CHAPTER 3

MATERIALS AND METHODS

3.1 Bee collection

The collection was done on Apis cerana workers from the northern part of Thailand to Southern Malaysia (Table 1). Ten bees from each colony were collected, killed and preserved in 70% alcohol. Locations, dates and other details were shown in appendix. The bees from each colony were separately put into glass vial and labelled.

3.2 Equipment, chemicals and computer programs

3.2.1 Equipment

1. dissecting microscope
2. forceps with very fine tips
3. needle
4. watch glasses
5. microscope slides
6. cover glasses
7. string
8. microcomputer: IBM PC/XT; PC/AT
9. digitizer: Houston Instrument Hipad™ model DT114
10. microslide projector: Bausch & Lomb Trisimplex® with special lenses: Zeiss Luminar® 25mm and 16mm

3.2.2 Chemicals

1. 70% ethanol
2. KOH
3. Erythrosin
4. Lignin Pink
5. Acid Fuchsin
6. Lactic acid
7. Carbolic acid
8. Glacial acetic acid
9. Euparal® (mounting medium)

3.2.3 Computer programs

1. Custom-written Basic program to capture coordinates from digitizer.
2. Custom-written program to convert coordinates to lengths and angles.
3. Custom-written program to calibrate the lengths of the images projected from microslide projector.
4. SAS (Statistics Analyzing Software) on IBM PC/AT

3.3 Dissection and making slides

3.3.1 Dissection

1. Ten bees were taken from each vial. Each of them was put into a numbered watch glass with 70% ethanol to keep the

bees soft and easy to dissect. Dissection was done under microscope (Fig.).

2. The body parts used for this study were: proboscis, fore and hind wing, hind leg, third and sixth sternites (count from the petiole), second, third and forth tergites (count from the petiole also).

3. The whole proboscis was pulled out consisting of postmentum, mentum, and glossa and in the way that the postmentum was still present and attached to the mentum.

4. Fore and hind wings were pulled out by firmly grasping at their attachment points with the attachment part of the wing was still present. The wings should not folded and every points to be measured must be present. In this study, only the right wing was used.

5. The parts of the hind leg to be used were femur, tibia and basitarsus. The leg was detached by pulling at the coxa or trochanter. Basitarsus was separated from femur and tibia. Coxa and trochanter were carefully removed from tibia. Femur and tibia were left together.

6. Thrid and sixth sternite: the sternites were the most difficult parts to pull out because they are easily broken. Every step had to be done gently and carefully.

The thrid sternite was separated by widening the hole at the petiole, slipping one prong of the forceps held in each hand into the hole and down the length of the inside with the other prong outside (one prong grips the sternite, the other grips the tergite) and pulling the abdomen apart.

The sixth sternite was pulled away from the rest

by using two forceps: one grips the sternite and the other grips the rest of the abdomen.

The muscle and connective tissue attached to the sternites had to teased off by using a small brush and forceps. This step had to be done carefully because it was easy to break the sternites while teasing away the tissue.

The sternites were boiled in KOH solution for 3-5 minutes to get rid of the wax and make the sternites a little clearer. KOH solution was prepared by adding 6-8 buttons of KOH and filling the container with 15-20 ml of distilled water. 50 ml beakers were used as the container of KOH solution.

3.3.2 Staining

Naturally the sternites are membranous with a very pale colour because they are poorly sclerotized. It is difficult to measure or digitize the sternites without staining them. Triple stain was used in this study. The composition is:

Triple stain solution

Triple stain solution was prepared by mixing one part of Triple stain and three parts of Essigs Aphid Fluids together.

Triple stain is composed of:

- Erythrosin solution:

0.036 grams Erythrosin in 1.8 ml distilled water

- Lignin Pink solution:

0.036 grams Lignin Pink in 1.8 ml distilled water

- Acid Fuchsin solution:

0.036 grams Acid Fuchsin in 0.9 ml distilled water

Essigs Aphid Fluid is commposed of

- Lactic acid	10 ml
- Carbolic acid	1 ml
- Glacial acetic acid	2 ml
- Distilled water	0.5 ml

After boiling, the sternites were put into triple stain for 5 minutes and then put back into 70% ethanol in order to get rid of the excess stain. Never keep the stained sternites in the alcohol too long because they would be destained (0.5-1.0 minute is supposed to be the best) (Fig.4)

3.3.3 Making slides

Preparing slides and body parts

1. All the processes of making slides were done under a dissecting microscope.

2. The body parts were separated into two sets:

Set 1

- Fore and hind wing,
- Third sternite,
- Hind leg (tibia, femur and metatarsus)

Set 2

- Third and fourth tergites,
- Sixth sternite

3. Twenty slides were required for each sample (ten bees)

The slides were placed in a slide tray (each tray contained twenty slides).

The first set of slides (ten slides) had to be prepared by glueing three vinyl props onto each slides as shown in the diagram.

Mounting slides

1. Set 1

- Put the legs among the vinyl props, add Euparal® and cover with the coverslip. Add more Euparal® at the edge, if needed.

_ Put the wings and sternite on Euparal® on the slide, add more Euparal® on top, eliminate air bubbles and place the coverslip on top.

2. Set 2

- Put the third and fourth tergites on Euparal® on the slide, try to keep the tergites unfolded, add more Euparal® to eliminate air bubbles, then place the coverslip on top.

_ Put the sixth sternite in Euparal® (next to the tergites), add more Euparal®, eliminate air bubbles and place the coverslip on top.

- Put the proboscis in Euparal® (next to the

sixth sternite), add more Euparal®, eliminate air bubbles and place the coverslip on top.

It is important to always arrange the body parts in the same orientation, especially the forewing, otherwise it is difficult to locate points when digitizing and mistakes will be made. Positions of the body parts can be adjusted with a probe and forceps.

Place the slides in the slide tray and put in an incubator (40°c) to set (harden) before measurement. An incubator is suggested, rather than a slide warmer, because the heat can warm every tray when many trays are used.

3.4 Digitizing

To make measurement, bee parts from a sample of ten bees were mounted on microscope slides and the mounted slides were projected by a Bausch & Lomb Trisimplex microslide projector onto a digitizer pad.

The crosshairs on the digitizer cursor were held above the specific points on the projected images of the bee parts. When the cursor's button was pressed, the location of that point, which was captured by a custom-written Basic program, was entered into the computer in a specific sequence. The points were located as coordinates in two dimensional space. it was important to keep the order correctly, otherwise the analysis can not be done properly. Another custom-written program reads the points and converts them into lengths and angles. The scale was calibrated by a custom-written calibration program to keep the measurement exact. In this study, the program that was written for analyzing the morphometrical

characters of Africanized and European honey bees, has been modified to analyze the eastern honey bee, Apis cerana. The classes or scores of pigmentation of tergite 2, 3, 4 and scutellum S, B, K are based on Ruttner et al., 1978. The characters used in this study are shown in Table 2

All procedures were performed on an IBM PC/XT microcomputer.

3.5 Analysis Procedures

The analyis was done on 129 samples of A. cerana. The samples were divided into 13 regions upon their distribution. Each group was given number as the regional code as shown in table .1.

Analysis of variance was performed by the ANOVA procedure in the statistics analytical software, SAS to test the differences among each region that each character was examined as a univariate character. Student-Newman-Keuls test (SNK test) was used to determined means differences of each characters within 13 regions.

The correlation between the bee body size and its appendage were plotted by SAS. Body size were represented by sternite length 3 and tergite 3 + 4. Appendages were represented by fore wing length, hind wing length and hind leg length. Correlation coefficient were calculated in SAS. The correlation plotted was done between: fore wing length against tergite 3 + 4; fore wing length against sternite 3 length; hind wing length against tergite 3 + 4; hind wing length against sternite 3 length; hind leg length against tergite 3 + 4; hind leg length against sternite 3 length.

Discriminant analysis was done among 13 geographical regions by CANDISC procedure in SAS that performed canonical discriminant analysis and then the scores were plotted as scattergrams. The first run of CANDISC was performed on 52 characters that each of them was used as a variable and 46 and 44 characters were examined in the second and third run. The pigment of sclerites and tergites were excluded in the second run and two more characters, postmentum and glossa were excluded in third run. Mahalanobis' distance (D^2) was calculated to indicate the similarity within 13 regions.

All samples were tested for their phenetic groups by clustering analysis which performed by FASTCLUS procedure in SAS. This procedure is provided to run many samples (> 100) which results the disjointed cluster. The same 52, 46 and 44 characters were used as did in discriminant analysis. The amount of cluster that have to be specified by the user are 3, 4 and 5 on each set of the characters.

All procedures were preformed on an IBM PC/AT computer.

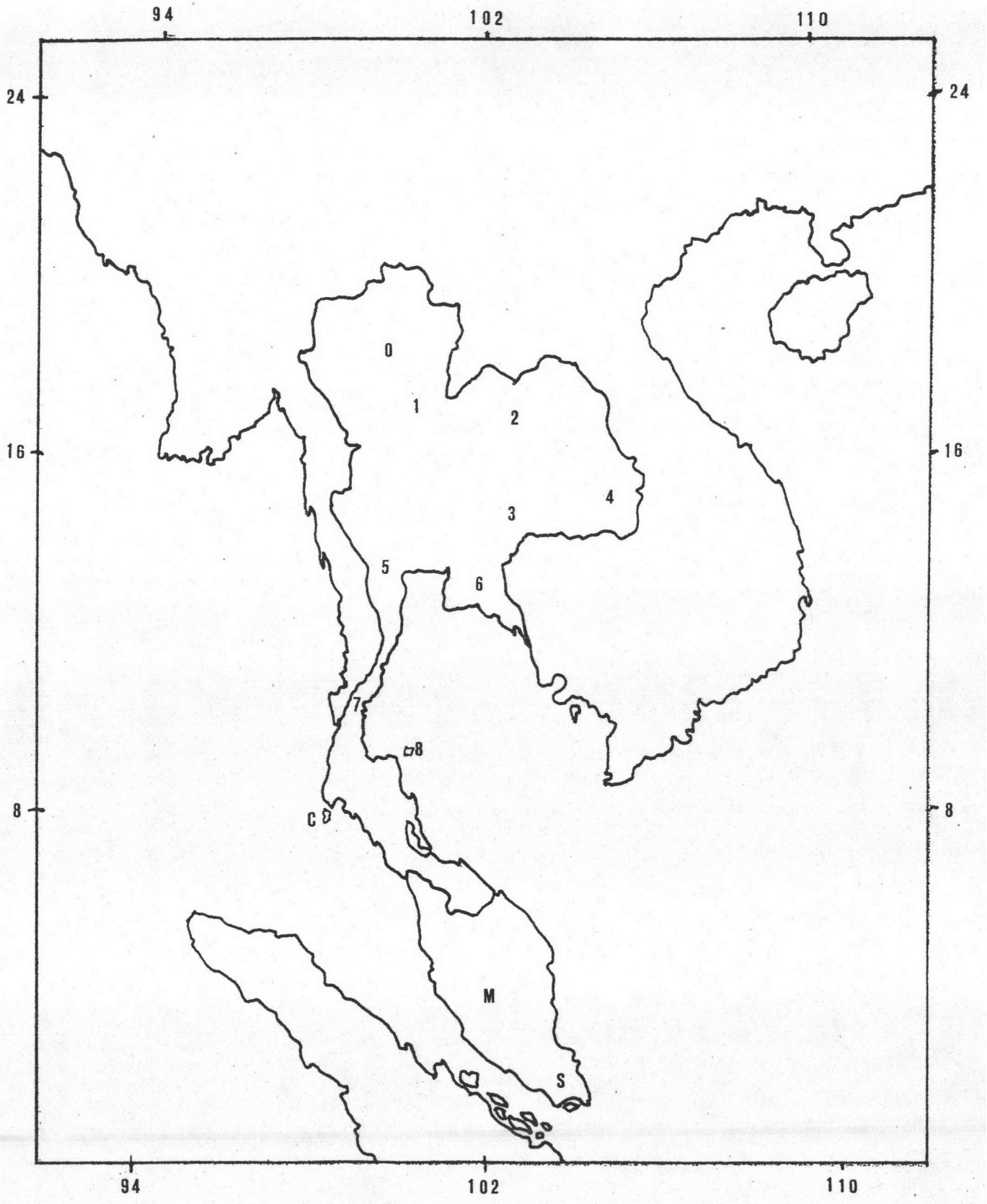

Fig. 1 Map of Thailand and Malaysian Peninsula showing collection locations of Eastern Honey Bee (regions are indicated in numbers and alphabets)

Table 1 Collection of Eastern Honey Bee (*Apis cerana*) in Thailand and Malaysian Peninsula

Region	no. of colony	Province	Country
0	19	Chiengrai, Chiengmai Lamphun, Lampang, Phrae	Thailand
1	10	Utradit, Phitsanulok	Thailand
2	18	Udonthani, Nongkhai, Khonkaen	Thailand
3	5	Nakhonratsima	Thailand
4	1	Ubonratchathani	Thailand
5	25	Samutsongkhram, Ratburi, Phetchaburi	Thailand
6	20	Chonburi, Rayong, Chanthaburi	Thailand
7	5	Chumporn	Thailand
8	5	Suratthani	Thailand
9	5	Songkhla	Thailand
C	5	Phuket	Thailand
M	5	Selangor	Malaysia
S	5	Johor	Malaysia

Table 2 Morphometric measurements on 58 charcaters of Apis cerana in Thailand and Malaysian Peninsula

Variable	Characters	Figure
FWLN	fore wing length	8
FWWD	fore wing width	8
HWLN	hind wing length	9
HWWD	hind wing width	9
HAMU	hamuli	10
AN20-AN43	angles 20-43	11
TBLN	tibia length	12
FELN	femur length	12
TRLN	basitarsus length	12
TRWD	basitarsus width	12
STLN	sternite 3 length	13
WXLN	wax mirror length	13
WXWDA	wax mirror width	13
WXWDB	distance between wax mirror	13
POST	postmentum	14
GLOS	gloossa + mentum	14
LPSEG	left proximal segment	14
LDSEG	left distal segment	14
RPSEG	right proximal segment	14
RDSEG	right distal segment	14
TER3	tergite 3 width	15
TER4	tergite 4 width	15
TOMA	tomentum A	15

Table 2 Morphometric measurements on 58 charcaters of Apis cerana in Thailand and Malaysian Peninsula

Variable	Characters	Figure
TOMB	tomentum B	15
ST6L	sternite 6 longitudinal	16
ST6T	sternite 6 transversal	16
PIG2	pigmentation of tergite 2	17
PIG3	pigmentation of tergite 3	17
PIG4	pigmentation of tergite 4	17
PIGS	pigmentation of sclerite S	18
PIGK	pigmentation of sclerite K	18
PIGB	pigmentation of sclerite B	18
CUBINDEX	cubital index	8
LPALP	left labial palpi	14
RPALP	right labial palpi	14
TONGUE	tongue length	14
FWINDEX	fore wing index	FWLN/FWWD
HWINDEX	hind wing index	HWLN/HWWD
TRINDEX	basitarsus index	TRLN/TRWD
WXINDEX	wax mirror index	WXLN/WXWDA
LPINDEX	left labial palpi index	LPSEG/LDSEG
RPINDEX	right labial palpi index	RPSEG/RDSEG
PINDEX	labial palpi index	
TER3_4	tergite 3 + 4	TER3 + TER4
LEG	hind leg length	TBLN + FELN + TRLN

Fig. 2 Dissecting microscope (10X-40X)

Fig. 3 Staining set

Fig. 4 Staining solution (Triple stain)

Fig. 5 Mounted slides (A: fore and hind wing, hind leg and sternite 3
B: Tongue, sternite 6, tergite 3 and tergite 4)

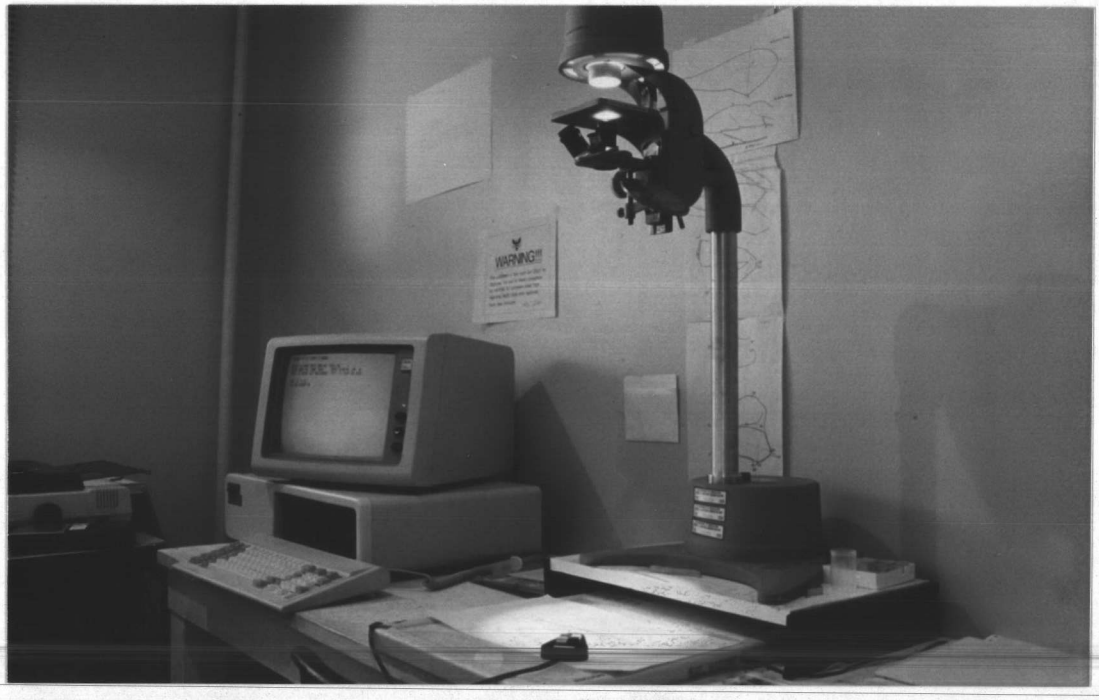

Fig. 6 Digitizing set (IBM PC/XT, microslide projector and
digitizer

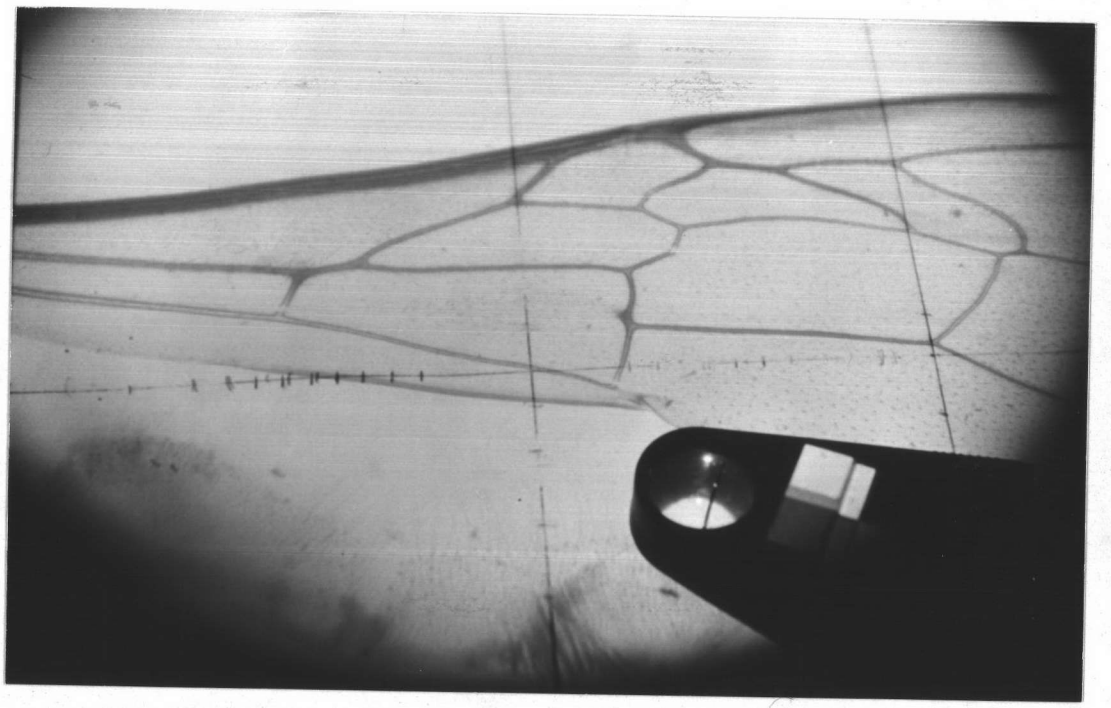

Fig. 7 Projected image from microslide projector onto digitizer pad

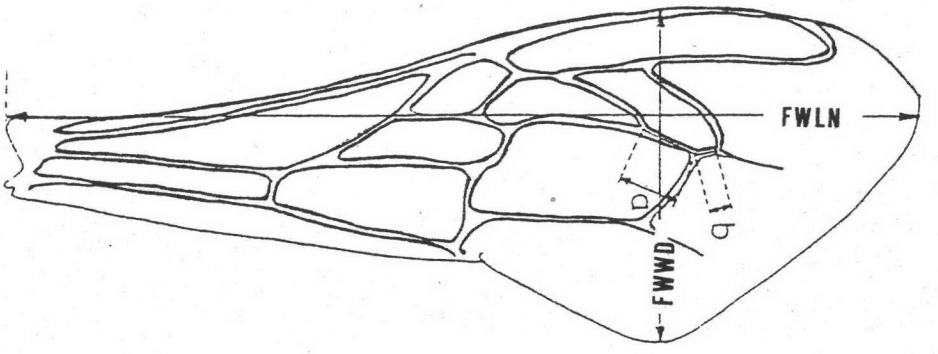

Fig. 8 Fore wing (FWLN: fore wing length; FWWD: fore wing width; Cubital index = a/b)

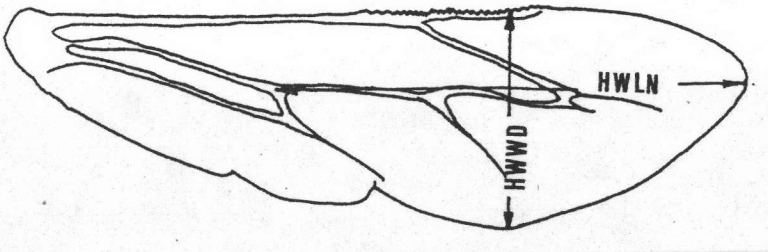

Fig. 9 Hind wing (HWLN: hind wing length; HWWD: hind wing width)

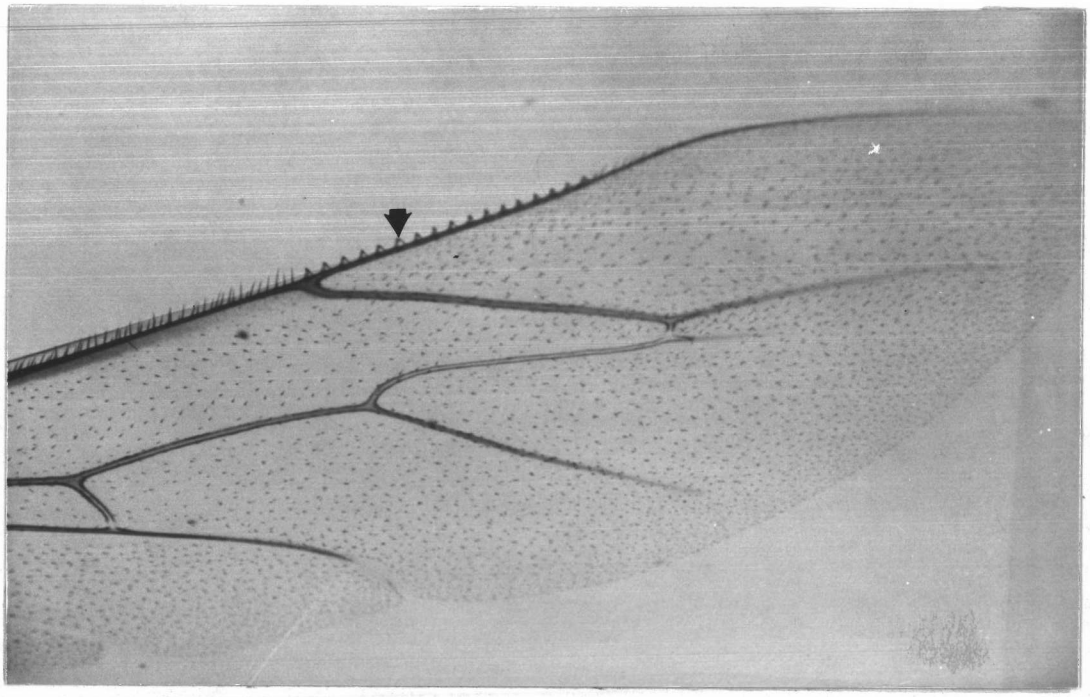

Fig. 10 Hamuli or wing hooks on hind wing

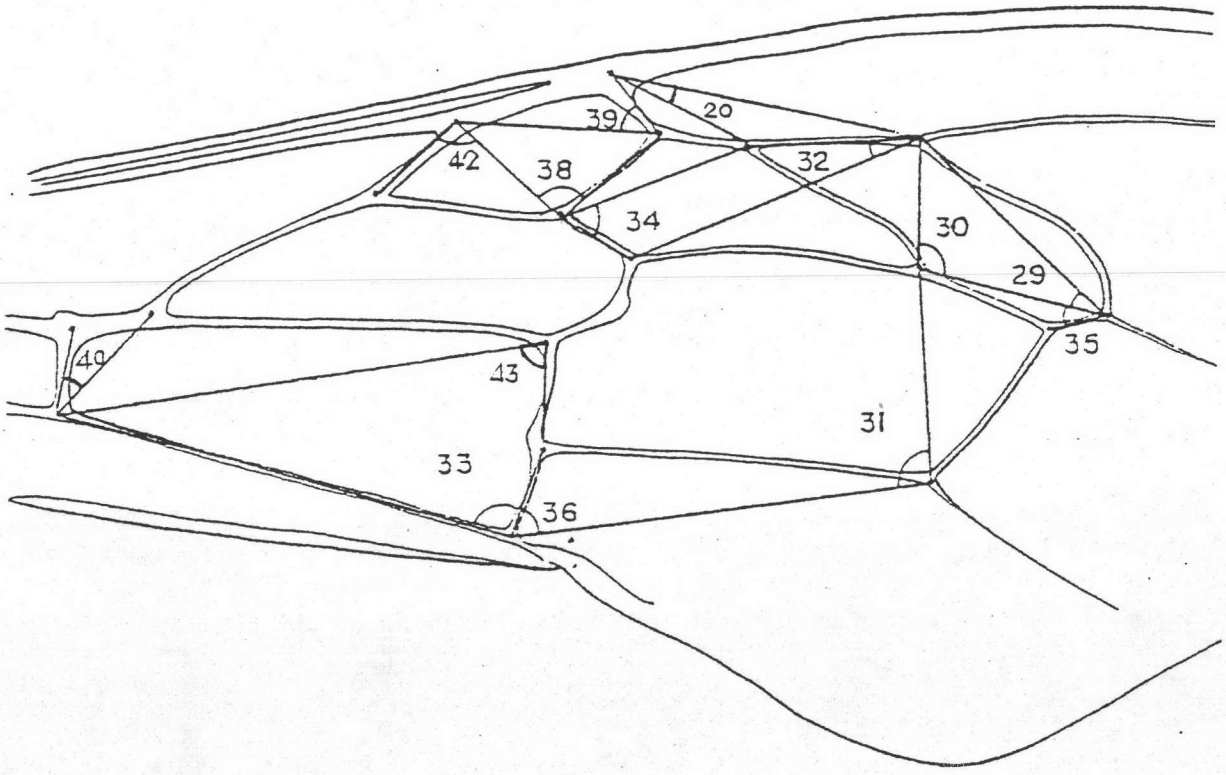

Fig. 11 Angles of wing venation (angles 20-43)

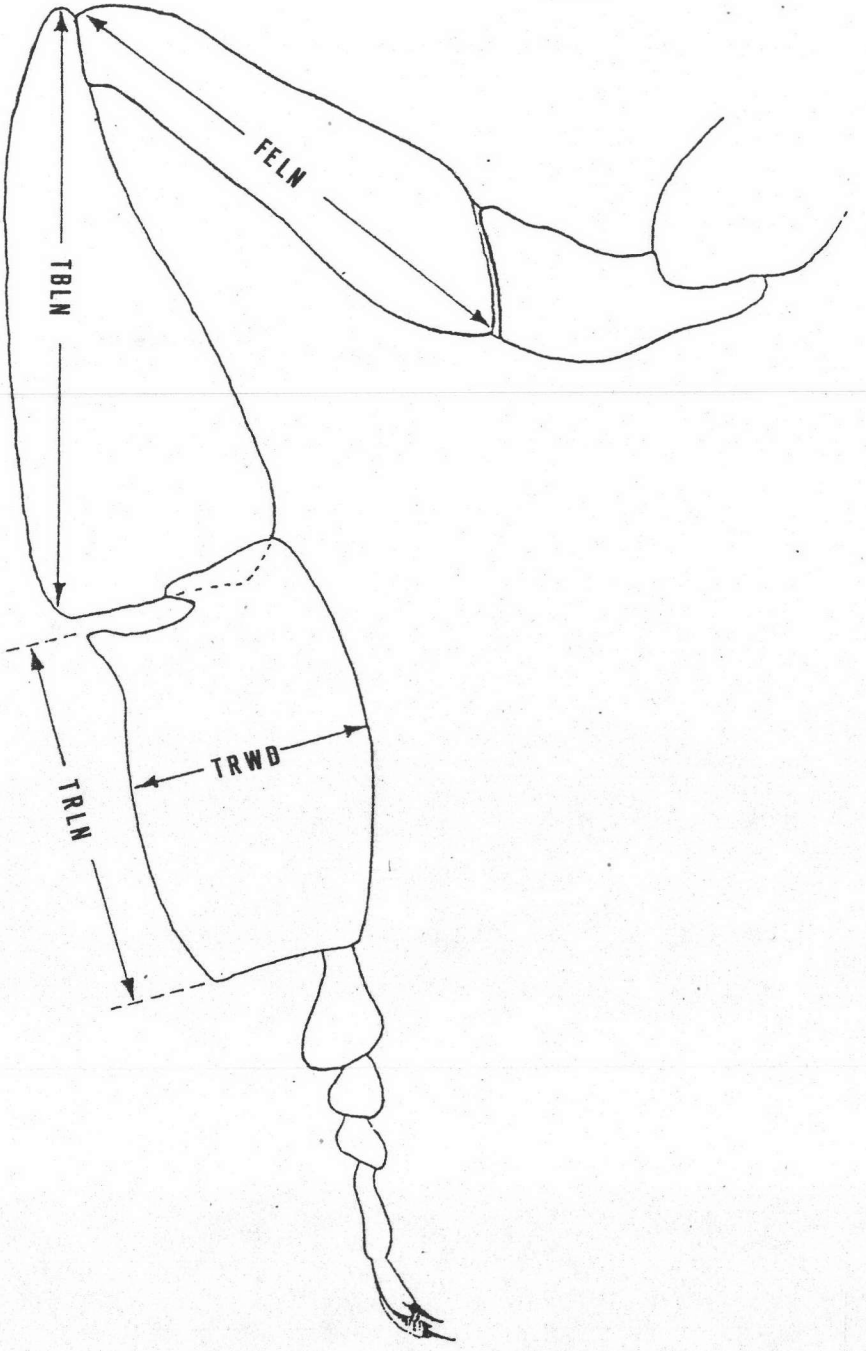

Fig. 12 Hind leg (FELN: femur length; TBLN: tibia length; TRLN: basitarsus length; TRWD: basitarsus width)

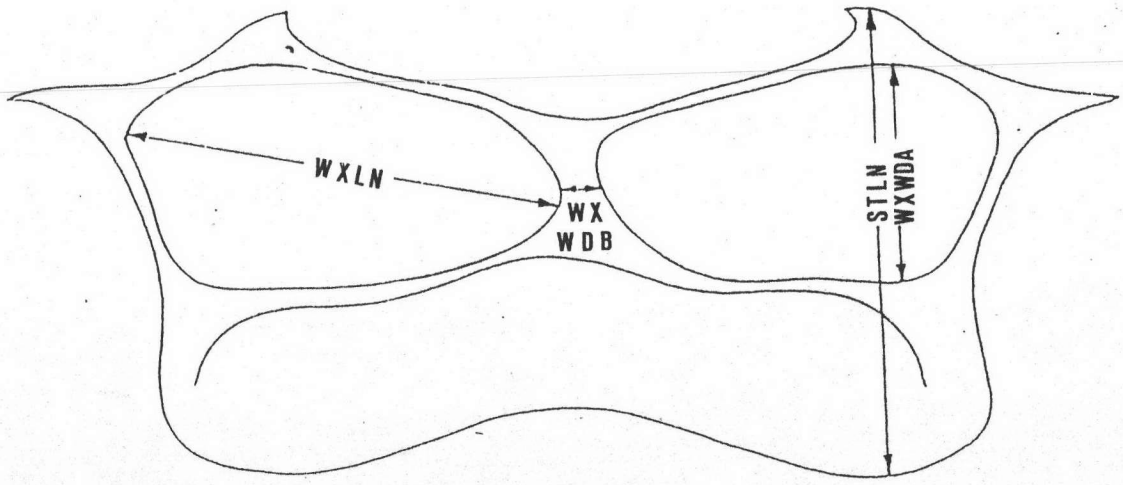

Fig.13 Sternite 3 (STLN: sternite length; WXLN: wax plate length; WXWDA: wax plate width; WXWDB: distance between wax plates)

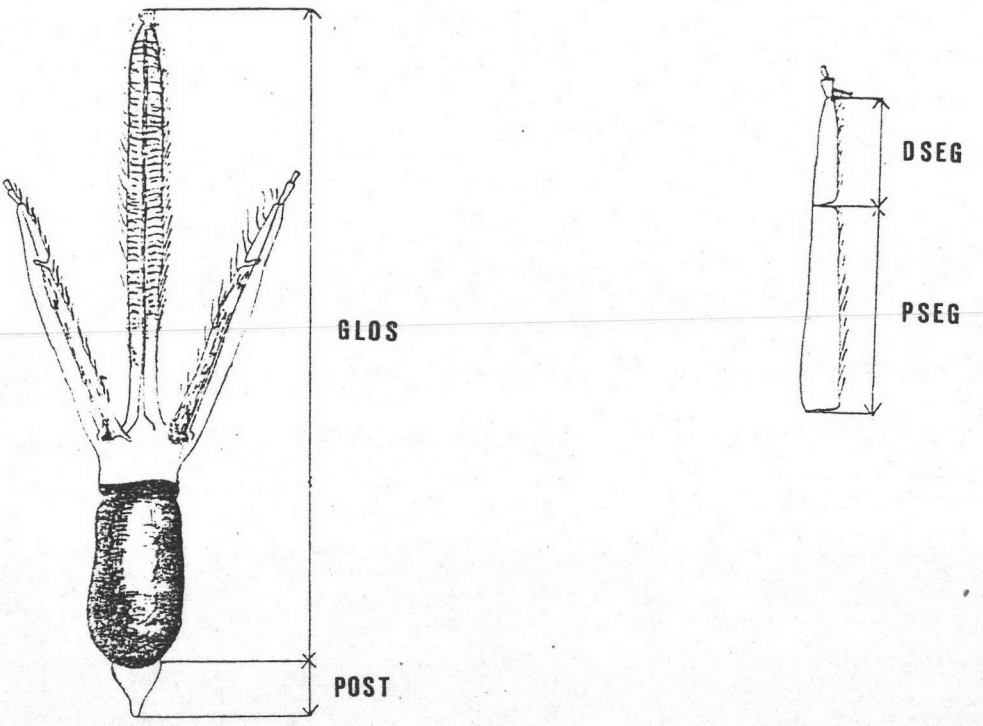

Fig. 14 Tongue (POST: postmentum length; GLOS: glossa+mentum length; DSEG: distal segment of labial palpi; PSEG: proximal segment of labial palpi)

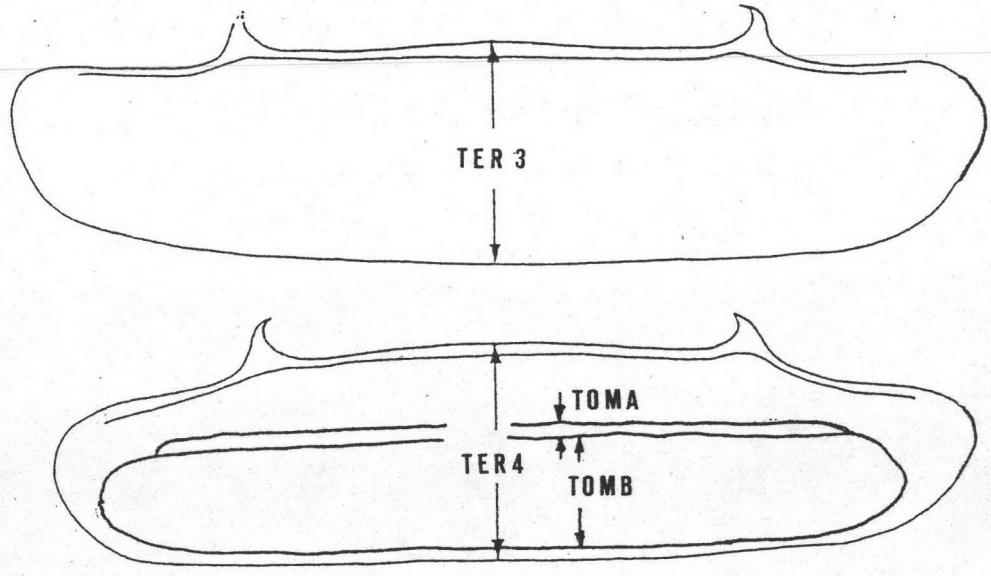

Fig. 15 Tergite 3 and 4 (TER3: tergite 3 width; TER4: tergite 4 width; TOMA: tomentum A width; TOMB: tomentum B width)

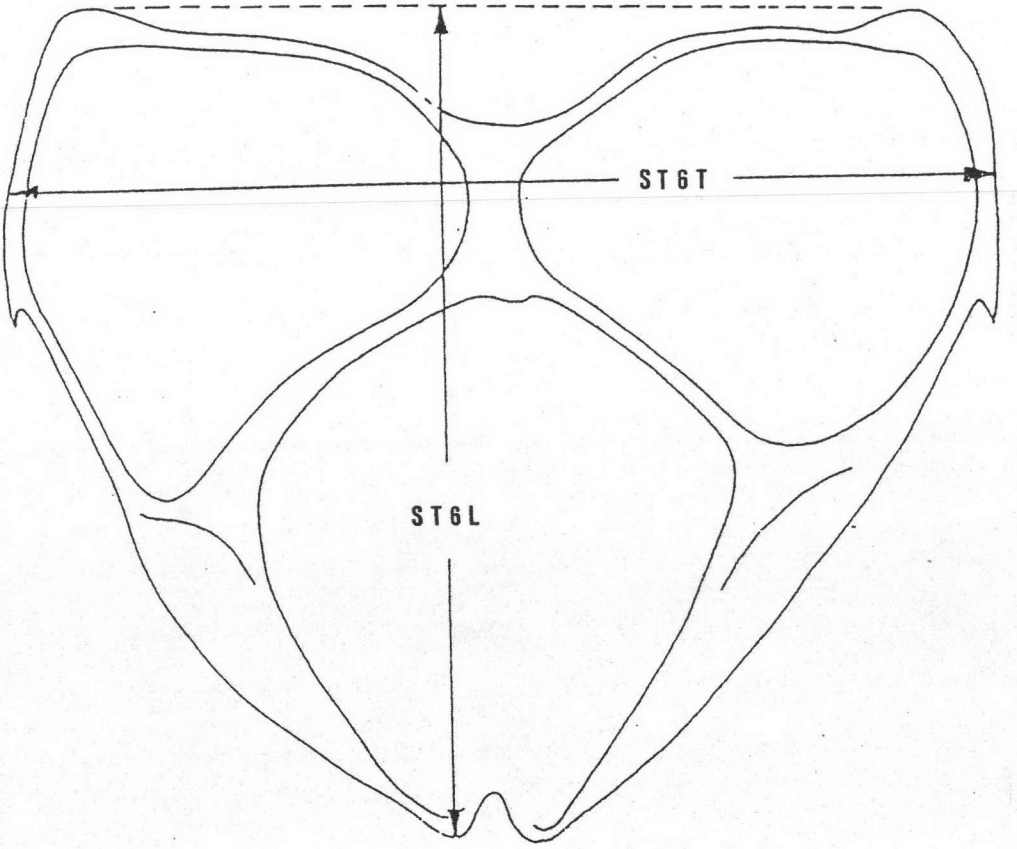

Fig. 16 Sternite 6 (ST6L: sternite 6 longitudinal; ST6T: sternite 6 transversal

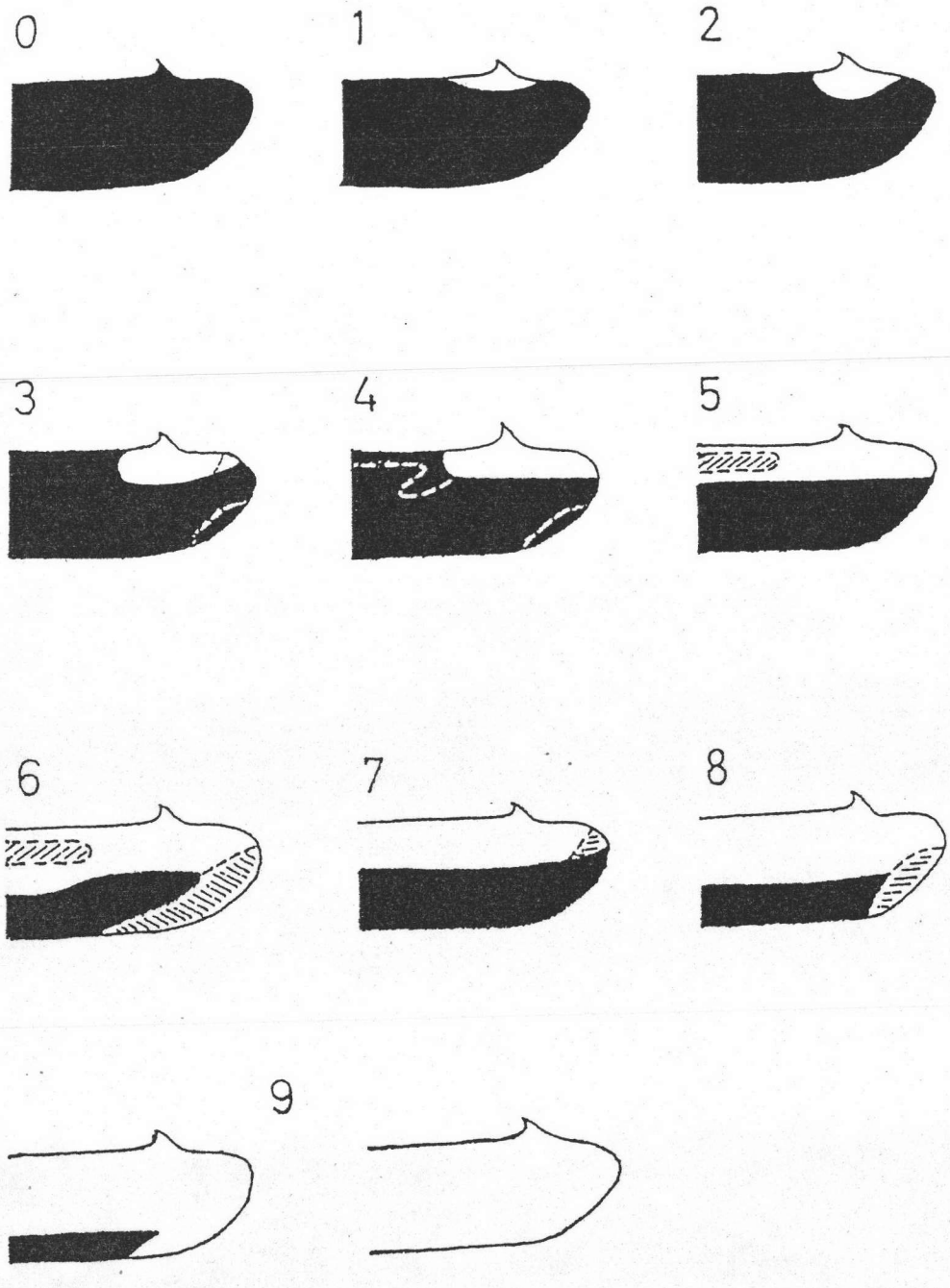

Fig. 17 Pigmentation of tergite 2-4,

classification 0 (dark) - 9 (yellow)

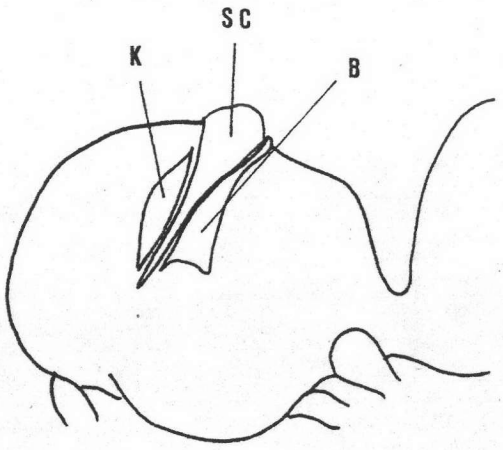

Fig. 18 Pigmentation of thorax

(Sc: scutellum - scale 0(dark) - 9(yellow);

B,K: metatergum and mesotergal sclerite - scale 0 - 5)