM: early and mature stage of digestive cells, DS: spent digestive cells, S_2E: early stage of secretory cell type 2, ST: stem cells.

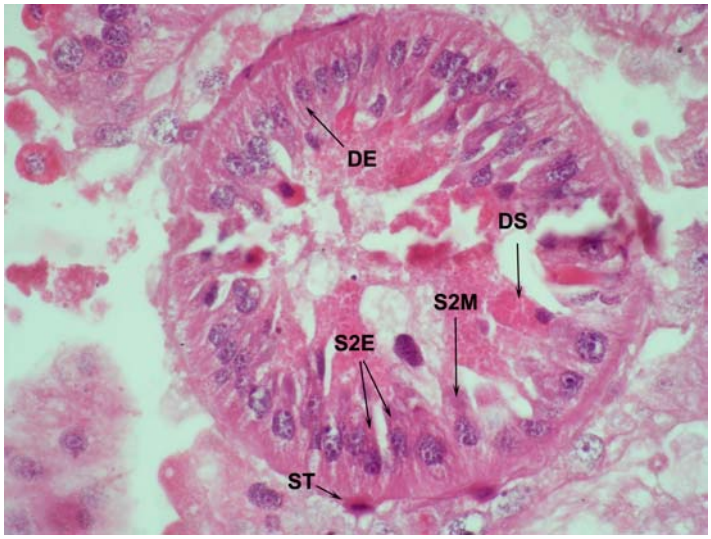


Figure 21 Stomach (STO) of tick *B. microplus*. Cross section, LM H&E DE: early stage of digestive cells, DS: spent digestive cells, S_2E: early stage of secretory cell type 2, S_2M: mature stage of secretory cell type 2, ST: stem cells.

DISCUSSION

Studies regarding the midgut of the ticks are important because this is the site where blood-borne parasites develop. Moreover, the midgut is an important part for the production of anti-tick vaccines that control cattle tick infestations. Numerous studies are currently under way aiming at finding the feeding processes of the cattle tick *B. microplus* (Canestrini) (Tatchell, 1964, 1968), (Grandjean and Aeschlimann, 1973), (Gough and Kemp, 1995). Lara et al (2003) studied about the pathway of haem detoxification in the midgut of the cattle tick *B. microplus*. There is some interest regarding the morphology of ticks but almost all were concentrated on the internal anatomy of Argas ticks (Roshdy, 1961, 1962, 1963, 1966; Saito, 2005) or only digestive cells of the midgut of *B. microplus* (Tatchell, 1968). As mentioned above, there are no data on the external morphology of the midgut of *B. microplus*, therefore in this study we use SEM in combination with LM to clarify the external structures and the cell components in each part of the *B. microplus* midgut. The present work was aimed at collecting new data on the morphology of the female midgut of *B. microplus* in order to provide information that would contribute to the future control of this parasite.

The number of midgut ventricular caeca is different in different ticks. Some ticks have 3 pairs of ventricular caeca (2 pairs arise from the anterior part and 1 pair from the posterior part) such as *A. (Chiropterargas) boueti* (Roshdy, 1962). Others have 4 pairs of ventricular caeca (2 pairs arise from the anterior part and 2 pairs from the posterior part) such as *A. (Ogadenus) brumpti*, *A. (Secretargus) transgariepinus* and *N. namaqua* (Roshdy, 1963, 1966; Shoura, 1984). Some ticks having 4 pairs of ventricular caeca have an additional branch on the median dorsal part between the 2 pairs of the anterior part such as *Argas vespertilionis* (Roshdy, 1961). In this study we observe *B. microplus* which has 7 pairs of ventricular caeca (5 pairs arise from the anterior part and 2 pairs from the posterior part). Moreover, the length of each ventricular caeca of the midgut in *B. microplus* is measured.

The morphology of the tip of ventricular caeca can be classified into 2 types. Type 1 has bifurcated ventricular caeca, such as *Argas vespertilionis* (Roshdy, 1961), *A. (Chiropterargas) boueti* (Roshdy, 1962), *A. (Secretargus) transgariepinus* (Roshdy, 1963) and *A. (Ogadenus) brumpti* (Roshdy, 1966). Type 2 has normal sacculated caecum at the end of ventricular caeca such as *N. namaqua* (Shoura, 1984) and we observe that *Boophilus microplus* belongs to this type.

The tip of ventricular caeca of a.l.1, a.l.2, a.l.3 and a.l.4 of *B. microplus* is extending towards the anterior while the tip of a.l.5, p.l.1 and p.l.2 is extending towards the posterior region of the body cavity. In the case of *A. (Chiropterargas) boueti* only the first pairs of ventricular caeca have the tip extending to anterior region of the body cavity, while the other caeca are extending to the end of the posterior region of the body cavity (Roshdy, 1962). The ventricular caecum a.l.3 of *Argas vespertilionis* and *A. (Ogadenus) brumpti* shows a retrograde curve and the tip of this caecum is extending towards the anterior region of the body cavity similar to a.l.5, p.l.1 and p.l.2 of the midgut of *B. microplus*. The species: *A. (Chiropterargas) boueti*, *Argas vespertilionis, A. (Ogadenus) brumpti* and *B. microplus* have a short but wide stomach, between the anterior and the posterior part. The anterior end of the stomach of some ticks: *Argas vespertilionis* (Roshdy, 1961), *O. (O.) savignyi* (Christophers, 1906), *O.(O.) moubata* (Hoogstraal, 1956; Grandjean and Aeschlimann, 1973) and *A. (Chiropterargas) boueti* (Roshdy, 1962) gives rise to a median dorsal part, which extends anterodorsally but is absent in *N. namaqua* (Shoura, 1984) and *Boophilus microplus* in the current study.

Balashov (1968) reported that the histological structure of the midgut wall of argasids and Nuttalliellidae is composed of digestive and undifferentiated epithelial cells (stem cells). The secretory cells contain large vacuoles with hemoglobin-filled inclusions and can be transformed into digestive cells. However, the secretory cells were not present in *N. namaqua* because they might have alreadny transformed into digestive cells (Shoura, 1984). In this study we found that the midgut of *B. microplus* contained 3 cell types: stem, digestive and secretory cells. The midgut digestive cells have been considered to have a common origin and to be derived from the stem cells,

as reported by Chinery (1964), Tatchell (1964), Guirgis (1971) and Balashov (1972). Tatchell (1964) and Balashov (1972) reported that only secretory cells (without classifying them into 2 types) have been found in *Argas (Persicargas) arboreus*. On the other hand, secretory cells of *B. microplus* can be classified into 2 types: S_1 and S_2. Granules of S_1 and S_2 are different and the two cell types can sometimes be found together in one caecum (a.l.1, a.l.4, p.l.1 and p.l.2). The S_1 and S_2 cells have different size, shape and ultrastructure. The cytoplasmic organelles of S_1 cells are virtually depleted by the time the granules matured, while the S_2 cells still retained their organelles although they were in the process of being reorganized. This evidence is confirmed for the secretory cells of *B. microplus* by Agbede & Kemp, 1985.

The midgut parts were categorized into 3 groups according to cell components in each part. STO, a.l.1 and p.l.2 are in group one. a.l.2, a.l.3 and a.l.5 are in group 2 and a.l.4, p.l.2 are in group 3. Results from the serial section in this investigation show that group 1 is composed of different cell components for each part, as follows: STO has scretory cells of type 2 in the early stage (S_2E), secretory cells of type 2 in the mature stage (S_2M), digestive cells in the early stage (DE), spent digestive cells (DS) and stem cells (ST) (Figure 21). The a.l.1 has S_1E, S_1M, S_2M, DE, DM, DS, ST and DHV (Figure 15). The p.l.1 has S_1E, S_2M, DE, DM, DS, ST, DHL and DHV. Group 2 (a.l.2, a.l.3, a.l.5) contains S_1E, S_1M, DE, DM, DS, ST and DHV (Figure 16 and Figure 17). Group 3 (a.l.4 and p.l.2) contains S_1E, S_1M, S_2E, S_2M, DE, DM, DS, ST, DHL and DHV (Figure 18 and Figure 20).

Even though the female ticks in this experiment are of the same size, because each of them has different amount of blood uptake we can identify 3 phases regarding the shape of the ventricular caeca. Small amount of content can not fulfill the ventricular caeca cavity and there is still enough space for extension due to an additional large amount of blood content. The surface folds of the constricted part are usually deeper than on any other part. The surface folds of the nodules in nodular phase are deeper than those of elongated-nodular and straight phase. Because of the small amount of blood content inside, the ventricular caeca has less volume and is less

extended. The different phases of the caecum of *B. microplus* midgut can be found in different time of feeding.

Numerous studies are aiming to find an efficient control strategy that would minimize the damages caused by these parasites such as anti-cattle-tick vaccine (Tellam et al., 1992; Willadsen, 1997). Recently, vaccines containing the recombinant *B. microplus* midgut antigen Bm86 have been developed. (Fuente, 1999). Antigens from partially engorged females of *B. microplus* have been used with variable success to immunize animals against tick infestation (Akhtar, 1999). Midgut of the ticks has been used as an immunogen. Agbede and Kemp (1985) suggested that antigens which are important for successful vaccination occur in the cells of the midgut ventricular caeca. The present study shows that all the ventricular caeca from a.l.1-p.l.2 have digestive cells but in different stage of development; early, mature and spent. Therefore this evidence suggests that the important antigens are located in the digestive cells as mentioned previously by Agbede and Kemp (1985). This result shows that we can collect specific ventricular caeca of the midgut without including the stomach, in order to get a cheaper extraction method and to decrease interfered substances from components that have excessive proteins.

CONCLUSION

The midgut consists of seven ventricular caeca and can be divided into two parts, the anterior and posterior part. The anterior part consists of 4 short pairs (a.l.1-a.l.4) and 1 long pair (a.l.5) of ventricular caeca, while the posterior part consists of 2 long pairs of ventricular caeca (p.l.1, p.l.2). The stomach (STO) is situated between the anterior and the posterior parts. A rectum forms from the ventral part of the stomach and enlarges becoming a rectal sac (RS). Even though the midgut is formed of 7 pairs of ventricular caeca and the stomach, it is possible to categorize them into 3 groups according to the cells composing each part. Group 1 consists of STO, a.l.1 and p.l.1 and contains S_1E, S_2M, DE, DM, DS, ST, DHL and DHV cells. Group 2 consists of a.l.2, a.l.3 and a.l.5 and contains S_1E, S_1M, DE, DM, DS, ST and DHV cells, while group 3 consists of a.l.4 and p.l.2 and contains S_1E, S_1M, S_2E, S_2M, DE, DM, DS, ST, DHL and DHV cells. This study shows that each ventricular caecum of the midgut of *B. microplus* can be identified distinctly by SEM and that the ventricular caeca can be identified by LM depending on the composing cells.

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APPENDIX

APPENDIX

1. Preparation of Bouin's Fluid Fixative

Saturated aqueous solution of picric acid	75ml
Formalin (~ 40% aqueous solution of formaldehyde)	25ml
Glacial acetic acid	5ml

Fixed tissue should be transferred to 70% alcohol. (Veterinary pathology, 2004)

2. Preparation of Eosin - Dye

Eosin, a red dye, properly used on well fixed material, stains connective tissue and cytoplasm in varying intensity and shades of the primary color, giving a most useful differential stain. The routine staining in histopathology is done with haematoxylin and much of the present knowledge of morbid histology has been gained from the study of H&E stained sections.

0.5% Aqueous Eosin	
Eosin Y, water soluble	5 gm
Distilled water	1000 ml

1% Alcoholic Eosin	
Eosin Y, water and alcohol soluble	10 gm
Distilled water	50 ml

Dissolve the eosin thoroughly in the water and then add;

95% Ethyl alcohol	940 ml

The eosin may be used as a stock solution diluted with an equal part of 95% ethyl alcohol, or it may be used in a solution of 1% strength.

Belonging to the Xanthene group of dyes, eosin is derived from fluorescein and is available in two main shades-as yellowish or as bluish (a deeper red). Eosin Y (yellowish) is most commonly used and is readily soluble in water, less so in alcohol.

A 5 % stock solution is convenient, using either tap water or distilled water as solvent, the alkalinity of the former being considered to give superior staining. In either case, moulds will grow in the solution. To prevent this, a crystal of thymol or a few drops of formalin may be added, or moulds may be removed by filtration. They do not affect the stain. 1 % Eosin is commonly used as a working solution although the exact concentration is not critical (Drury R.A.B. and Wallington E.A., 1967).

3. Preparation of Harris' Alum Hematoxylin

A powerful and selective nuclear stain gives sharp delineation of nuclear structure. For this reason, Harris' haematoxylin is used widely as a nuclear stain in exfoliative cytology and when staining sex chromatin with haematoxylin. Stain for 2-5 minutes.

Appendix Table 1 Preparation of Harris' Alum Hematoxylin

Substance	Quantity
Haematoxylin	1 g
Absolute alcohol	10 ml
Ammonium or potassium alum	20 g
Distilled water	200 ml
Mercuric oxide	0.5 g

Source: Wallington (1967).

Dissolve the haematoxylin in the alcohol and add to the alum, previously dissolved in hot water. Bring quickly to boil and add the mercuric oxide, when the solution will turn dark purple. Cool rapidly under the tap, filter before use. Mallory (1938) recommends the addition of 8 ml glacial acetic acid to the above, after cooling,

to sharpen nuclear staining although the stain is generally used regressively. The stain should be prepared in a flask of sample size on account of the frothing that takes place on addition of the mercuric oxide (Drury R.A.B. and Wallington E.A. 1967).

4. Preparation of Phosphate Buffer Solution (Sorensen)

M/15 sodium phosphate, dibasic; Na_2HPO_4 (m.w.: 142). Dissolve 9.465 g in distilled water and make up to 1 liter.

M/15 sodium phosphate, monobasic; NaH_2PO_4 (m.w.: 136). Dissolve 9.08 g in distilled water and make up to 1 liter.

M/10 solutions maybe used, but these give slightly lower pH values; these differences are not usually of significance in histological techniques.

Take the amounts of each solution as shown in Appendix Table 2. The mixtures may be diluted with distilled water up to 1 liter (Hayat M.A., 1974).

Appendix Table 2 Formula of phosphate buffer solution

pH	**M/15 NaH_2PO_4**	**M/15 Na_2HPO_4**
8.0	4	96
7.6	12	88
7.2	27	73
6.8	50	50
6.4	71	29
6.0	88	12
5.6	95	5

Source: Hayat (1974**)**

5. Preparation of Prefixative for Sem

2 grams of paraformaldehyde powder dissolve in 25 ml distilled water. Mix and heat at 65 ºC and add 1-3 drops of NAOH to dissolve residual paraformaldehyde. Cool down, add 5 ml of 50% glutaraldehyde and dilute with phosphate buffer pH 7.2-7.4 up to 100 ml (Karnovsky M.J., 1965).

6. Preparation of Post Fixative For SEM

2% Osmium tetroxide in distilled water mixing with the phosphate buffer pH 7.2-7.4 (1:1) (Karnovsky M.J., 1965).

7. Tick collection

Refering to laboratory of parasitology department of Kasetsart University and the study of Agbede, 1986 citing Wharton and Utech, 1969, the ticks can be categorized in 4 groups depending on their body size as follows:

Group 1 less than 3 mm. = unfed-fed 1 day
Group 2 3-4.5 mm. = fed 3 days
Group 3 4.5-6 mm. = fed 5 days
Group 4 more than 6 mm. (semi engorge) = fed 7-9 days

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