THE RELATIONSHIP BETWEEN THE WOODWASP SIREX NOCTILIO F. AND THE WOOD-ROT FUNGUS AMYLOSTEREUM SP.

A Thesis Submitted

by

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STATEMENT

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university, and no material previously published or written by another person, except when due reference is made in the text of the thesis.

C.B. Boros
January, 1968

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I. SUMMARY

Dissections of larvae of both sexes of Sirex noctilio confirmed Parkin's (1942) observation that the gut in Siricidae is simpler than is usually found in larvae digesting wood. The variation in the distension of the salivary reservoirs in these larvae suggests that saliva is collected and then released in quantity, possibly for extra-intestinal digestion of the symbiotic fungus Amylostereum.

Neither on anatomical nor on mycological grounds was it possible to confirm the claim of Clark (1933) that mycangia occur in the hind-gut of female larvae.

Paired hypo-pleural organs have been found in second instar larvae of S. noctilio. With each moult the cuticular layers of the organ carrying the fungus are shed, the newly secreted organ being distinctly longer and having more pits, towards the center of the organ, the pits usually having two internal partitions. The coils of fungus within the pits are, just prior to ecdysis, set in a waxy matrix which dissolves in xylene. Rarely there are indications of a second smaller organ on the posterior fold of the meta-thorax, as has been found on Tremex columba (Stillwell, 1964).

Stained serial sections of the larva showed that exocuticle is present only in the tips of the spines. Consequently during moulting the septa of the hypo-pleural organs collapse exposing

the contents of the pits. On moist freshly-cast, pre-pupal exuviae, the contents of the pits form a glistening ridge. Individual wax packets can be removed from this firm ridge and soaked in xylene to expose the fungus.

Dissections of the reproductive system of the adult females of S. noctilio showed that there are three distinct sets of accessory glands: the paired mucous glands, the median oil sac, and the unicellular glands. The mucous and oil glands begin secretion in the teneral adult. The mucus is probably an acid mucopolysaccharide-protein complex. The oily secretion contains five fatty acids, one being a major component.

Pure cultures of Amylostereum were obtained from excised hypopleural organs, from slivers of wood from larval tunnels, from the walls of the pupal chamber and from wood fragments in the pupal chamber and on the surface of the pupa. Negative results from all region of the larval gut indicate that if the fungus is eaten, it is digeste rapidly, possibly extra-intestinally.

Cultures taken from pupal and adult females showed that the inter-segmental sacs became infected with Amylostereum only after the teneral adult became active.

The activity of the teneral adult female appeared to be an example of adaptive infective behaviour.

Francke-Grosmann's (1957) hypothesis that wax packets pass along the ovipositor to the inter-segmental sacs, neglects the role of insect secretions in the infective process. Experiments have shown that oil and mucus applied separately or mixed together, caused a marked increase in the vegetative growth of the fungus and probably assisted the release of the fungus from the wax packets. The presence of insect secretions which stimulate the vegetative growth of the fungus, and possibly function as fungal attractants, as suggested by Parkin (1942) for the hypopleural organs, would be valuable in maintaining the symbiotic relationship whether the sacs are infected by wax packets, or by fungus growing in from the pupal chamber Buchner (1965).

II. INTRODUCTION

(i) Discovery of Mycangia.

Büchner (1928, 1930) established that the adult females of Urocerus gigas (L.) carried the oidia of basidiomycete fungi within a pair of inter-segmental sacs at the base of the ovipositor. He found similar structures in several species of Siricidae and in Xiphydria camelus (L.).

Following Büchner (1928), Cartwright (1929) reported that the fungus was inoculated into the wood during oviposition, giving rise to the mycelium lining the larval tunnels.

Clark (1933) claimed that there were glands in the hind gut of female larvae of Sirex noctilio which corresponded with the inter-segmental sacs of the adult and carried the same fungus. This claim was not substantiated by other workers. Neither Müller (1934) nor Francke-Grosmann (1939) was able to culture the fungus from the larva.

Parkin (1942), working with Sirex cyaneus F. and U. gigas, reported that some of the larvae, which he correctly assumed to be females, carried the fungus in highly specialised cuticular organs. By reason of their location on the posterior side of the hypo-pleural fold of the first abdominal segment, he called them hypo-pleural organs.

(ii) Description of the Mycangia.

(a) The inter-segmental sacs of the adult female were described by Francke-Grosmann (1939). Paired invaginations of the membrane between the seventh sternite and the modified eighth sternite envelop club-like swellings on the bases of the first pair of valvulae, Sections of these club-like swellings show that they contain masses of unicellular glands, each with a single large duct which opens into the inter-segmental sac. Francke-Grosmann (1939) surmised that these structures allowed freer movement of the first pair of valvulae of the ovipositor and carried the fungus as a secondary function.

(b) Parkin (1942) described both the external appearance and the internal anatomy of the larval hypo-pleural organs. Viewed from the surface, these organs are fusiform and slightly curved towards the ends. They contain a series of pits in which tangled bundles of fungal threads may be found. On histological grounds Parkin (1942) assumed that the modified hypodermis underlying the organ was secretory, but was uncertain of its precise function.

The youngest larvae in which he was able to detect these organs were one quarter to one third grown. He assumed that the cuticular parts of the organ were shed at each moult and observed that the number and size of the pits increased as the larva grew.

He suggested that these organs could be used not only to sex larvae, but to identify the various species by using the ratio of length to width, as well as the size, number and arrangement of the pits.

Rawlings (1953) confirmed that only the females have these organs when he reared the larvae of S. noctilio in two groups according to the presence or absence of these organs.

The significance of these mycangia on the female larvae only was not understood for several reasons. The males were able to complete their development without them, and they were absent from the female pupae which both Francke-Grosmann (1939) and Parkin (1942) had found were free of the fungus. They did not appear to have any connexion with the infection of the inter-segmental sacs.

(iii) Method of Fungal Transfer.

(a) Larva/Adult.

Francke-Grosmann (1939) suggested that the inter-segmental sacs became infected with fungus growing from the wall of the pupal chamber during the quiescent phase of the teneral adult. She based this hypothesis on the correlation she observed between the developmental stage of the adult female, and the condition of the fungus in the inter-segmental sacs. She found only a few mycelial strands in the sacs of newly-moulted adults, whereas

the sacs of females boring through the wood were distended with large balls of growing mycelium. By the time the females emerged from the wood, they carried only masses of short oidia.

Francke-Grosmann (1957) suggested that the fungus carried in the hypo-pleural organ was transferred to the inter-segmental sacs of the adult female. This transfer involved the formation of wax packets containing the fungus within the pits of the hypo-pleural organ during the pre-pupal stage. During the final moult the packets were shed from the organ and could be found lying in the pupal chamber, and on the pre-pupal skin. Francke-Grosmann found that when these sticky plates were placed on the ovipositor they were passed along the moving shafts either in the direction of the inter-segmental sacs or towards the tip of the ovipositor. She postulated that some of the waxy packets scattered about the pupal chamber must come into contact with the moving shafts of the ovipositor of the female. On reaching the inter-segmental sacs they would give rise to a new growth of the fungus. When attempts to culture the fungus proved unsuccessful unless the plates were deliberately damaged, she made a further suggestion that the vigorous movement of the shafts of the ovipositor would damage most of the waxy plates they touched. Francke-Grosmann claimed she has found the remains of these plates in the inter-segmental

sacs of infected females.

She found that woodwasps sometimes complete their development in timber which is too dry for the growth of the mycelium to continue, yet the adults invariably carried the fungus. She claimed that the protective waxy layer around the fungus during the critical pre-pupal and pupal stages ensured the continuation of the association of woodwasp and fungus in the next generation regardless of the moisture content of the wood.

(b) Larva/Larva.

Parkin (1942) assumed that as the part of the hypopleural organ which contains the fungus is cuticular, the symbiotic association would be broken at each moult, and consequently that the hypopleural organs of the newly moulted larvae might be reinfected by hyphae growing in from the wall of the tunnel. He also raised the questions of how the fungus was attracted to the organ, and whether the secretions of the hypodermal cells were a nutrient source for the fungus.

(iv) Role of the Fungus.

(a) Larval establishment.

The powdery whiteness appearing around the tunnels two days after oviposition has been attributed to the drying action of the fungus on the wood. Morgan & Stewart (1966a) have

observed that females select logs of intermediate moisture content for oviposition, and that larval mortality increases sharply in logs which fail to dry out in the usual way. They have suggested that this modification of the microenvironment by the fungus aids the establishment of the larva.

In cases of heavy attack, it is possible that the fungus weakens the trees so that the resistance mechanisms against the larva are ineffective, Titze (1965). King (1964) has shown that the fungus produces substances which are toxic to pine seedlings.

(b) Larval nutrition.

Büchner (1928) suggested that the fungus growing in the larval tunnels predigested the wood for the larvae.

Cartwright (1929) reared a newly hatched woodwasp larva for three weeks and another larva, half grown, for three months, on a culture of Stereum sanguinolentum. This result indicated that the larvae might not eat wood under normal conditions either.

In 1934, Müller made a comparative analysis of the fungus-infested wood and the frass around larvae of U. gigas and U. phantoma (F.). He found that the frass contained fewer pentosans and less cellulose than the infested wood. Without similar information on the composition of healthy wood, it is impossible to decide whether the enzymes of the larva or the fungus brought

about this change.

Francke-Grosmann (1939) carried out enzyme tests with the digestive juices of the larvae of Sirex juvencus (cyaneus) (L.) which provided evidence for assessing the role of the fungus in larval nutrition. She found that while these digestive juices had no effect on cellulose, hemicellulose or wood, they caused rapid disintegration of the fungal mycelium, indicating that the fungus could be digested by the larva.

Stillwell (1966) succeeded in rearing adult females of S. juvencus which were free of the fungus. The eggs of these females, both fertilised and unfertilised hatched but all died in the first instar. Whether these larvae died from starvation or some other cause cannot be determined from this experiment, but it indicated that the fungus may be necessary for the survival of the larvae in the wood.

(v) Specificity of Relationship.

There have been conflicting opinions regarding the specificity of the relationship between woodwasps and their associated fungi. This controversy can be attributed to the difficulties involved in identifying the fungus carried within the mycangia, and the incorrect but credible assumption that the fungus cultured from the rotting wood around the pupal chamber would be the fungus

carried by the emerged female.

After culturing from the inter-segmental sacs of S. gigas and S. cyaneus, Cartwright (1938) claimed that S. sanguinolentum was the only fungus involved in the association.

Francke-Grosmann (1939), made cultures from the surrounding wood and the inter-segmental sacs of S. noctilio, S. juvencus, U. gigas, U. augur and Tremex fuscicornis. Her results indicated that the different species of woodwasp were not always associated with the same species of fungus, but that with each species of woodwasp, one fungus seemed to be dominant.

Parkin (1942) reported that he and Cartwright isolated only S. sanguinolentum from S. gigas and S. cyaneus in England. Talbot, (1964); King, (1960) have shown that only one fungal symbiont, identified as a species of Amylostereum Boidin, is associated with S. noctilio in Australia.

Stillwell (1960) reported that S. sanguinolentum was associated with woodwasps in New Brunswick and Nova Scotia because this fungus was isolated from deteriorating wood near the pupal chamber. Subsequently, attempts to isolate the fungus from the adult female were unsuccessful.

Working from the key devised by Nobles (1948), Stillwell

(1966) claimed that Cartwright's identification of S. sanguinolentum was invalid. He found that sub-cultures of the original isolates resembled S. chailletii, as did the cultures from S. noctilio in New Zealand. Francke-Grosmann (personal communication to Stillwell) considers the fungus from S. juvencus in Germany, to be similar to the New Zealand fungus. Stillwell put forward the suggestion that

S. noctilio in New Zealand

S. juvencus in Germany

S. cyaneus) in England

U. gigas

U. gigas flavicornis F.) Canada

U. albicornis F.)

S. cyaneus)

may be associated with the same fungus which is S. chialletii.

III. MATERIALS AND METHOD

A. Anatomy

(i) Larval Anatomy.

The anatomy of the larva has been studied from dissections and stained serial sections.

(a) Dissections and serial sections.

Larvae used for dissection were stored in ground-up horse-radish and kept under refrigeration. To obtain a clearly defined outline of the organs surrounded by masses of white fat body, the dissections were stained with Fat Red and Methylene blue. Sixteen female larvae and twelve male larvae were dissected.

The larvae used for serial sectioning were fixed in Lillie's neutral buffered formalin and dehydrated in ethyl alcohol. They were cleared in benzene and embedded in paraffin wax M.P. 60°C containing 1% ceresin.

Serial sections were cut on a Reichart rotary microtome at 5 microns and 8 microns. The mounted sections were treated with a modified Gram-Weigert stain (Leach, 1940) found by Fernando (1960) to stain fungus selectively. Lower's Trichrome stain, which differentiates the layers in the cuticle was also used. Seventeen female and three male larvae were sectioned and stained for investigation.

(b) The hypo-pleural organ.

1. The surface view of the hypo-pleural organ was studied from excised organs mounted either directly in Berlese's fluid, or in Sira after dehydrating and clearing. Measurements taken from these permanent mounts were used for calculating length: width ratios of the organ.

Stained and mounted squashes of the whole organ were examined to determine the gross appearance of the fungus within the pits. The fungus was stained with Aniline Blue, and the surrounding wax with Sudan IV, Oil Blue N and Magdala Red. The squashes were mounted in glycerine jelly.

2. To obtain a three dimensional concept of the structure of the organ and its development, larvae of all sizes and all stages were sectioned transversally and also longitudinally in both horizontal and vertical planes. Details of the layers in the larval cuticle were worked out from these sections which were stained with Lower's Trichrome. Observations of a moulting larva and serial sections of a late pre-pupa provided additional information on the changes taking place in the organ from instar to instar.

(c) Wax packets.

1. Cast skins were moistened with a drop of water and stretched out on a glass slide. The area between the third and fourth spiracle was examined. The waxy contents of the hypo-pleural organ form an opaque, finely corrugated, glistening ridge on the moist, recently moulted skin of the pre-pupa. Individual wax packets can be separated from the ridge as they adhere to the surface of a blunt, cylindrical needle rolled against them.

2. Packets were mounted in glycerine jelly and Berlese's fluid for examination and measurement under the compound microscope.

3. To find whether these packets were coated in wax only, or whether there was a cuticular envelope as well, they were tested with wax colorants, wax solvents, cuticular stains and fungal stains.

As the number of packets available was limited, and these small packets were difficult to manipulate, experiments on ten packets from which some results were obtained have been listed in Table I.

During experiments with wax solvents, the fragile packets were placed in excavated blocks containing either chloroform (Carnoy's fluid), di-ethyl ether, ethanol or xylene. Some of the packets were sketched and measured before and after this treatment. They were examined during the experiments which ranged in time from 10 minutes to eighteen hours.

During the staining operations, the packets were kept in glass rings fixed onto slides, while the stains and clearing agents were pipetted into and drawn out of the ring. Sudan IV and Magdala Red were used as wax colorants. The cuticle was stained with Lower's Trichrome, and the fungus bundles with Methyl Green, Phloxine B and Aniline Blue. The results have been listed in Appendix I.

(ii) Female Reproductive System.

(a) Pupa.

The anatomy of the female pupa was studied from stained serial sections using the same method as in Section III (i)a. When cutting longitudinal sections of late stage pupae, the cutting surface of the block was painted with a 1% solution of celloidin in a mixture of equal volumes of alcohol and ether, before each section. The film of celloidin kept the brittle shafts of the ovipositor of late stage pupae in place.

(b) Adult.

As the tough exoskeleton, and the ball of brittle mucus in the abdomen of fixed and dehydrated adult females made sectioning extremely difficult, the anatomy of the abdomen of both sexes was studied from dissections stained with Fat Red and Methylene Blue, and details of the reproductive systems were worked out from permanent mounts which were stained and cleared.

B. Secretions of Accessory Glands.

Secretions of both accessory glands were obtained free of contamination with cellular material as indicated below:

(i) The Oil Gland.

The oil gland is a narrow median sac loosely attached to the anterior wall of the mucous duct. Once the sac-like gland had been separated from the mucous duct, the narrow neck could be severed, and the entire structure removed from the abdomen of the adult female. To obtain the contents, the sac was placed on a slide and opened with a lateral incision. The oily contents were tested with 0.02% Nile Blue sulphate which remains blue if acidic lipids are present. The oily droplets were taken up with a 1 micro-litre pipette. As less than one microlitre was obtained from each female, thin layer chromatography was the most suitable method for analysis. Microscope slides were dipped in a mixture of 35 grams Keiselguhr and 100 ml. chloroform, and the solvent was allowed to evaporate at room temperature. The slides were spotted with 0.5 µl of the oily secretion; the standards used were similar volumes of 10% cholesterol in ethanol, oleyl alcohol, and tributyrin. The chromatograms were run in the solvent mixture petroleum ether, di-ethyl ether, glacial acetic-acid (90+10+1), and the chromatograms were developed by the following

procedures.

(a) They were placed in a chamber of iodine vapour to reveal neutral lipids.

(b) They were sprayed with 2',7' di-chlorofluorescein and then examined under u/v light to observe neutral lipids.

(c) They were sprayed with a saturated solution of antimony trichloride to see whether the spot with the same rf as cholesterol was a steroid.

(d) They were sprayed with 0.04% Bromo-Thymol-Blue (pH range 2.8-4.6) , adjusted with $NaHCO_3$ until at the point of changing from greenish-yellow to blue, to detect fatty acids.

(ii) The Mucous Gland.

Once the oil gland has been removed, the mucous duct is clearly visible. To obtain the contents of the mucous reservoir, the duct was cut, and the reservoir with glands attached was lifted out of the abdomen. The membranous wall of the reservoir was peeled away from the firm clear secretion of the glands.

(a) Histochemistry.

The first tests on the mucus were carried out to find whether proteins, carbohydrates and fats were present.

To test for protein, the unfixed mucus was smeared over a slide, and a few drops of Millon's reagent were added, (see

Pearce (1960) p. 791. The slide was warmed over a bunsen burner until the reagent reacted with the mucus, which was then rinsed in distilled water.

The Periodic Acid Schiff technique, see Pearce (1960) p. 832, was used to test for carbohydrates. Mucus was smeared over two slides, and only one slide was placed in 0.5 per cent aqueous periodic acid before both were treated with the Schiff's reagent. Results were the same whether the mucus had been "fixed" in chloroform for half an hour or left unfixed.

To see whether the PAS reaction could be prevented by acetylation, and so confirm the carbohydrate nature of any reacting groups, the method of Pearce (1960), p. 832 was carried out.

The mucus was not fixed when tested with a saturated solution of Sudan Black B in 70 per cent ethyl alcohol. The pigment dissolves in lipids but can be washed out in acetone. Acetone-fast staining occurs when the pigment stains protein and is not indicative of lipids. The mucus was tested with 0.02% Nile Blue which remains blue if acidic lipids are present.

Further histochemical tests, see Pearce (1960) p. 236, were carried out to confirm the provisional identification of the mucus as an acid mucopolysaccharide-protein complex.

(b) Acid hydrolysis.

An acid hydrolysis of the mucus was prepared for further analysis by paper chromatography. Approximately 50µg of fresh mucus was sealed in an ampoule with 1 ml 1NHC at 100°C over-night. The hydrolysate was evaporated to dryness three times to remove traces of the acid. Unidimensional ascending chromatography on Whatman No. 1 filter paper was carried out in Phenol + Water (4 + 1), and dried in air for forty-eight hours until the smell of phenol had disappeared. The standards used were 0.2 M galactosamine HCl, glucosamine, chondrosamine, galacturonic acid, glucuronic acid, D-galactose and D-glucose.

The chromatograms were revealed in the following reagents:

Ninhydrin (for detecting amine groups).

The strips were dipped in 2 per cent ninhydrin in ethanol and incubated at 100°C for 10 minutes.

Silver Nitrate (for detecting sugars).

(a) The strips were dipped in a solution of 2 grams of silver nitrate dissolved in 20 ml. water and diluted with acetone to 1 litre.

(b) After drying, they were dipped in fresh ethanolic 0.5N Sodium hydroxide.

(c) The papers were rinsed in distilled water.

(d) The papers were fixed in a solution containing 1.5% sodium metabisulphite, and 10% sodium thiosulphate, and given a final rinse in water.

Acetylacetone-Dimethylaminobenzaldehyde Reagent of Block, Durrum and Zweig (1958) p. 209, which reveals free hexosamines as cherry-red spots and N-acetylglucosamine as purple-violet spots.

p-Anisidine HCl for revelation of uronic acids see Block, Durrum and Zweig (1958) p. 182.

C. Culturing the Fungus.

Except for experiments in Section VI, (iv), the special medium developed for the Sirex fungus, NDY/6 with 2 p.p.m. of o-phenyl phenol, has been used in all culturing experiments.

All experiments were carried out at a constant temperature of 22°C.

(i) Cultures made from the Larva.

(a) The Gut.

In an attempt to verify the claim of Clark (1933) that there were glands in the hind gut of female larvae which carried the Sirex fungus, cultures were made from twenty females and ten male larvae. The larvae were rinsed three times in sterile water, and then dissected dry. The gut was cut off anteriorly across the narrow oesophagus and posteriorly in front of the anus. The hind-gut was cultured separately from the mid-gut in an attempt to see which regions were giving results.

(b) The Hypo-pleural Organ.

To obtain cultures from the hypo-pleural organs of female larvae, the organ was cut out. Underlying fat body was scraped off before the organ was cultured. No attempt was made to sterilise the external surface of the organ.

(c) Larval tunnels.

Cultures were made from slivers of wood sliced from the larval tunnels with a sterile scalpel.

(ii) Pupa and Adult.

(a) Wood scrapings in pupal chamber, the pre-pupal skin.

Cultures were made from the wood lining the pupal chamber, from wood scrapings, from the surface of the frass and sometimes from the pre-pupal skin.

(b) Ovipositor and inter-segmental sacs.

Very few specimens were available for these experiments and inevitably some were contaminated.

To study the onset and development of fungal inoculation of the inter-segmental sacs, female pupae and adults at different stages of development were cut out of the wood.

Cultures were made from the head, thorax, abdominal sclerites, tip of the abdomen and ovipositor. The ovipositor was cut off at the junction of the saw sheath and its base. The sheath was cultured separately from the distal end of the valvulae. The proximal parts of the valvulae were lifted out of the sheath base and cut off as close to the body as possible.

The membranous and muscular connexions between the sub-genital plate and the inter-segmental sacs were severed, so that the sacs could be used for culturing too. In this way it was possible to see from which areas of the body and which regions of the ovipositor the fungus could be cultured at the different stages of development of the female.

(iii) Germination of Wax Packets.

To see whether the individual wax packets would germinate when ruptured, ten packets were cultured on the special medium for the Sirex fungus. Five of these packets were damaged with a sharp needle. The rough maps drawn of each plate, showing the relative positions of the wax packets, made the subsequent inspection of these plates easier.

(iv) Effect of mucous and oily secretions on the fungus.

(a) Stock cultures.

The effect of these secretions on the vegetative growth of the fungus was investigated as follows:

Stock cultures of the fungus were established on NDY/6. Isolates punched out of stock cultures with a cork borer of diameter 0.3 mm were sub-cultured on plates of 2% water agar, on which the vegetative growth is sparse.

Three days later, a sparse corona of fungal threads of radius two to three millimeters, could be seen growing around each isolate. At this stage, secretions of the accessory glands were placed 2 a

away from each isolate. Altogether there were 4 different treatments replicated four times in this experiment-

(1) only the oily secretion was used

(2) " " mucus " " "

(3) a mixture of the two secretions was used

(4) there were no secretions on the control plates.

The plates were examined daily for a week.

A similar experiment was carried out using macerated club glands. The presence of cellular material not normally accessible to the fungus made it difficult to assess the results. Ideally, the oily droplets occurring in the inter-segmental sacs of teneral adults should have been used. The shortage of females at this stage made this approach impossible.

(b) Wax packets.

In an attempt to see whether undamaged wax packets could be stimulated to germinate by these secretions, twelve packets were placed on water agar and left for three days before they were given four different treatments. This method ensured that no damaged packets were tested with the secretions.

(1) The packets were coated with drops of the oily secretion.

(2) They were covered with mucus.

(3) They were coated with a mixture of oil and mucus.

(4) They were not treated at all.

The plates were examined daily for two weeks.

D. Infective Behaviour.

A teneral adult female, which had recently emerged from a pupa placed in a small test tube lined with filter paper, was kept under close observation for three hours to see whether there was a display of adaptive infective behaviour.

IV. ANATOMY OF SIREX NOCTILIO

(i) Larva.

(a) Dissections and Serial Sections.

Parkin (1942) drew attention to the structure of the gut of Siricid larvae which is much simpler than is generally found in larvae digesting wood. The possibility that the larvae of Siricidae feed either on the fungus lining their tunnels, or on wood which has been attacked by fungus, made the digestive system the focus of attention during these studies which have revealed that there are extensive, branched salivary glands, throughout the body cavity (see Figure 1a). There is considerable variation in the size of the salivary reservoirs, some being wider than the mid-gut, others being as narrow as the oesophagus. A possible interpretation of this variation is that the reservoirs discharge their entire contents in one dose. Morgan (pers. comm.) has suggested that the mandibles fit together to form a cup in which scrapings of wood and fungus might be pre-digested by saliva. Although Francke-Grosmann (1939) found traces of mycelium in the gut of larvae, the gut contents of specimens of Sirex noctilio examined in this study can best be described as a milky fluid containing coarse particles.

There are differences between the larvae of S. noctilio

Figure 1.

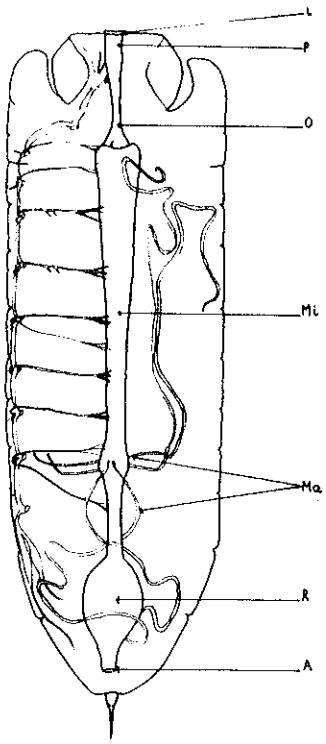
a. Gut and main tracheae of larva of *S. noctilio*.

l	labrum
p	pharynx
o	oesophagus
Mi	mid-intestine
Ma	malpighian tubules
R	rectum
A	anus

b. Salivary glands and central nervous system of larva of *S. noctilio*.

csd	central salivary duct
r	salivary reservoir
sg	branched salivary gland
br	brain
s•	sub-oesophageal ganglia
t_1	first thoracic ganglia
a_1	first abdominal ganglia

c. Gut and salivary gland of larva •f *S. noctilio* after Maxwell.



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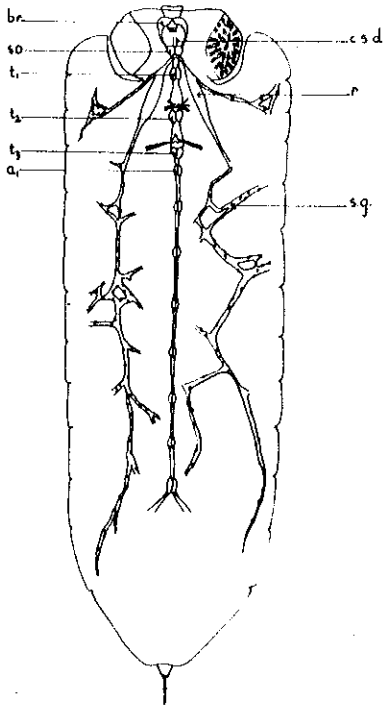

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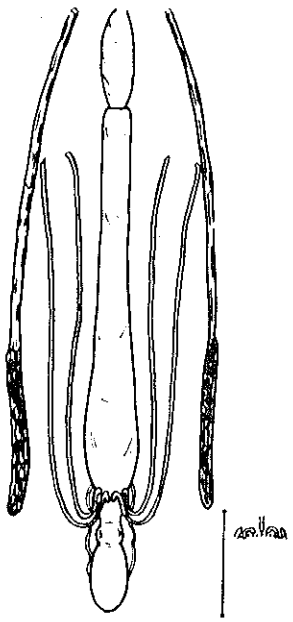


c

FIG. 1

Figure 2.

Female larva of S. noctilio showing the position of the hypopleural organ (h.p.o.)

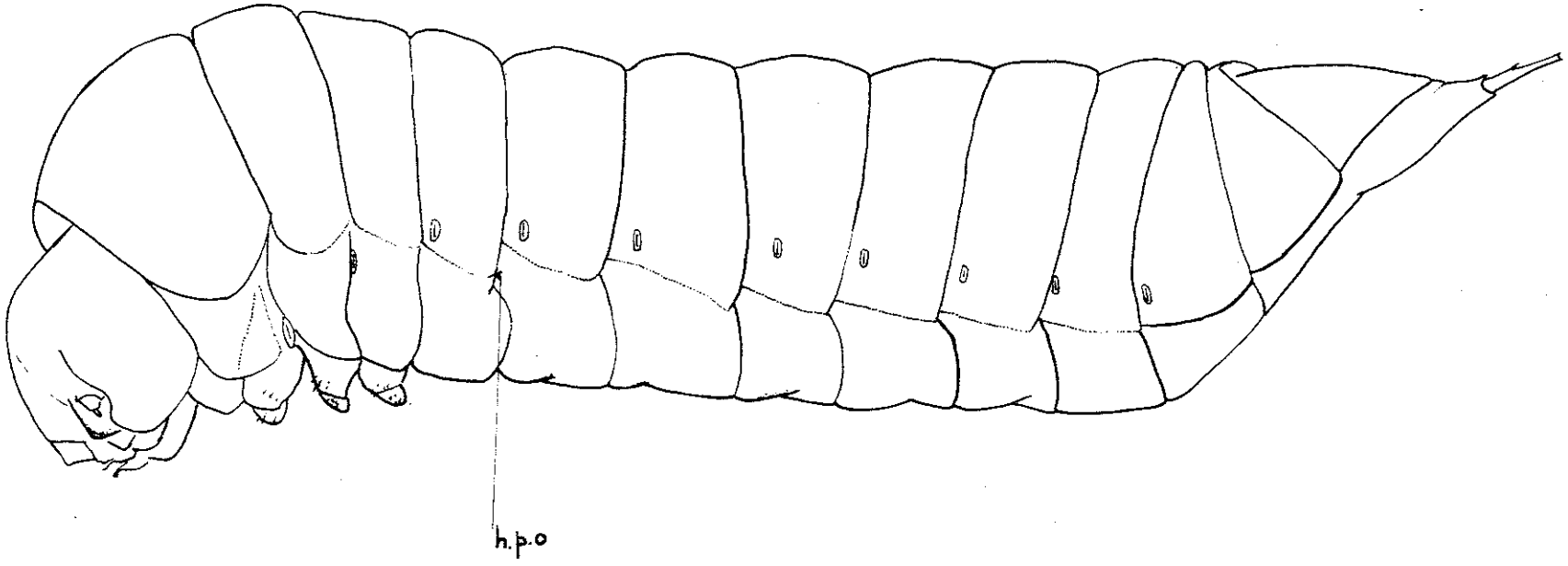

h.p.o

Figure 3.

a. Surface view of larval hypopleural organ showing arrangement of pits, according to Parkin.

A *Sirex cyaneus*

B *S. gigas*

b. Surface view of larval hypo-pleural organ of *S. noctilio* mag. 90x.

c. Dark bundles of fungus within the pits of the hypo-pleural organ of *S. noctilio* mag 330x.

d. Surface view of two damaged pits of the hypo-pleural organ of *S. noctilio*. The third pit is lying on its side.

e. An isolated pit, showing a projection of the scalloped base partially dividing it. Mag. 330x.

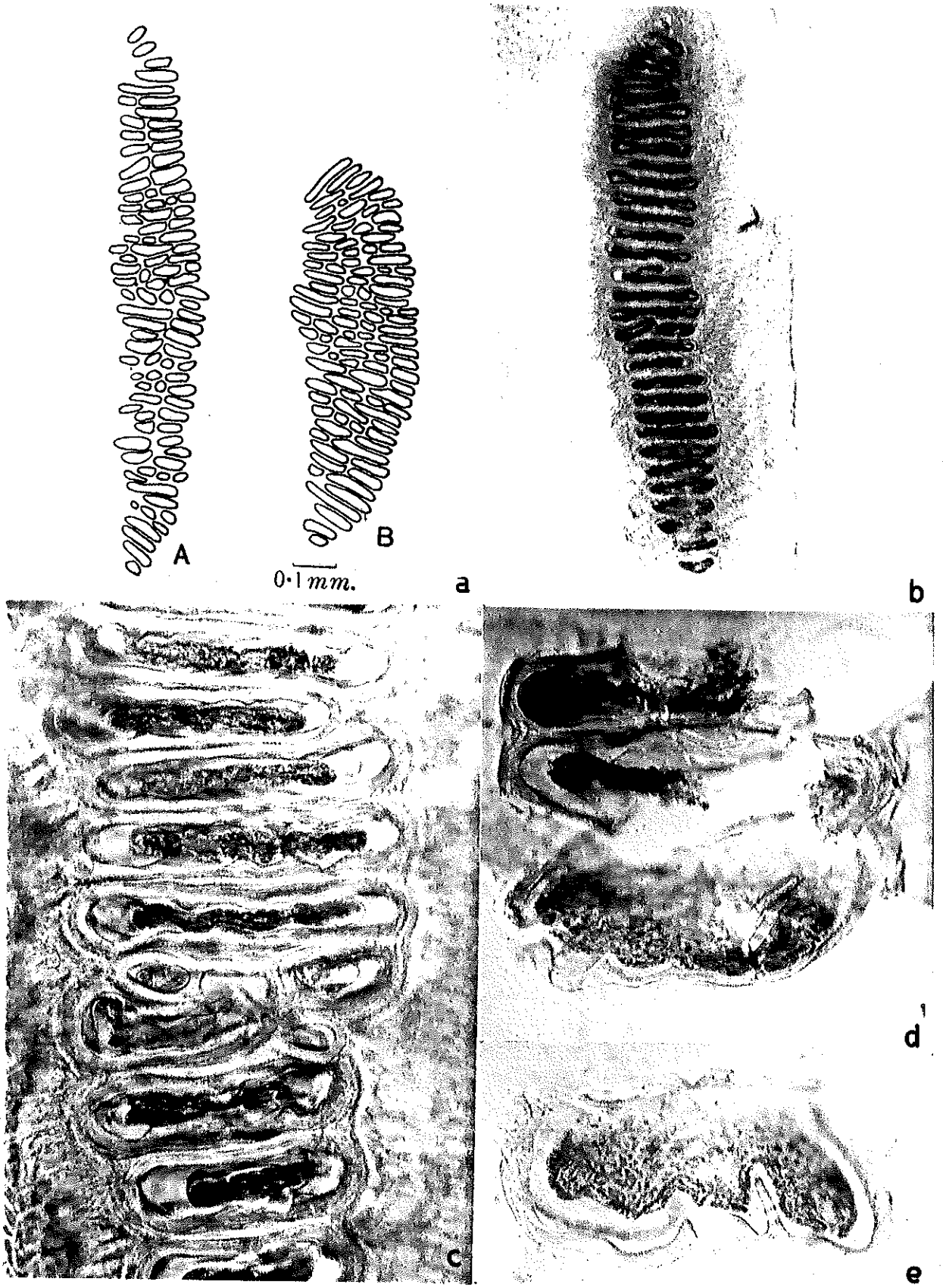


FIG. 3

examined during this study and those described by Maxwell (1955). Whereas the larvae obtained from logs of Pinus radiata in Tasmania have six malpighian tubules and extensive branched salivary glands, those obtained from Pinus sp. in England and described by Maxwell, have eight malpighian tubules and "slender squared salivary ducts widening posteriorly into rectangular glandular body". (Compare Fig. Ia with Fig. Ic).

Attempts to locate the glands in the hind-gut described by Clarke (1933) were also unsuccessful. Serial sections show there are six longitudinal pads lining the internal surface of the rectum which has a rich tracheal supply. Considering the dryness of the frass and the fluid condition of the contents of the mid-gut, these pads might be the sites at which water is resorbed.

The structure of the hypo-pleural organ, which was worked out from serial sections cut vertically, sagitally and horizontally, will be discussed in (i)b.

(b) The Hypo-pleural Organ.

1. The position of the hypo-pleural organ on the posterior surface of the pleuron of first abdominal segment of female larvae is shown in Figure 2.

Compared with the drawings of the hypo-pleural organ of U. gigas and S. cyaneus (Fig. 3a) first published by Parkin (1942),

the surface appears to be a series of narrow pits. Under high magnification (Fig. 3c) the darkened coils of fungus are clearly visible within the pits.

Parkin (1942) suggested that larvae of closely related woodwasps species could be identified from the arrangement of the pits and the ratio of the length to the width of the hypo-pleural organ. He gave the measurements of the hypo-pleural organ of almost fully grown larvae of S. cyaneus and U. gigas. From these measurements it can be seen that the length/width ratio would be approximately 5:1 for S. cyaneus and 3:1 U. gigas.

Rawlings (1953) has shown that this ratio is 6:1 for larvae of S. noctilio.

Morgan F.D. (Manuscript) has shown that the size of the larva varies with the moisture content of the wood. As the hypo-pleural organ is extended with each moult and its size is also related to the size of the larva, only when rearing conditions are identical, are comparisons between the hypo-pleural organs of similar-sized larvae of different woodwasp species possible. As environmental conditions vary widely, the usefulness of this method of identification is limited.

Figure 4.

a. Waxy lump squashed out of the hypo-pleural organ of S. noctilio. The arrow indicates a clamp connexion on the mycelial thread trailing from the lump. Mag. 1750x.

b. Similar lumps with the edge of the matrix dissolved by xylene. Mag. 1750x.

FIG.4

Figure 5.

Eight consecutive horizontal sections taken near the center of the hypo-pleural organ of a large larva of S. noctilio.

a. A major pit sub-divided by a central partition.

b. The sub-division on the left is closing up. A third sub-division is forming on the right.

c & d. The closure of the lift sub-division is completed.

e. Another sub-division is forming on the right.

f. Three sub-divisions are of approximately equal size.

g. The left sub-division has closed up, the right sub-division is partially obscured.

h. The thin wall blocks off the entire pit.

FIG. 5

Figure 6.

a. Vertical longitudinal section of the hypopleural organ of a second instar larva of S. noctilio, showing eight major pits. The thick septa carry stout spines. Mag. 400x.

b. Vertical longitudinal section of the hypo-pleural organ of a large larva of S. noctilio showing twenty-five pits, most of them carrying fungus. Mag. 80x.

c. Vertical longitudinal section of the hypo-pleural organ of a pre-pupa of S. noctilio, showing the shrunken septa without any lumen. Mag. 160x.

FIG. 6

Figure 6d.

Longitudinal section of female larva of *S. noctilio* showing the main hypo-pleural organ and a smaller organ on the anterior segment.

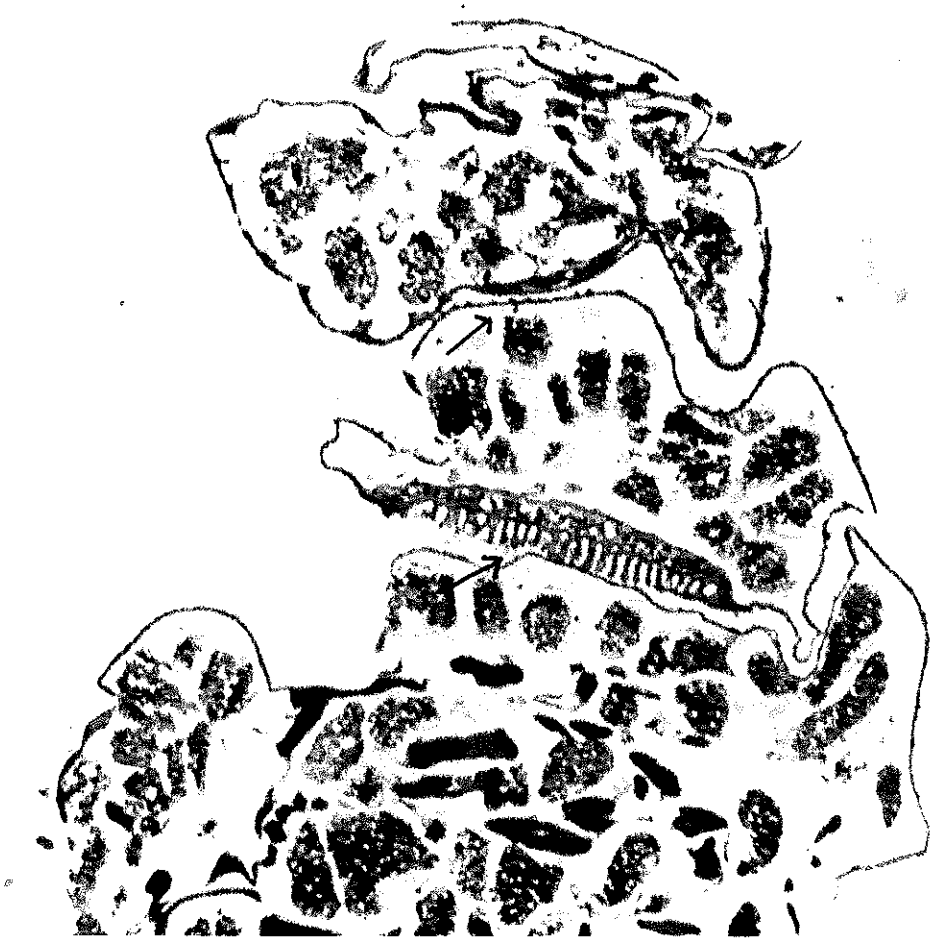


Table I indicates the variation in length/width ratio of the hypo-pleural organ of a few different sized larvae of S. noctilio.

TABLE I.

S. noctilio - measurements of hypo-pleural organ.

♀ larva	length	width	ratio	
1	4.1 units	0.9	4.5	from same small larva
2	4	0.85	5.1	from same small larva
3	8.4	1.2	7	
4	9.0	1.0	9	
5	5.5	0.9	6	
6	8.6	1.2	7.1	
7	7.6	1.2	6.3	

Gross appearance of the fungus.

Squashes of the organ, show the contents as waxy lumps, discoloured with a tangle of fungus. The wax readily colours with Sudan IV and Sudan Brown. After some of the wax has been dissolved away the fungus can be stained with Aniline Blue and Phloxine B. Fig. 4a, shows a strand of fungus with clamp connexions, extending from a waxy lump squeezed out of the organ.

On one occasion, the secretion within the pits came streaming out as oily droplets, and not as a firm lump.

In serial sections of the organ, from which all wax has been the removed, a few growing tips can sometimes be seen protruding from the tightly-coiled, mycelial balls. Fig. 6b.

2. As would be expected from the external appearance, in vertical longitudinal sections the organ resembles a comb, the

teeth of the comb being the septa between the pits (Fig. 6b). Horizontal longitudinal sections made during this study indicate that towards the centre of the organ, the pits usually have two internal partitions. (Fig. 5). There may be as many as four of these partitions. Parkin (1942) has shown that similar subdivisions of the major pits occur in S. cyaneus.

There is often considerable variation in the structure of the organs, not only from larva to larva, but also between the two organs on a single larva.

Stillwell (1965) described an additional pair of hypo-pleural organs in T. columba situated on the posterior fold of the metathorax. These organs were smaller than the principal pair on the first abdominal segment and were not as heavily infected with fungus. In only one of the seventeen female larvae of S. noctilio could an additional pair of hypo-pleural organs be detected. (Fig. 6d). It was also situated on the posterior fold of the metathorax. It is doubtful whether these smaller organs would be visible in external examinations, as they could be overlooked even in sections. There was no sign of fungus in the cavities which in these sections, did not appear to open to the exterior.

This discovery leads to the suggestion that perhaps there were once cuticular organs on all body segments of larval Siricidae. Although Yuasa (1923) lists the types of glands opening to the

exterior in larval Tenthredinoidea, he does not give details of structure which are required to develop this idea.

Serial sections of larvae about 3 mm long (presumably in their second instar) show that the hypo-pleural organ is already present at this early stage. Parkin (1942) was unable to find these organs either in sections or dissections until the larvae were $^{1}/4$ to $^{1}/3$ grown. He realised that the size of the organ and the number of pits forming it increased as the larva grew. The small larvae of S. noctilio showed only from 8-10 cavities (Fig. 6a). Moreover, the openings to the cavities are wider in proportion to their depth in the small larvae than they are in the older larvae. (Fig. 6b). No cultures were made from the hypo-pleural organs of these small larvae, but the contents took up the fungal stain Methyl Green.

Layers in the Cuticle.

The layers in the insect cuticle are clearly differentiated by Lower's Trichrome stain. The extremely thin epicuticle, when visible, is stained red, the exocuticle becomes pale yellow, the meso-cuticle turns orange and the endo-cuticle is coloured green. The cellular hypodermis, with orange nuclei is stained pale purple.

In sections of the hypo-pleural organ in the inter-moult condition prepared for examination under the light microscope and stained with Lower's Trichrome, the epicuticle could not be detected.

The exocuticle is absent from the cuticle of the hypo-pleural organ except for the tips of the spines. According to Lower (1964) the exocuticle is absent from the cuticle of most immature insects.

The meso-cuticle forming the bases of the spines and the outer visible layer of the cuticle stained strongly with Orange G.

The green endo-cuticle is the innermost layer of the cuticle. In the region of the hypo-pleural organ the purplish cells of the hypodermis are elongated, spindle-shaped. On histological grounds, it is likely that they are secretory cells.

Moulting.

From the description of the cuticle which reveals that there is no tanned or sclerotised layer (exocuticle) in the integument of the larva, it is to be expected that the pits of the hypo-pleural organ will collapse as the supporting endocuticle is digested away by the moulting fluid. Serial sections of a moulting pre-pupa show that the endocuticle, both within the septa of the hypo-pleural organ and in the cuticle generally, has been digested. Only the orange meso-cuticle remains. It is also evident from these sections that, as the sides of the septa have shrunk together, the external openings of the pits of the hypo-pleural organ have become wider (Fig. 6c).

Figure 7.

a. Hypo-pleural organ of S. noctilio on cuticle removed during the early stages of moulting and stained with Aniline Blue. Mag. 115x.

b. Seven coiled bundles found in the region of the hypo-pleural organ on a cast larval skin. Mag. 140x.

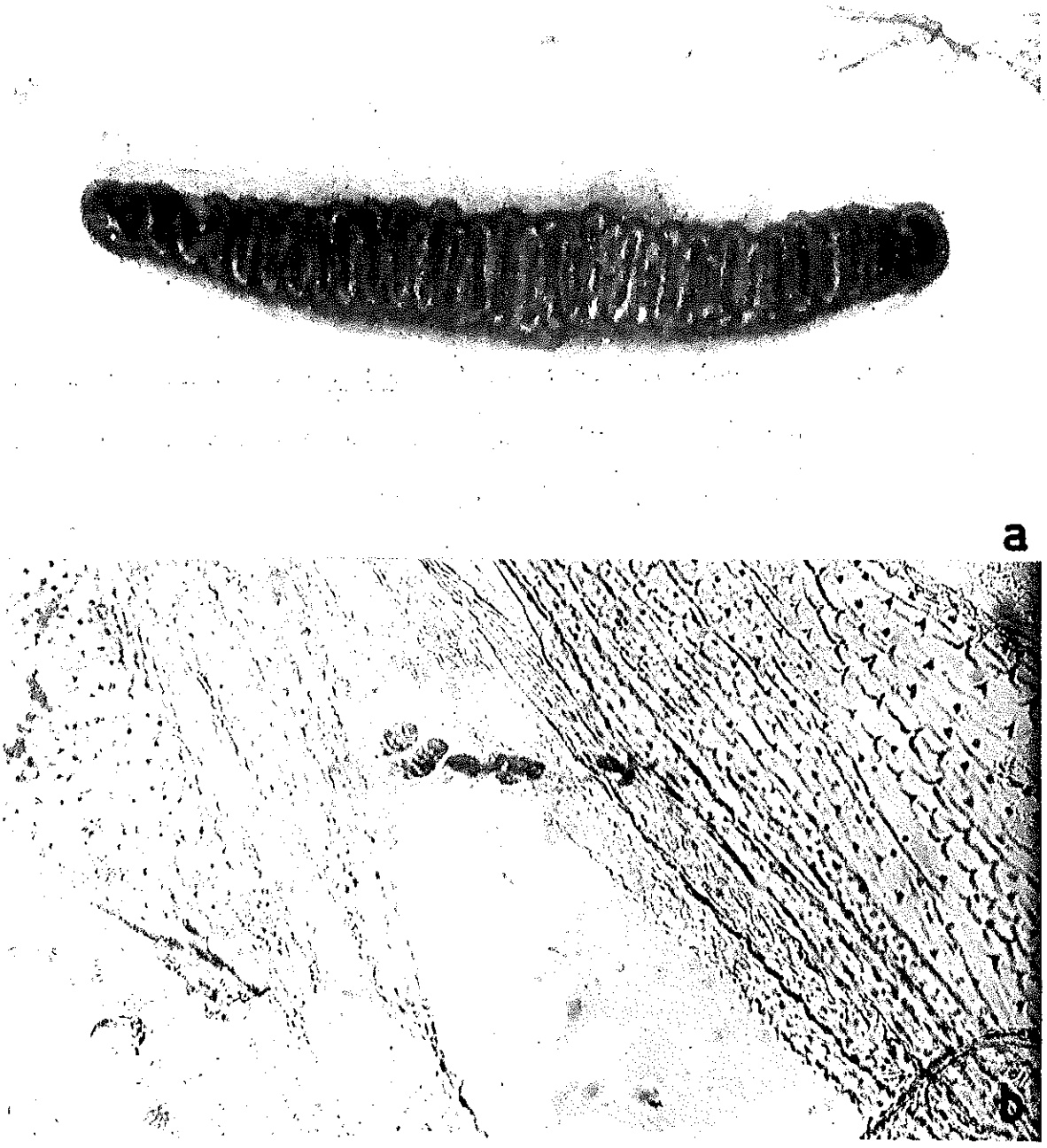


FIG. 7

Parkin (1942) first speculated about the changes which would take place in the hypo-pleural organ during moulting. He assumed that the cuticular pits which contain the fungus would be shed during a larval/larval moult, in this way breaking the association between the larva and the fungus at each instar. Observations were made during this study on a moulting larva preserved in formalin. These showed that his assumption was correct. In this larva the old cuticle had become detached in the region of the head, but had not yet ruptured, and was distended like a balloon. After excising one of the hypo-pleural organs it became apparent that the old cuticle could simply be lifted off. Fig. 7a shows this piece of cuticle with the pits of the hypo-pleural organ still clearly defined but without any surrounding glandular issue (Fig. 3b). The darkened coils of fungus are visible within the pits. It is suggested that this hypo-pleural organ had not collapsed because the moulting process was incomplete and therefore the septa of the pits were still supported by endocuticle.

On the soft cuticle of the new exoskeleton, the shallow cavities of the new organ could be seen. Measurements taken under high magnification showed that the new organ was slightly longer than the old one. The cavities appeared free of fungus and attempts to stain any contents with the fungal stain Aniline Blue were unsuccessful.

Fig. 7b presents further evidence that the association between larva and fungus is broken at each moult. It shows the torn remains of the hypo-pleural organ on a skin cast during a larval/larval moult. Seven tightly coiled bundles can be seen at one end of the organ. These bundles are of the same order of magnitude as the pits in the hypo-pleural organ, and could be the fungal contents of the organ.

Francke-Grosmann (1957) states that the wax packets contained within the pits are forced out of the hypo-pleural organ by the contraction of muscles at the time of the pre-pupal/pupal moult. No evidence can be produced from this study either to confirm or criticise her statement, but during the moulting process, the waxy contents of the pits of the hypo-pleural organ become exposed as the septa of the hypo-pleural organ shrink and collapse. A waxy ridge is clearly visible on the first abdominal segment of the quiescent, female pre-pupa. Only in moist, recently cast skins is it usual to find intact this ridge of wax packets, which flakes off at the slightest touch. (Fig. 8a). Fig. 8d shows a group of seven wax packets which was displaced from the sides of the first abdominal segment to the centre of the second abdominal segment of the exuviae by pressure applied to the coverslip used in mounting the specimen.

(c) Wax Packets.

1. Examinations of cast skins show that an elongate, elliptical area of short parallel wrinkles is often all that remains of the septa of the hypo-pleural organ. Sometimes a few pits near the tip of the organ are still clearly defined on the cast skin. (Fig. 8b & 8c).

2. Under high magnification the outer layer of the wax packets appears to be stratified (Fig. 8d and Fig. 9c). There are two possible interpretations of this observation. Either the stratifications represent numerous scales which will flake off the packets or they represent the progressive but discontinuous deposition of wax around a bundle of fungus.

3. Examinations of numerous wax packets, stained, unstained and after treatment with wax solvents, provide evidence to substantiate the second interpretation. (See Appendix I and Figure 10).

(ii) Female Reproductive System.

Francke-Grosmann (1939) described the comparative morphology of the ovipositors of X. spectrum, U. gigas and S. juvencus and illustrated the reproductive system and the inter-segmental sac of S. juvencus. The same general description applies to the morphology and anatomy of these structures in S. noctilio. However, a more precise knowledge of the structure of the ovipositor and accessory glands is required for an appreciation of later discussions regard-

Figure 11.

Ventral view of ovipositor of *S. noctilio* adult.

is	inter-segmental sacs
cg	club gland
Ivl	first valvula
VIIIs	eighth stermite (triangular plate)
IXt	ninth tergite (quadrate plate)
b	base of sheath (oblong plate)
ss	saw-sheath

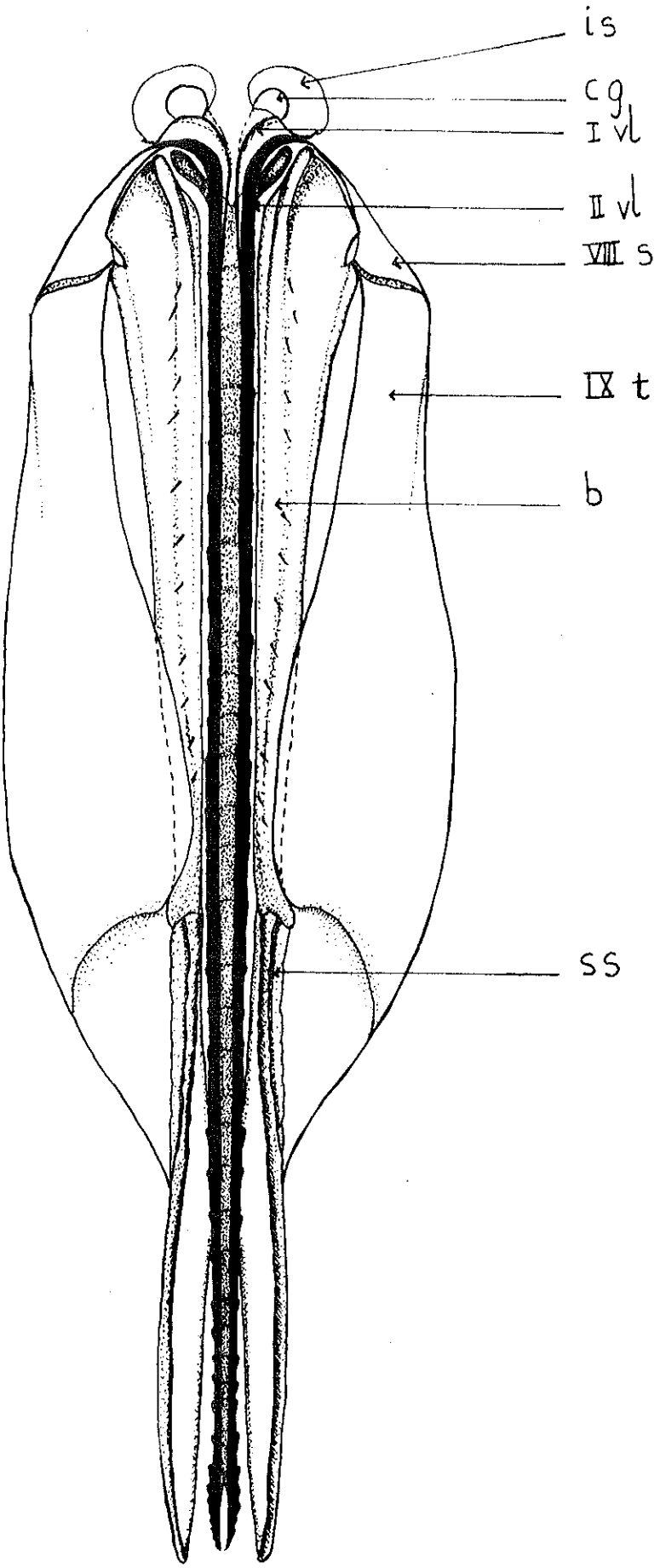

is
cg
I vl
II vl
VIII s
IX t
b
ss

Figure 12.

Lateral view of ovipositor of S. noctilio.

9t	ninth tergite
8s	eighth stermite
Ivl	first valvula, branched
is	inter-segmental sac
cg	club gland
Ilvl	second valvula

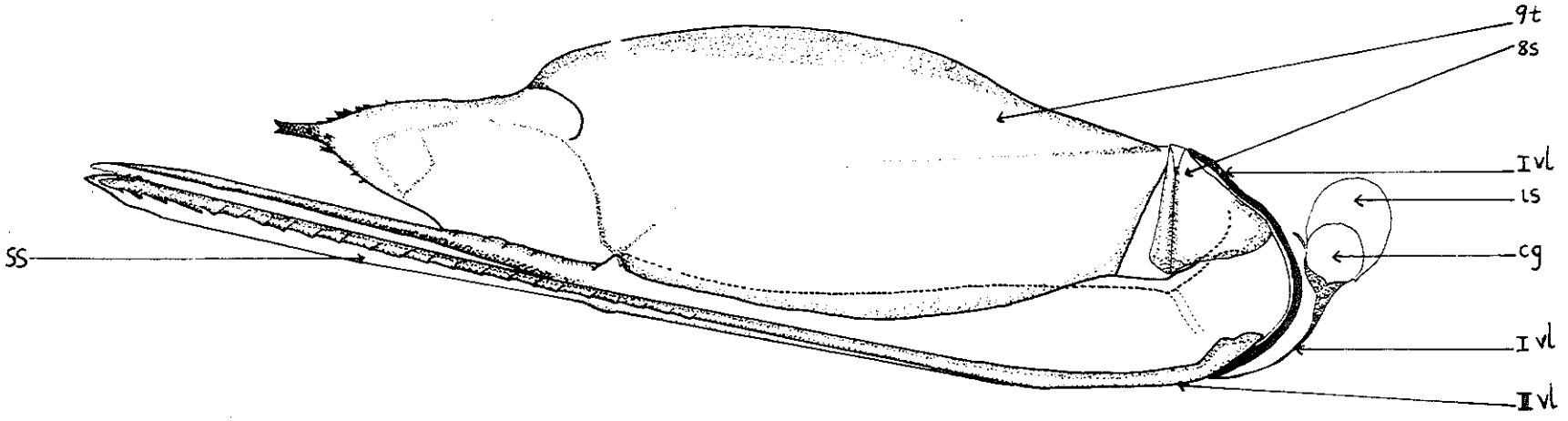

9t
8s
Ivl
ls
cg
I vl
I vl
ss

Figure 13.

The first valvula of the ovipositor of S. noctilio.

VIII st — eight stermite

is — inter-segmental sac

cg — club gland

Figure 14.

Section of the club gland and inter-segmental sac of a pupa of S. noctilio, showing the unicellular glands with stout ducts opening into the sac.

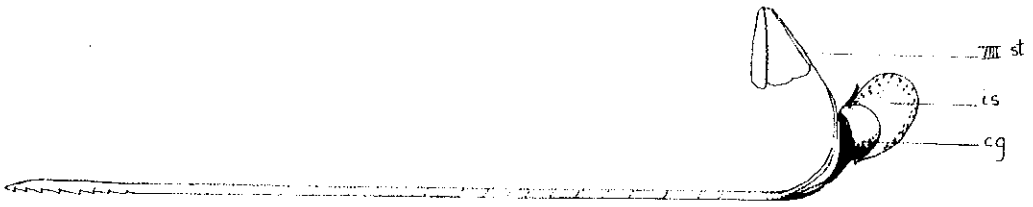


FIG. 13

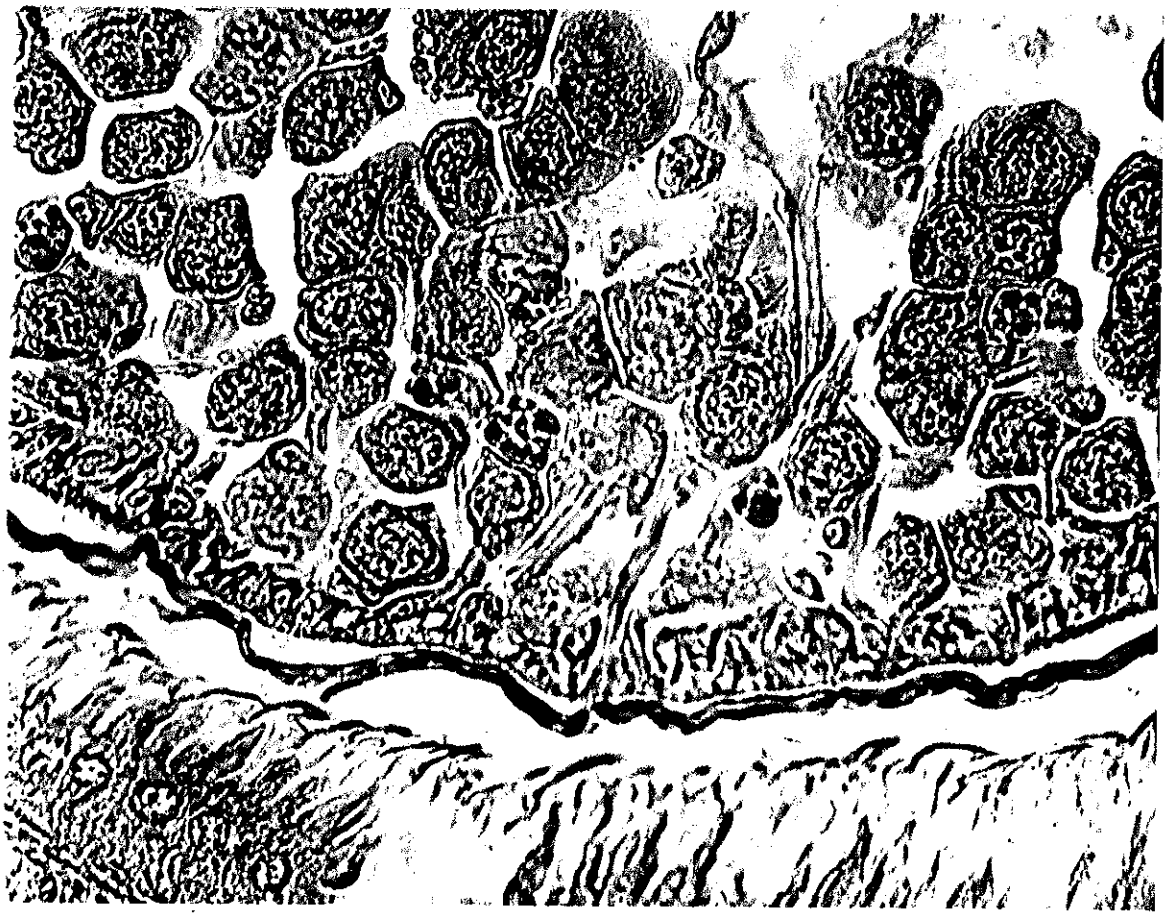


FIG. 14

Figure 8.

a. Glistening ridge of wax packets on the moist pre-pupal exuviae of S. noctilio. Mag. 72x.

b. The wrinkled remains of the hypo-pleural organ after the wax packets have sloughed off the pre-pupal exuviae. Two terminal pits are clearly defined. Mag. 56x.

c. Three terminal cavities is all that remains of the hypo-pleural organ on a cast pre-pupal exuviae. Mag. 400x.

d. A group of seven wax packets sloughed off the pre-pupal exuviae of S. noctilio and viewed end-on. Mag. 200x.

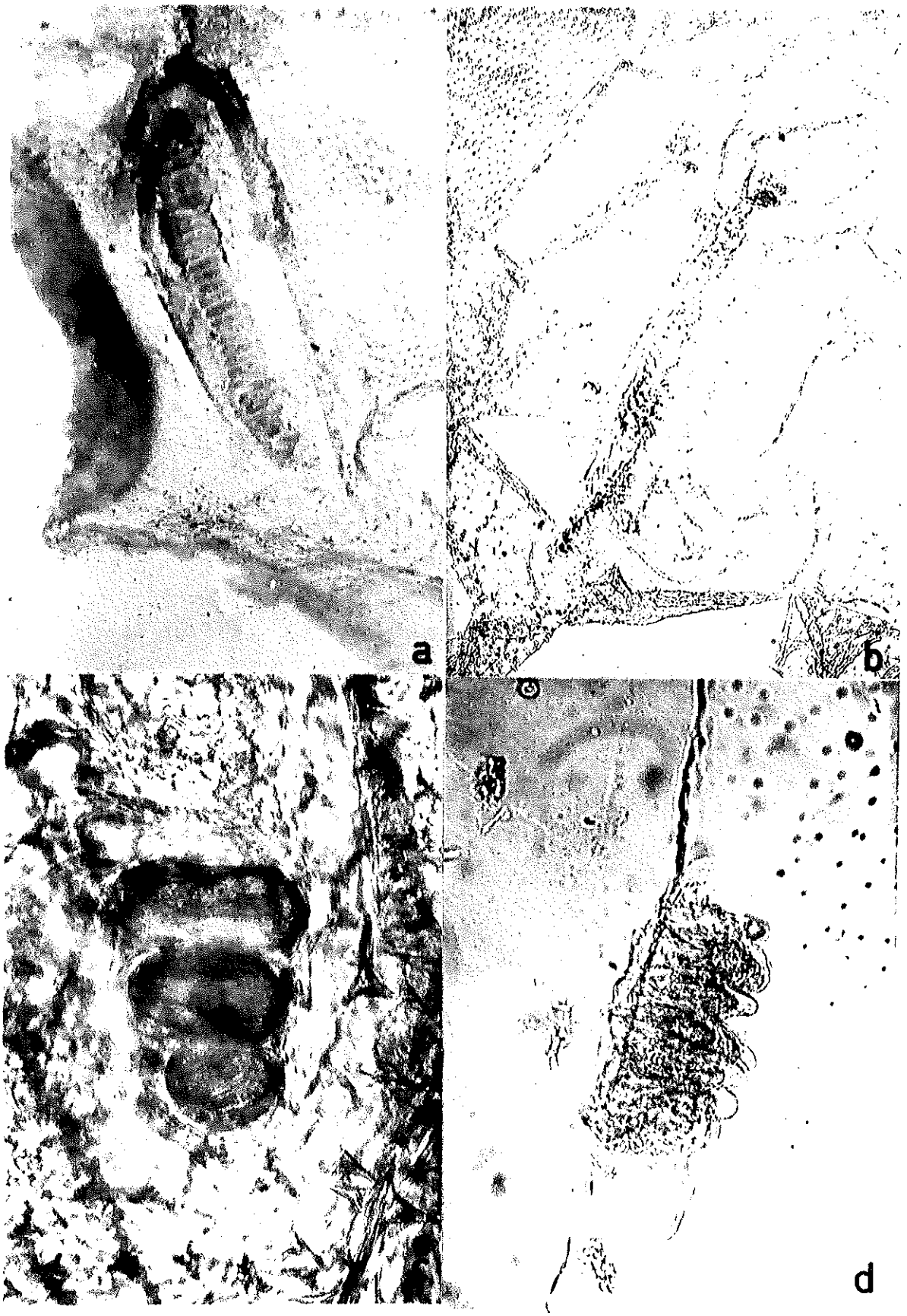


FIG. 8

Figure 9.

Three wax packets of S. noctilio viewed from the side to show the scalloped base with a thick coat of wax, and the flat top edge formed at the surface of the pit. Mag. 650x.

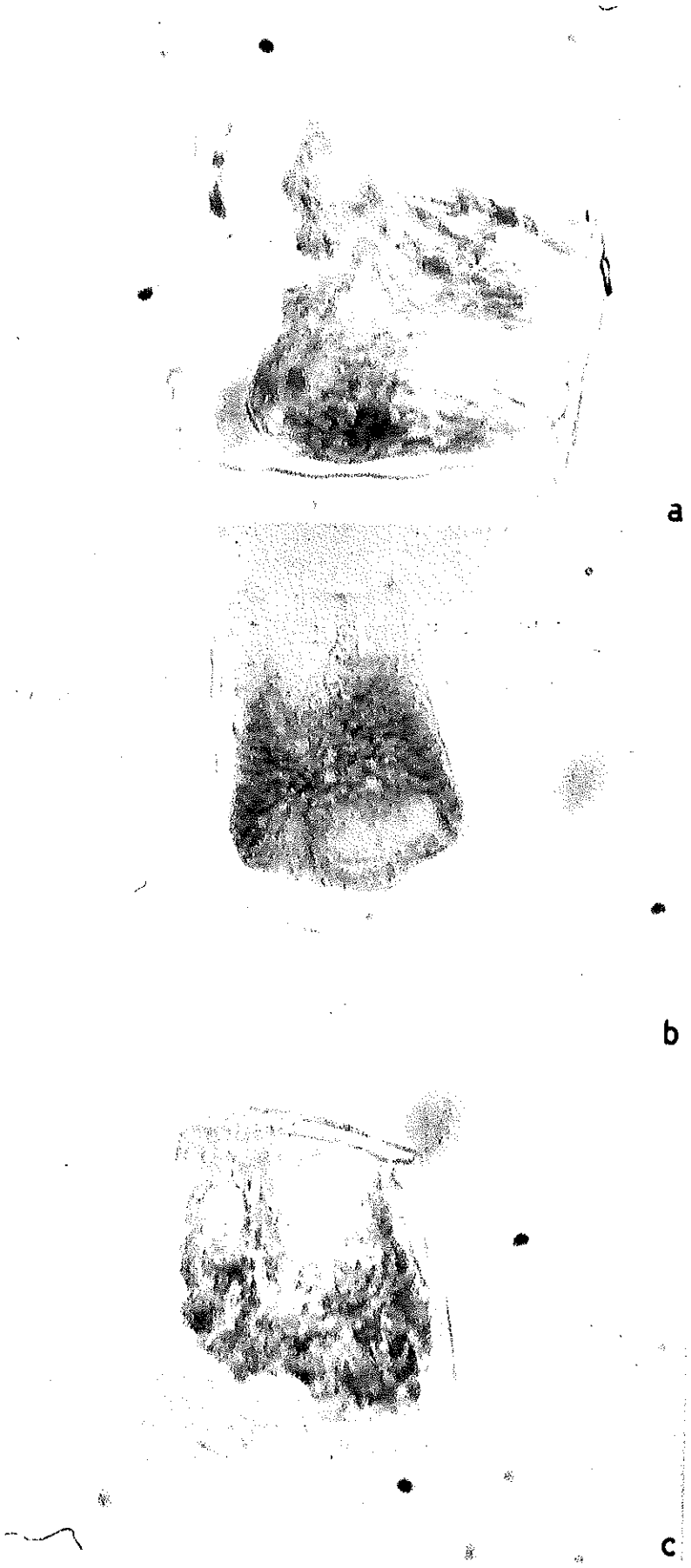


FIG. 9

Figure 10.

The fungal contents of the wax packets which have been treated with wax solvents.

c. mag. 200x

the rest mag. 1,000x.

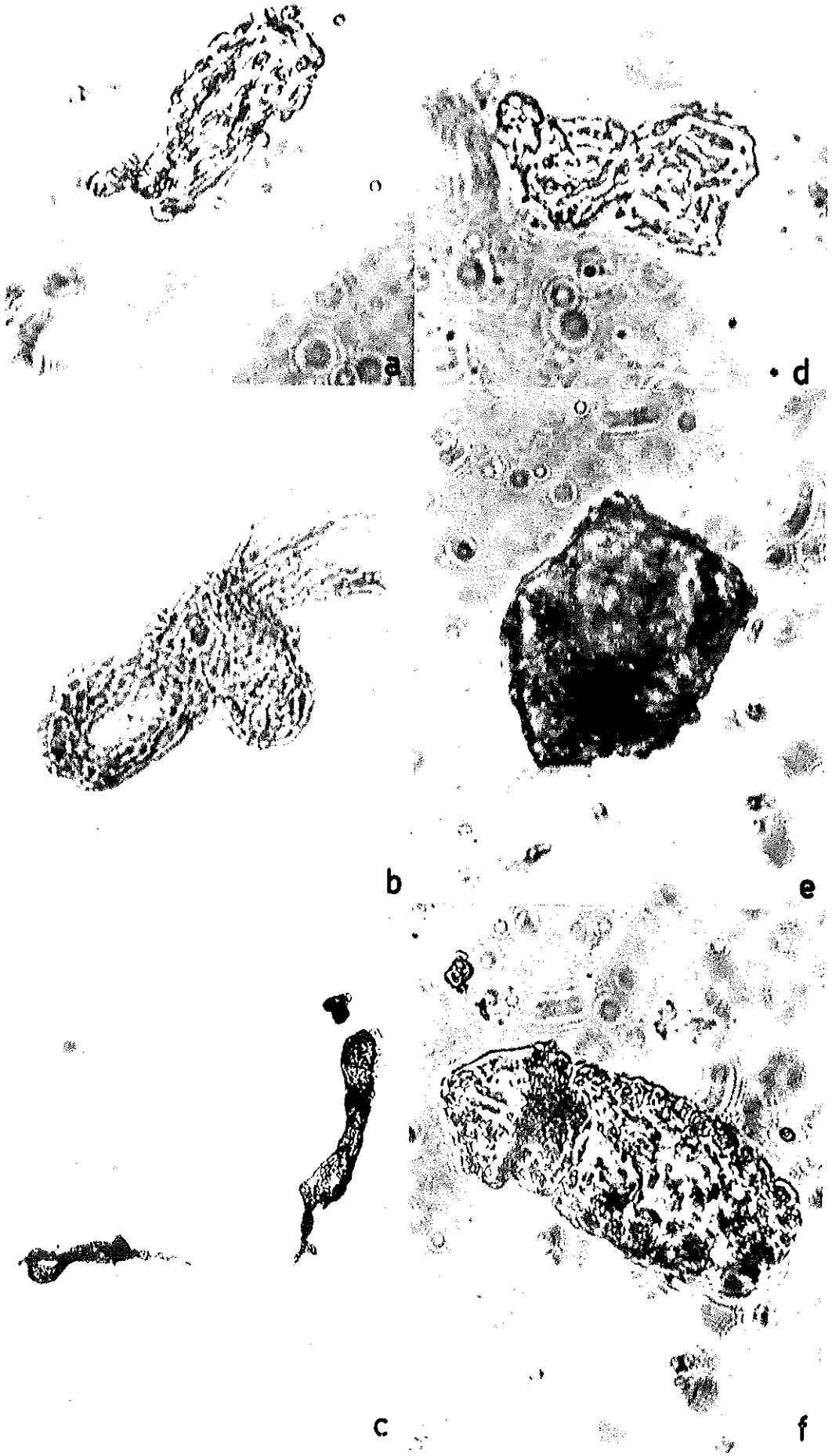


FIG. 10

Figure 15.

Ventral view of bases of first and second valvulae.

is	inter-segmental sac
cg	club gland
Ivl	first valvula
Ilvl	second valvula
VIIIst	eighth stermite

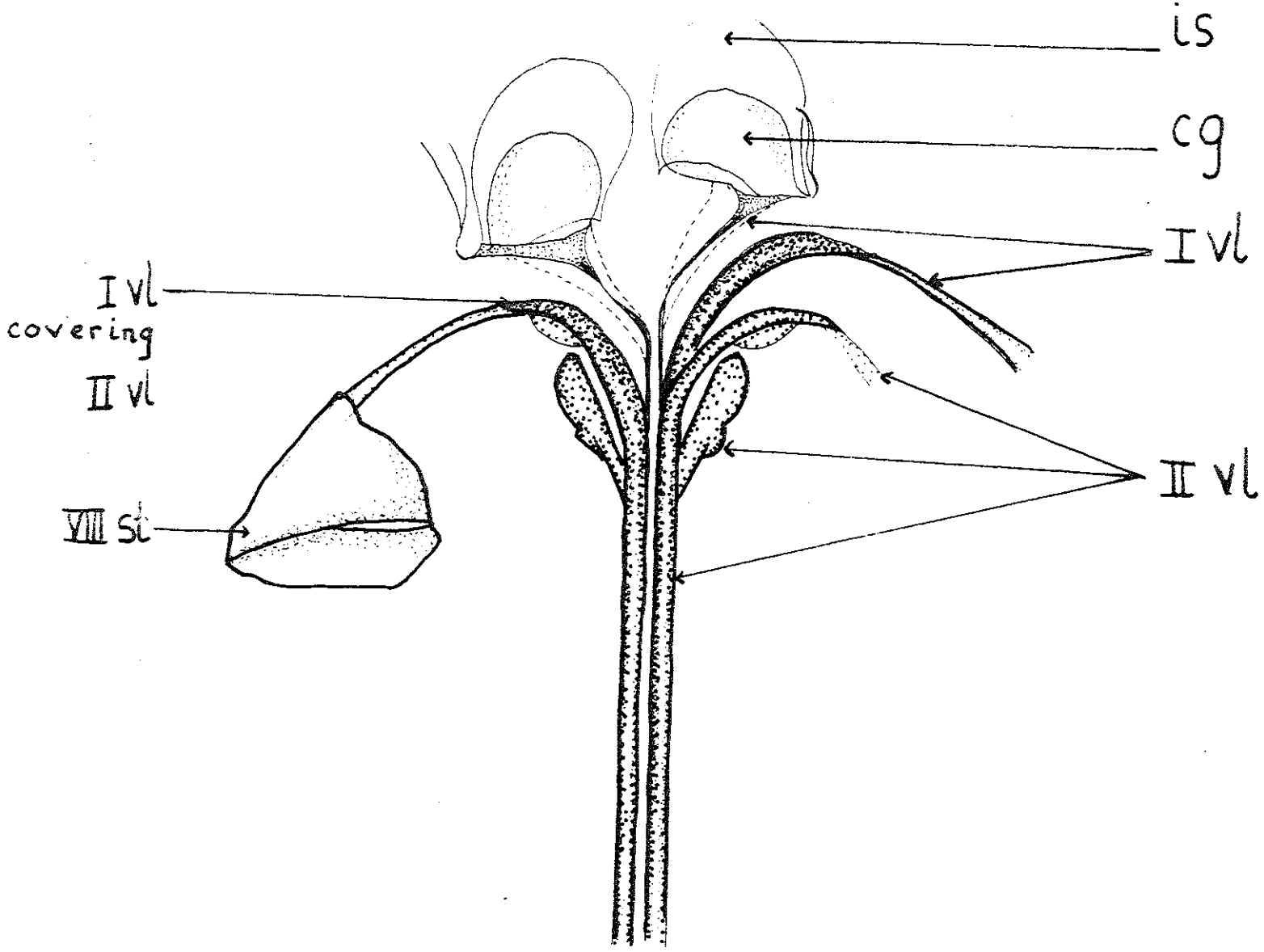
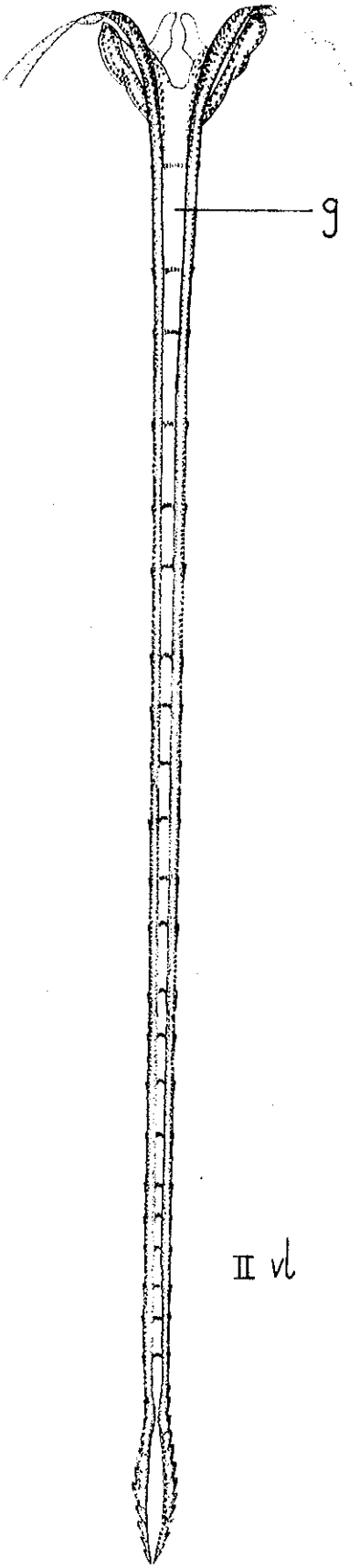

is
cg
I vl
I vl
covering
II vl
II vl
VIII st

Figure 16.

Ventral view of fused second pair of valvulae of S. noctilio.

g ventral groove



II vl

Figure 17.

Dorsal view of base of the fused second pair of valvulae and the inner surface of the base of the sheath.

b ridge on sheath base

p peg which articulates with second pair of valvulae.

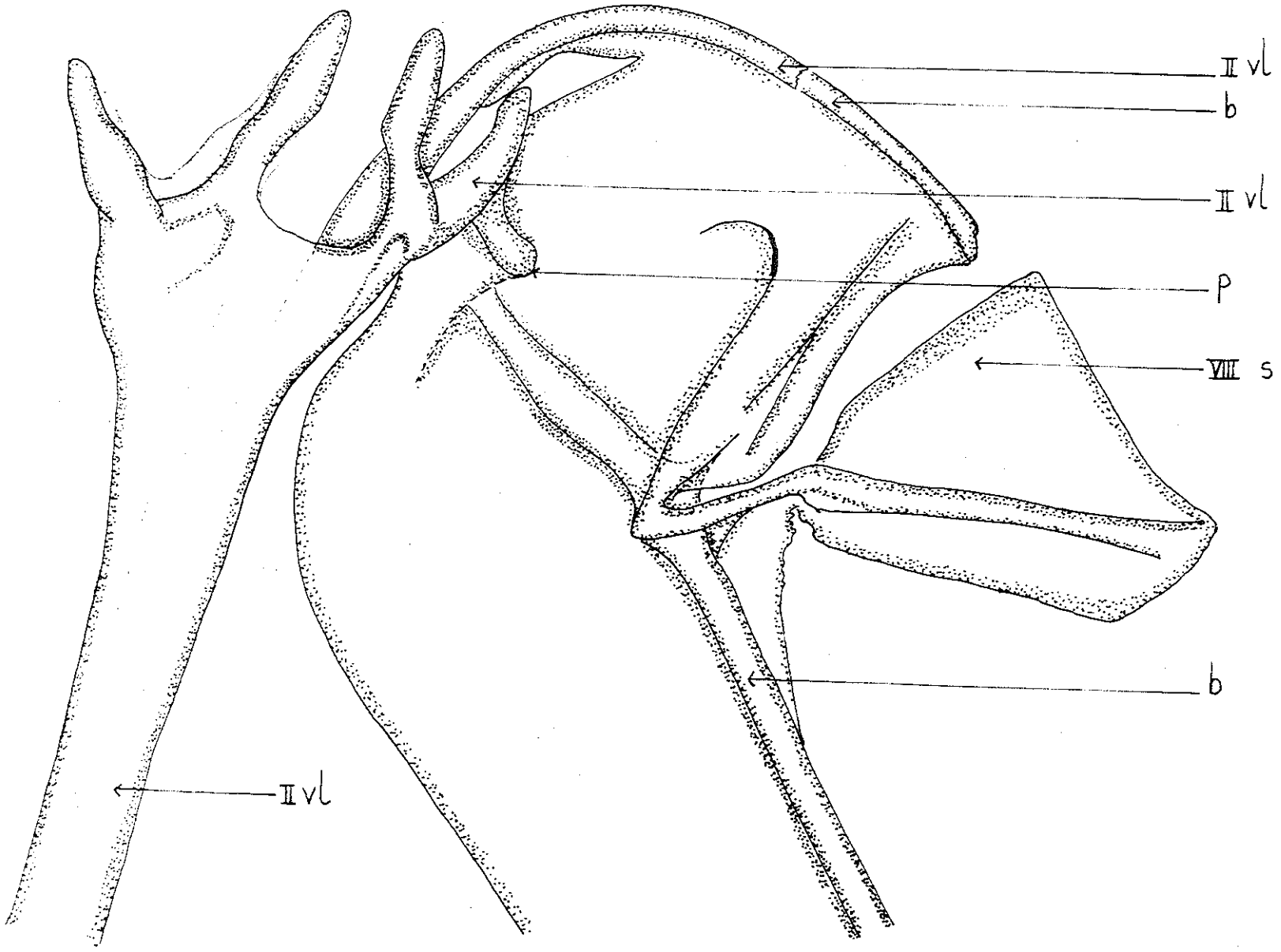

II vl
b
II vl
P
VIII s
b
II vl

Figure 18.

Inner surface of ovipositor sheath.

p peg.

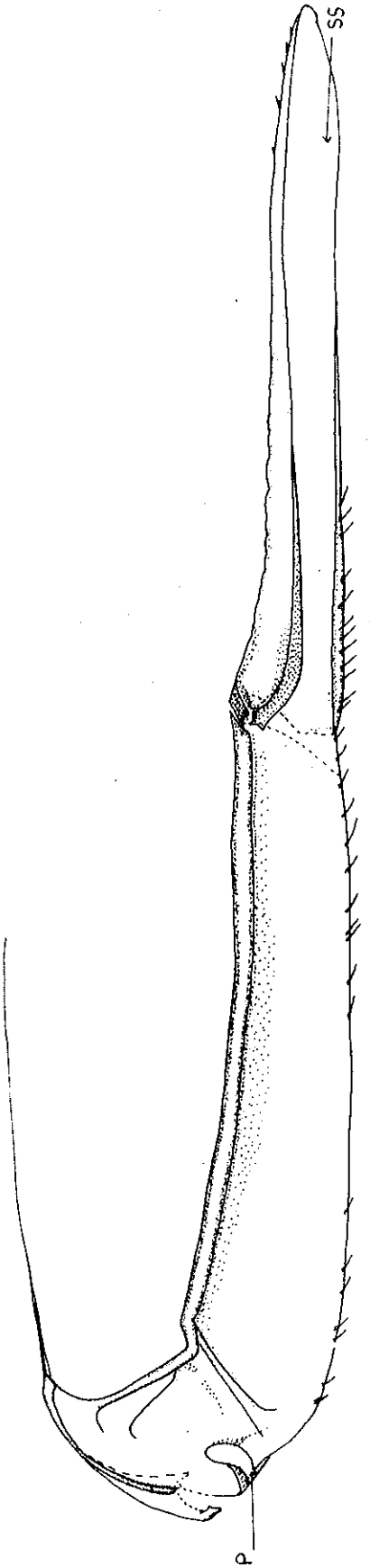

SS
P

Figure 19a.

Tracing made from a photograph of a longitudinal section of a female pupa of S. noctilio.

mu gl	mucous glands
oil	oil gland
sp	spermatheca
od	oviduct
is	inter-segmental sac
cg	club gland
VII s	seventh stermite (sub-genital plate)
I vl	first valvula

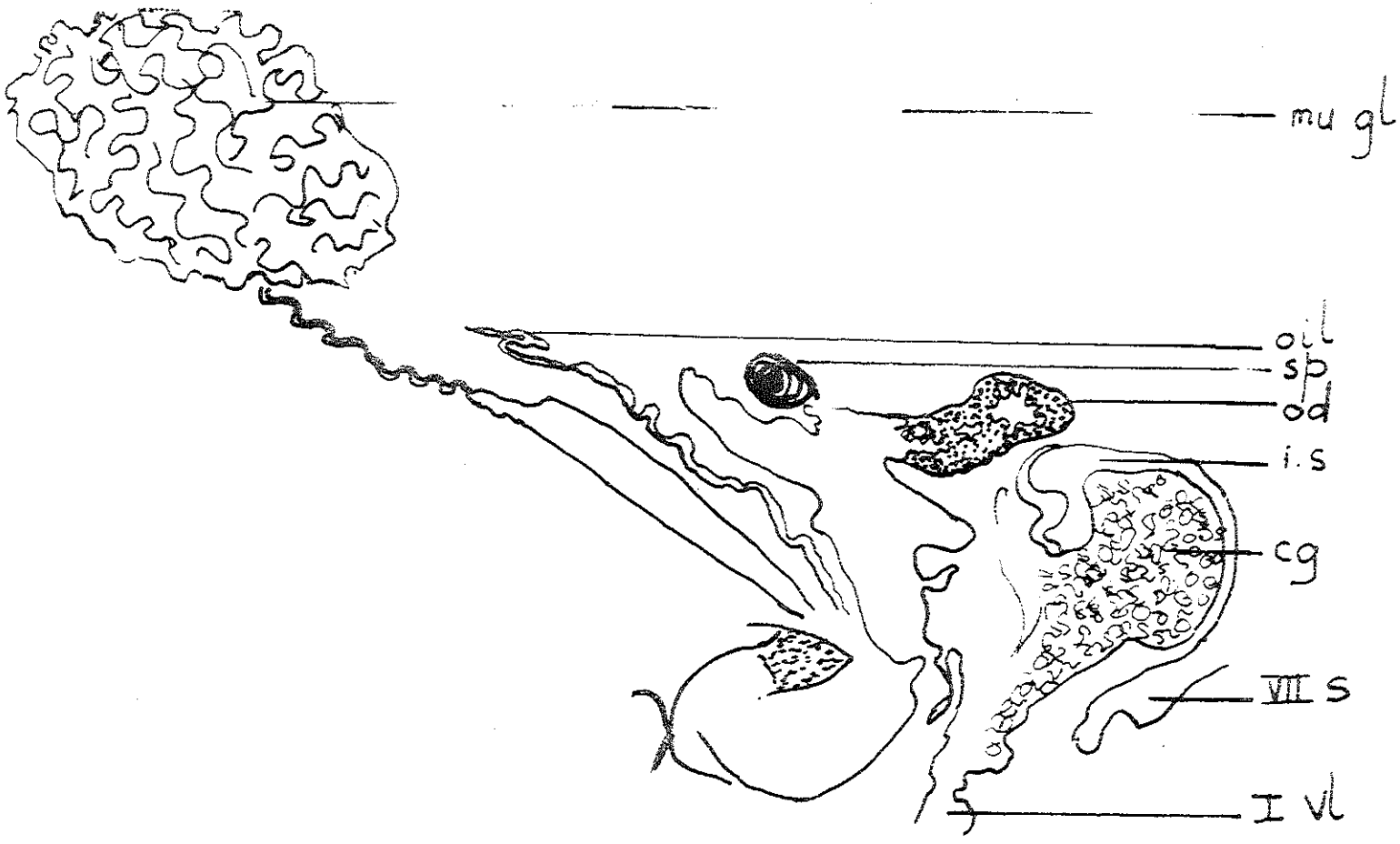

mu gl
oil
sp
od
i.s
cg
VII s
I vl

Figure 19b.

Lateral view of female reproductive system of an adult female of S. noctilio.

ov	ovary
oil	oil sac, distended
mu	mucous reservoir, distended
sp	spermatheca
is	inter-segmental sac, distended
cg	club gland
I vl	first valvula

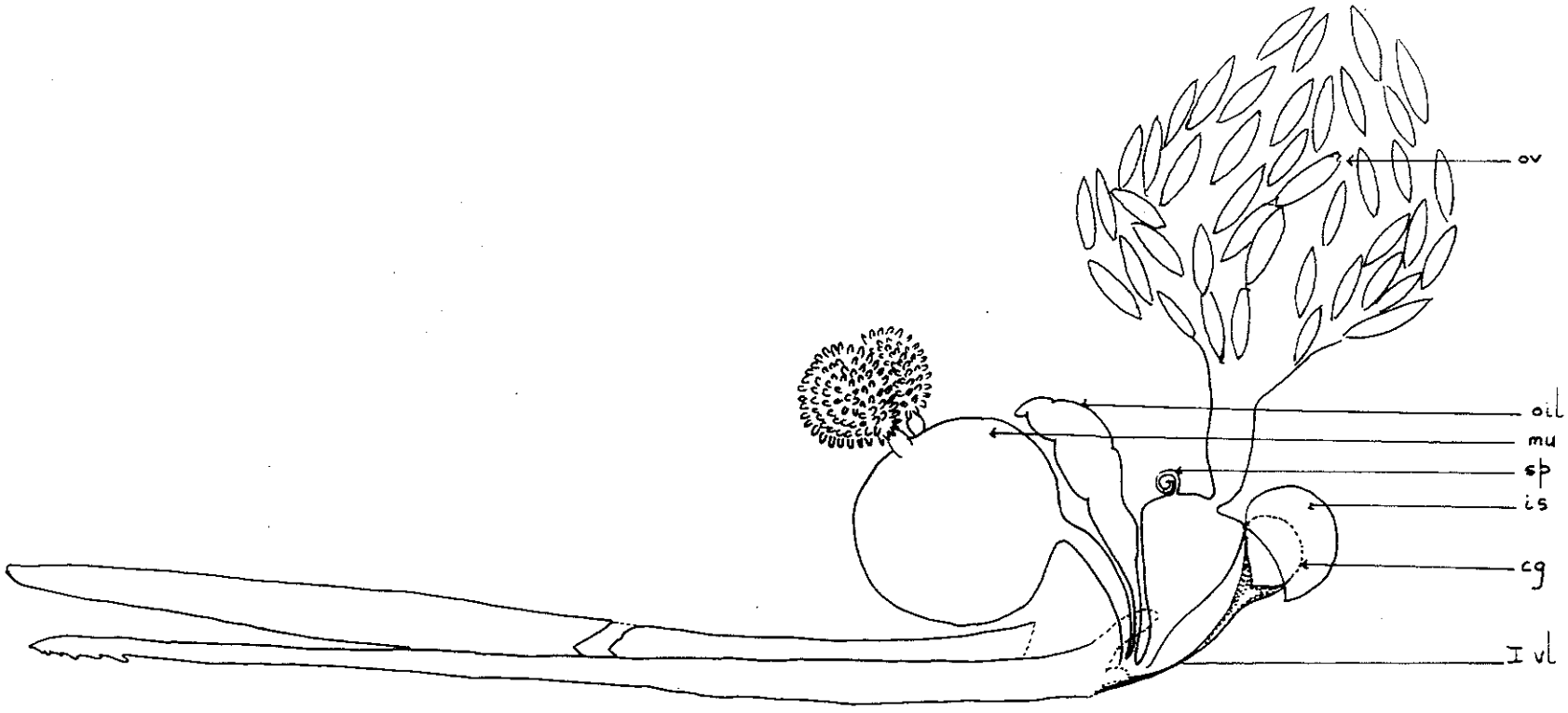
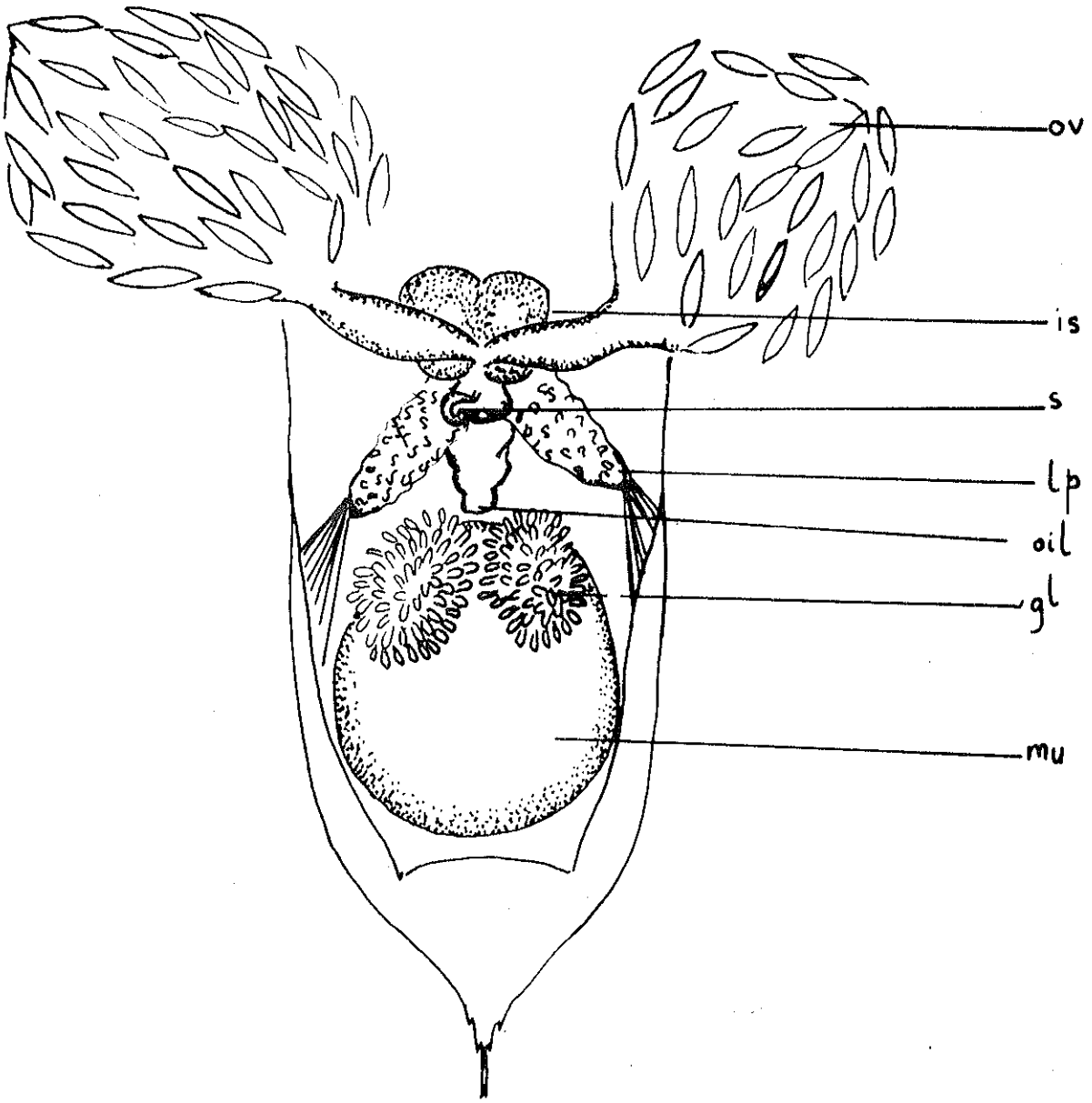

ov
oil
mu
sp
is
cg
I vl

Figure 19c.

Dorsal view of female reproductive system of adult *S. noctilio*.

ov	ovary
s	spermatheca
lp	lateral pouch
oil	oil sac
gl	mucous gland
mu	mucous reservoir


ov
is
s
lp
oil
gl
mu

ing the infection of the inter-segmental sacs.

As in all Hymenoptera, the ovipositor of S. noctilio is made up of three pairs of basal plates and three pairs of shafts or valvulae, but it is modified for boring tunnels in sapwood. (Figs. 11 & 12). The three pairs of basal plates support several compact sets of muscles on their inner face, and function as the motor apparatus of the ovipositor and its sheath. The basal plates of the ovipositor are derived from the exoskeleton of the eighth and ninth segments. The eighth sternite is displaced dorso-laterally and is modified to form the triangular plates on either side of the base of the ovipositor. The ninth tergite, (quadrate plate) covering most of the posterior region of the abdomen, is produced into the body cavity along its dorsal margin as a flat apodeme to which the muscles of moving the sheath, and the shafts of the ovipositor are attached. The base of the saw-sheath (oblong plate) is formed from the modified ninth sternite. These basal plates are shown in Figure 11 as VIIIs, IXt and b respectively.

The first and second pairs of valvulae are connected to the basal plates by curved arms. The first pair of valvulae is divided proximally into two arms. (Fig. 12). The outer arm curves outwards and upwards to articulate with the apex of the triangular plate. The inner arm curves upwards into the body cavity. Its swollen base or club organ is covered by a pouch of the inter-segmental

membrane, known as the inter-segmental sac, described in the introduction. Figure 14 illustrates the anatomy of the club organ, showing details of the unicellular glands within the club with long, wide ducts opening into the inter-segmental sac.

The second pair of valvulae is fused dorsally and curled under along its edges to form the two channels within which the first pair of valvulae slide back and forth as the female drills in the wood. (Fig. 15). Figure 16 shows the first valvulae of the left side pulled some distance out of the second valvulae. The curved arms of the second valvulae are continuous with the base of the saw-sheath. (Fig. 17). The saw-sheath and base encloses the drilling shafts of the ovipositor. (Fig. 18).

There are three pairs of accessory glands associated with the female reproductive system. Two pairs of these glands have ducts opening into the ovipositor. They are the paired bunches of finger-like glands on the dorsal surface of the mucilage reservoir, and the median sac-like gland, which is not depicted by Francke-Grosmann (1939) in her diagram of S. juvencus. It is suggested from these studies that the mucous reservoir and its glands are homologous with the poison sac and glands of the honeybee which also opens by a narrow neck into the swellings of the second pair of valvulae. According to Snodgrass (1956) the neck of the poison sac is kept open by folds in the chitinous lining

which form interrupted rings. Similar ridges can be seen lining the duct of the mucous reservoir in sections of the female pupa of S. noctilio in Figure 19b.

The oil sac lies loosely attached to the anterior wall of the mucous duct. It opens into the ovipositor at the point where the first pair of valvulae diverge. It appears that the oil sac of S. noctilio and the alkaline gland of the honey bee are homologous structures. During the pupal stage the looped thick walled oil sac of S. noctilio has an extremely narrow lumen. Snodgrass (1956) describes the alkaline gland of the honey bee as a thick walled convoluted tube opening directly into the base of the "bulb".

Figure 19a and Figure 19b show the differences in the relative sizes of the reservoirs of these two pairs of accessory glands of S. noctilio during the pupal and adult stage. The mucous reservoir of the adult is an enormous, spherical sac, whereas in the pupa it is difficult to detect it connecting the conspicuous finger-glands and the clearly-defined mucous duct. The oil sac changes from a thick-walled looped tube with a narrow lumen in the pupa to a turgid, distended conical sac in the mature adult.

A pair of lateral pouches appears to arise from the membranous posterior wall of the vagina of S. noctilio. These pouches extend dorsolaterally towards the point of articulation of 8S and 9T.

Figure 20.

Spermatophores of S. noctilio obtained from the upper vas deferens.

Mag. 100x
480x
1400x

FIG. 20

They are attached by strong muscles to the dorsal apodeme of 9T and to the inner surface of 8S. (Fig. 19c). The surface of the pouches is covered in a mass of white cells which are thought to be homologous with the lubricating glands of the honey bee. Koschevnikov (1900) who discovered these glands in the honey bee, assumed that their secretions lubricated the basal plates of the sting. The glands are unicellular, each opening by a duct into a pouch of the membrane between 8T and 9T.

Apart from the accessory glands already described, the female reproductive system is made up of large paired ovaries occupying most of first six segments of the abdomen. The short paired oviducts and the even shorter common oviduct lead into the vagina through a T-shaped opening. Chitinous rings support the walls of the oviduct. The anterior and dorsal surfaces of the vagina are thick and firm, whereas the posterior wall is thin and membranous. The narrow coiled spermatheca is situated on the dorsal wall of the vagina. During the pupal stage, the spermatheca has virtually no lumen and appears in sections as a tightly coiled ball (Fig. 19b). Saline squashes of the spermatheca of the mated female contain spermatophores, innumerable discarded tails and spermatozoa.

Spermatophores similar to those found in the spermatheca can be obtained by pricking the uncoiled section of the vas deferens of the adult male.

After 30 seconds fixation in osmium vapour, the heads of the spermatozoa appear as dark specks clustered inside the dome-shaped gelatinous capsule of the spermatophore. The tails are spotted with dark granules. (Fig. 20)

V. SECRETIONS OF THE ACCESSORY GLANDS

(i) Oil.

The transparent colourless contents of the median glandular sac formed discrete drops on the glass slide. When frozen, the oily secretion became cloudy. Innumerable needle-like, crystals visible in a beam of polarised transmitted light, appeared to be the cause of this cloudiness. Some crystals remained when the oil regained room temperature and the cloudiness disappeared.

Droplets of the secretion were coloured by Oil Blue N and Sudan IV showing that they contained lipids. Threads pulled from a lump of mucus did not stick to slides which has been smeared with the oily secretion. The secretion gave an acidic reaction when tested with drops of 0.2% Nile Blue. When thin layer chromatograms of the oily secretion were treated with iodine vapours to reveal lipids, five distinct spots, became visible, with an additional faint spot near the solvent front. The spot lying second from the origin was much larger and darker than the others, indicating that there is a single major component in the oil.

As this major component appeared to have the same rf as cholesterol, further slides were developed in $SbCl_3$ to test for steroids. Only the standard reacted with this reagent, so

probably there are no steroids in the oily secretion.

Chromatograms were also developed in 2'7' Di-chlorofluso-cein to test for non-polar lipids. Under U/V light, four fluore-scent green spots appeared indicating the presence of both sat-urated and unsaturated non-polar lipids. In chromatograms freshly sprayed with Bromo-Thymol-Blue, five yellow spots appeared against a place blue background, showing that the oily secretion contains at least five fatty acids.

In laboratory tests, threads of mucus harden within minutes on exposure to the air. Adult females of Sirex noctilio oviposit over a period of a fortnight without any apparent trouble from hardening mucus. Dissected shafts of the ovipositor glisten with oil and threads of mucus did not stick to glass slides which had been smeared in oil. Possibly the oily secretion acts as a non-stick lubricant for the moving shafts of the ovipositor.

It seems likely that this film of oil keeps the inner sur-faces of the ovipositor free from contamination. Wax packets kept for a month in drops of the oil secretion showed no signs of surface contamination. When transferred from the oil to plates of agar, several packets developed surface contaminants, mainly yeasts.

The presence of at least five fatty acids in this secre-

tion suggests that it might supply nutrients for the fungus inoculated into the sapwood as well as for any crops of fungus associated with the ovipositor. Gaut (pers. comm.) has shown that in the presence of unsaturated fatty acids supplied experimentally as nutrients, the rate of growth of cultures of Amylostereum spp. increases as the C-chain length of the acids increases from 10 to 18. However, the intensity of the spots of the chromatograms revealed in iodine vapour, indicates that the fatty acids in the oily secretion of the woodwasp are probably saturated, see Stahl (1965) p. 150.

(ii) Mucus.

The mucus is clear and colourless. It turns deep amber in dead specimens. The firm ball of mucus can be drawn out into sticky threads which become brittle within a few minutes.

These threads can take up water rapidly and become sticky again. A ball of mucus takes up water slowly, however, Thus, two hours after an equal volume of borate buffer pH 10 had been added to it, it was still firm, although all the buffer was absorbed overnight.

A thin smear of mucus on a slide disappeared within twenty minutes in 0.1N KOH. The smooth shiny surface of the gel became fluffy after 2 hours in 8M urea, indicating that hydrogen bonds

were being broken down. The gel dispersed rapidly with some frothing in 6N HCl.

When tested with Millon's reagent, which reacts with Tyrosine, the mucus gave a positive reaction by turning orange. These results indicated that proteins might also be present.

The PAS test for carbohydrates was inconclusive. Once the fixed mucus had been oxidised with periodic acid, it reacted with fuchsin to produce a dense purple-pink colour, possibly indicating that carbohydrate was present. As the reaction with periodic acid was not blocked by acetylation, it was not possible to confirm this result.

When testing for lipids with a saturated solution of Sudan Black B in 70% alcohol, the mucus became black. Nevertheless, this colouring did not wash out in an acetone rinse, and thus the result could not be attributed to the presence of lipids. It seemed likely that the secretion contained protein capable of staining with sudan black B.

Using both the standard method of Pearce and the method of Kramer and Windrum, listed by Pearce, the mucus was tested with toluidine Blue for metachromasia. The mucus was not fixed. In both cases the mucus became blue (β-metachromasia). According to Pearce acid mucopolysaccharides should turn pink.

When the mucus was treated with 0.0004M methylene blue buffered at pH 2.6 it was able to bind the dye even at this pH, showing that it was strongly basophilic,

When tested with Alcian Blue, the mucus turned clear blue-green, which is the colour reaction shown by acid mucopolysaccharides.

Both mucus that had been incubated with hyaluronidase, and a control piece that had been submerged in 0.85% saline, turned blue when stained for twenty minutes in 0.5% Toluidine Blue. Half an hour later, the colour in the blob of mucus treated with hyaluronidase had vanished. Hence the hyaluronidase had affected the reaction between the mucus and toluidine blue. Possibly, this could be explained as the hydrolysis of some of the hyaluronic acid in the mucus by the hyaluronidase.

Although unsatisfactory results were obtained using PAS and Toluidine Blue and inconclusive results were available from the test with hyaluronidase, the results obtained using Alcian blue, Methylene blue, Sudan Black B and Millon's reagent, support the provisional identification of the mucus as acid mucopolysaccharide protein complex which is corroborated by the results from the chromatograms of the acid hydrolysate.

The paper chromatograms of the acid hydrolysate of the mucus

were developed with four reagents. Attempts were not made to determine precise Rf values, nor to identify components unequivocally, but the chemical nature of the compounds in the hydrolysate was investigated and standards were used to verify the colour reactions produced by the reagents.

Two bands in the chromatograms of the hydrolysate developed with phenol, water, reacted with ninhydrin, showing the presence of amine groups in the hydrolysate, and corroborating the indication of protein obtained with Millon's reagent.

When the solvent system methyl ethyl ketone: Proprionic acid:Water (60+20+20) was used, eight bands in the chromatograms reacted with ninhydrin.

When chromatograms of the hydrolysate were developed in phenol, water, and subsequently were dipped in $AgNO_3$ for detecting sugars, five black bands were revealed.

A single orange band appeared on experimental chromatograms sprayed with Acetylacetone - Dimethylaminobenzaldehyde reagent, showing the presence of hexosamines in the hydrolysate.

Three bands in the experimental chromatograms gave the same colour reactions with p-anisidine HCl as the standards glucuronic and galacturonic acid.

Taken together, these results indicate that the mucus is a protein-carbohydrate conjugate. A further test for sulphate groups was carried out on the hydrolysate. After adding a few drops of $BaCl_2$ to the hydrolysate, a beam of light was transmitted through it. The fine white precipitate confirmed the presence of sulphate groups.

The presence of proteins, uronic acids, hexosamines, oligosaccharides and sulphate groups in the mucus, is consistent with its being an acid micopolysaccharide-protein complex.

King (1966) mentioned that the germination of the arthrospores must be rapid, as two days after oviposition she could find no trace of them near the eggs. It has been assumed that the mucus is a source of nutrient for the fungal oidia inoculated into the sapwood by woodwasps. Tests carried out in Section VI substantitate this assumption.

VI. CULTURING

(a) Cultures from the larval gut.

Attempts to culture Amylostereum from the hind gut of female larvae were unsuccessful. Clarke (1933) mentions that he dissected three female larvae under sterile water when removing sections of the gut for culturing whereas the larvae in this study were cut up dry. As the larval cuticle is not sterile, his results must be considered suspect. His cultures could easily have been contaminated with scrapings of fungus from the tunnel.

Yeasts were cultured from fore, mid and hind-gut of both male and female larvae, in all but a five of the larvae. Diplodia and Trichoderma were obtained from these remaining larvae, indicating that these yeasts and fungi are not digested by the gut fluids.

(b) Cultures from the hypo-pleural organ.

Pure cultures of Amylostereum were obtained from three excised organs. These organs, together with the underlying agar and the mycelium were cleared and mounted. The mycelium could be seen growing out of the pits. There were numerous cystidia near the margin of the cultures, confirming the identity of the fungus (see Talbot, 1964).

In two instances the excised organs were over run with Trichoderma, and the cultures were discarded. Although a third organ

was also contaminated, threads of a white mycelium with clamp connexions could also be detected.

(c) Cultures made from larval tunnels.

Of the 108 slivers of wood shaved off the surface of larval tunnels, only seven produced pure cultures of the symbiotic fungus. The rest were contaminated with either Diplodia or Trichoderma. Sub-cultures could have been made from the stray threads of basidiomycete fungus sprouting from the surface of the wood, however, King (1964) had already made many isolates of the symbiotic fungus from the tunnels.

(ii) a). The dense white mycelium lining one of the pupal chambers created the impression that the inter-segmental sacs could readily be infected by the invasion of fungus from the surrounding growth. Cultures were taken from the wood in an attempt to identify the fungus. These were over-run with contaminants but a stained and mounted sliver of wood showed a basidiomycete fungus growing among the contaminants.

Further information was obtained from the cast pre-pupal skin found in another pupal chamber.

On one side of the skin, a series of dark patches were apparent in the characteristically elliptically shaped region of the hypo-pleural organ. Unlike the usual glistening ridge of wax packets, these dark patches had a crumbly appearance, and were separated from each other by regularly arranged transverse folds reminiscent of collapsed septa. Threads of a clamp fungus were separated from fragments taken from these patches. Examination of the stained mount made of this culture showed that the mycelial threads originated from the dark patches. Scattered over this skin were numerous tiny scrapings of wood which were encrustered with the mycelium a basidiomycete fungus. See Fig. 22a. Cultures made from these scrapings developed cystidia, indicating that the fungus was Amylostereum sp.

(ii) b. Ovipositor and inter-segmental sacs.

No fungus could be cultured from the inter-segmental sacs of any pupae. The youngest adults used during these experiments were newly moulted and therefore soft and brown. Attempts at culturing the symbiotic fungus from the inter-segmental sacs were unsuccessful. The symbiotic fungus could sometimes be cultures from the external

surface of the body. In one case the shafts of the ovipositor gave a positive result.

Adults which had blackened but not started boring out of the pupal chamber gave similar results, except that the fungus was not obtained in cultures made from the shafts of the ovipositor.

In these females, the abdomen was packed with fat body, the white inter-segmental sacs contained a few droplets of oil, and the accessory glands showed slight secretory activity.

Detailed results were obtained from a female which had started boring. Separate cultures were made of the inter-segmental sacs, the club glands, the tip of the abdomen, the ovipositor sheath, the distal ends of the ovipositor, the bases of the first pair of valvulae, the surface of the frass and the wood lining the pupal chamber. Five days later, the only development was the contaminants growing from the wood. By the 12th day a tuft of fungal threads with damp connexions could be seen growing from the tips of the valvulae. Cultures of the symbiotic fungus were obtained from the tip of the abdomen. After three weeks there was still no sign of any growth from the other parts of the ovipositor and sacs.

Cultures made from the sacs and ovipositor shafts of females which were chewing through the reddish bark, from females with their heads visible within the emergence holes and from females

Figure 21.

Germinating wax packets of *S. noctilio*.

a. Mag. 120x.

b. Fungal threads are growing from the side damaged with a sharp needle. Mag. 650x.

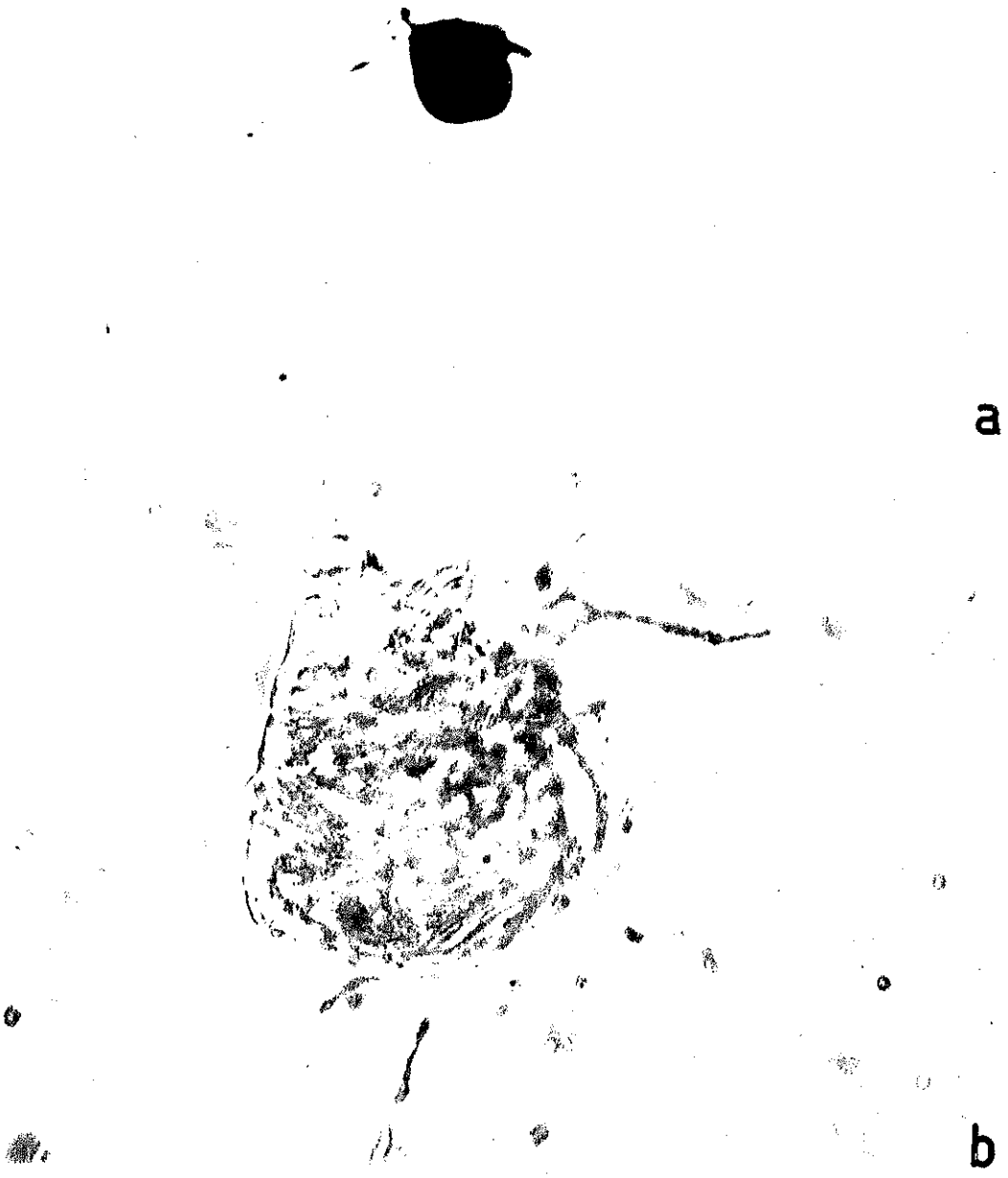


FIG. 2 1

Figure 22a.

Fungus growing on scrapings of wood in pupal chamber.

Figure 22b.

Loose fungal threads found in inter-segmental sacs of adult female which had started boring.

FIG.22

Figure 23.

Experiments to show the effect of the secretions of the female accessory glands on the vegetative growth of the fungus.

a. Corona around isolate after three days on Water Agar.

b & c. Controls.

d. Effect of mucus on the vegetative growth of the fungus.

e. Effect of oil on the vegetative growth of the fungus.

f. Effect of a mixture of oil and mucus on the vegetative growth of the fungus.

FIG. 23

which had emerged, all developed the symbiotic fungus. Unlike the results obtained from the teneral adults, no fungus could be cultured from the surface of head, thorax or abdomen. This suggests that they were tunnelling faster than the fungus was advancing, so reducing the surface contamination.

Females near the bark carried dense balls of fungus in their distended sacs. Only oidia were found in the sacs of emerged females.

(d) Wax packets.

None of the ten undamaged wax packets germinated on plates of agar. A week later, five of these packets were pricked with a needle. Within twenty-four hours they appeared to be germinating. Two days later the mycelium with clamp connexions were clearly evident.

(iv) a) As shown in Fig. 23d, e, f both the mucus secretion and the mixture of oil with mucus caused an increase in the vegetative growth of the fungus. Similar results were obtained using only the oily secretion.

The entire semi-circle of mycelium near the secretion was affected. The increase in vegetative growth was apparent before the growing front of the mycelium reached the mucus secretion. The possibility that volatile components of the mucus were affecting the fungus seems unlikely, even if the volatile substances were

being released continuously. During the course of the experiment, the mucus adsorbed water from the agar and became slacker and it seems more likely that some fraction of the mucus was adsorbed onto the agar, causing this reaction.

(b) In one experiment using wax packets obtained from recently shed pre-pupal skins, one packet covered with a mixture of oil and mucus germinated after two days giving a pure culture of the symbiotic fungus. There was no development from the controls which suggests that these secretions may release the fungus from the wax packets.

Figure 24.

Side view of the abdomen of a female pupa of S. noctilio showing the exuvial cap on the tip of the ovipositor.

VII. INFECTIVE BEHAVIOUR

The success of a mutualistic relationship depends on mechanisms ensuring the continuity of the association. The mechanisms which achieve the transfer of the microorganisms to the next generation may be fortuitous but inevitable, or there may be special adaptations of the insects' behaviour.

Observations upon a teneral adult female of S. noctilio indicate that the infection of the inter-segmental sacs may result from specialised activity occurring before boring commences.

Soon after the final moult, when the body was soft, and the antennae hung down beside the legs, the abdomen was rotated vigorously. Later the darkening valvulae could be seen sliding back and forth within the pale sheath, while the sub-genital plate was turned down to expose the inter-segmental sacs and the bases of the valvulae, which receive the ducts of the oil and mucous glands.

When the third pair of legs had strengthened, the female was able to brace herself while deflecting the last three segments of the abdomen upwards through an angle of 30°. While the abdomen was held momentarily in this attitude, the sub-genital plate was flicked away from the ovipositor, then snapped down again. The movement of the valvulae continued throughout this time. With

alternating periods of rest, this behaviour was repeated intermittently during the two hours the female was under observation.

How the larval hypo-pleural organ becomes infected after each moult is still open to speculation. The suggestions of Parkin and Morgan have been mentioned in the introduction.

DISCUSSION

Two significant facts revealed by the dissections of larvae are the form of the salivary glands, which are extensively branched with large reservoirs, and the fluid condition of the contents of the gut. These observations, in conjunction with the repeated failure of attempts to culture the fungus from the larval gut, prompted Morgan's suggestion (1966) that the fungus is digested extra-intestinally by saliva discharged into the cupped mandibles. Further evidence to support this theory, and the associated view that wood scrapings do not pass through the gut, might be gained from watching feeding larvae to see whether droplets of saliva are discharged and sucked back again.

The woodwasps occurring in Australasia have been identified from living and dead specimens at all stages of development, whereas Maxwell (1955) obtained many of her specimens from museums. It is reasonable to attribute the anatomical differences between the larvae of S. noctilio obtained from Tasmania and those from the N. Hemisphere described by Maxwell (1955), to the fact that her specimens were not correctly identified.

The studies on the development of the hypo-pleural organ have answered many of the questions raised by Parkin (1942) con-

cerning the changes which take place during moulting. Now that hypo-pleural organs have been found in second instar larvae, sections of first instar larvae and late embryos are required to complete the series, with the possibility of showing how the pits are formed in the cuticle.

The anatomical studies on pupal and adult females not only revealed the existence of the median oil sac, the paired lateral pouches covered with white patches of glandular cells, and the remains of spermatophores in the spermatheca, but they also showed details of the structure of the club gland, and the inter-accessorial sacs.

The stimulating effect of the oil and mucus on the vegetative growth of fungal cultures has provided experimental evidence in support of suggestions that insect secretions might be involved in the symbiotic relationship. These experiments were crude, though effective. Quantitative estimates of the limiting dose of secretion required to stimulate the vegetative growth of the fungus, could be assessed from the increase in dry weight of liquid cultures to which graduated doses of the secretions had been added.

The provisional identification of the chemical composition of the mucus of the adult females as an acid mucopolysaccharide-protein complex, and the oil as a mixture of at least five fatty

acids, has led to further speculation on the role of these secretions in the symbiotic relationship, which must be considered in conjunction with the common host of insect and fungus - the pine tree.

Krainsk (1966) identified the contents of the first oviduct gland of Cynips foliae as acid mucopolysaccharide-protein globules associated with mucopolysaccharides-phospholipid-protein globules. She maintains that this secretion, which is smeared over the surface of the egg before it is laid, functions together with the chitinous egg envelope to protect the embryo from the "noxious action of the vegetal medium". Possibly the mucus surrounding the siricid egg serves this function, as well as providing the arthrospores with a rich source of nourishment.

While it has been demonstrated that the oily secretion prevents the mucus from sticking to glass and, therefore, might act as a non-stick lubricant for the shafts of the ovipositor, the presence of at least fine fatty acids for this one function is unlikely. The stimulating effect of the oil on the vegetative growth of the fungus indicates that it must also be important for fungal nutrition.

The information revealed by experiments with these two secretions emphasizes the need for similar work on the secretions

of the club glands which are closely associated with the fungus. Presumably they are concerned with the establishment of the fungus and its development within the sacs which culminates in the formation of arthrospores. Stillwell (1966) succeeded in infecting the inter-segmental sacs with fungus from the hypo-pleural organ. This suggests that the secretions of larval and adult mycangia are similar both chemically and in their effect on the fungus.

Mechanisms of Infection.

In support of Francke-Grosmann's (1957) hypothesis, wax packets of S. noctilio which had been damaged deliberately, germinated within twenty-four hours, whereas undamaged packets did not germinate. Contrary to Francke-Grosmann's claim, no trace of wax packets could be detected in inter-segmental sacs of females, although stained squashes of sacs were examined from females at all stages of development. It seems reasonable to assume that wax packets can be passed along the ovipositor only where the first and second pair of valvulae slide against one another, and that they will be stranded near the base where the valvulae diverge. It is at this point that the ducts of the oil sac and mucous glands open into the ovipositor. The oil and mucus have been shown to stimulate the vegetative growth

of cultures of Amylostereum. In one instance a pure culture of the symbiotic fungus was obtained from a wax packet which had been covered with a mixture of oil and mucus. This evidence suggests that wax packets which have reached the base of the ovipositor might be provided with a growth medium, and possibly a chemical release mechanism, enabling Amylostereum to grow the short distance to the inter-segmental sacs.

Francke-Grosmann (1957) maintains that the waxy coating around the fungal packets preserves the fungus when low moisture content within the wood prevents fungal growth and explains the successful inoculation of the i.s. sacs of adult females which emerge from packing cases and other examples of dry wood.

It is possible that attempts to explain the infection of the inter-segmental sacs in terms of experiments carried out on newly moulted, firm wax packets deals with an unnatural situation. In a few cases, cultures of the symbiotic fungus have been obtained from the amorphous, crumbling remains of wax packets on old exuviae. If this waxy coat normally deteriorates with time, it will not always "preserve" the fungus in wood with a low moisture content. In these situations the stimulating effect of the secretions of the accessory glands and possibly the club gland of the teneral adult will assume greater significance. Even when low moisture content

prevents fungal growth, the fungus will not be killed. The symbiotic fungus has been cultured many times from the minute wood scrapings scattered over the surface of the pupa and pupal chamber. These scrapings must also be considered as a possible source of fungus for the infection of the inter-segmental sacs. Recent attempts at culturing the fungus from the ovipositor of females at different stages of development indicated that there might be a progressive growth of the fungus from the tip, along the shafts, to the sacs. Perhaps the fungus grows away from the ruptured or crumbling packets at the tip rather than the base of the ovipositor.

Recent observations suggest that the teneral adult female exhibits infective behaviour when the sub-genital plate is flicked open simultaneously with deflection the abdomen upwards.

One can only speculate whether the action of the sub-genital plate moves wax packets from the base of the ovipositor into the sacs, or whether fungal fragments are scraped off the walls of the pupal chamber in this way. Büchner (1965) claims that the sacs can be infected by fungus growing in from the pupal chamber. In either case, the oil and mucus would provide a rich growth medium.

Apart from Parkin (1942) speculation that fungus grows into the newly formed hypo-pleural organ of the female larva after each

moult, Morgan (1966a) placed glass sheets over cut pine logs to observe larvae tunnelling. He observed that when the body is stretched out, the hypo-pleural organ becomes exposed. Having seen scraps of fungus caught on cuticular spines, he suggests that fungus could be scraped from the walls of the larval tunnel, so infecting the organ.

Permanent mounts of larval cuticle made during this study show that the cuticular spines are finer and smaller in the inter-segmental fold than on the remainder of the cuticle. The thickened rim around the openings of the major pits appear to be devoid of spines, see Figs. 3b, 3c and 7b. Possibly secretions from the hypo-pleural organs direct the growth of fungal fragments caught on larger spines towards the pits.

The role of the fungus in the modification of the micro-environment of egg and larva, and in larval nutrition, has been reviewed in the introduction. It has been assumed that the fungus derives benefits through being dispersed by the insect and placed directly into the wood of a suitable host without having to penetrate any protective tissues. The present series of experiments have demonstrated that the oil and mucous secretions of the adult female woodwasp stimulate the vegetative growth of the fungus. It is possible that these secretions may be anti-bacterial and toxic to other wood-rotting fungi and it seems that this line of investigation could be pursued further.

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APPENDIX I

Packet	Shape and Measurements	Wax Solvent	Time	Result	Stain	Result	Interpretation
1					Oil Blue N	Faintly Blue	Surface could be waxy
2					Sudan Black B	Colour was washed again by acetone	Surface is coated with a lipid.
3	Max. width 78µ	Absolute Ethanol	15 mins.	Max. width and outline unchanged			If there is a lipid coating to these packets, it has not been removed by this treatment.
		Carnoys Fluid	5 mins.	Outline unchanged			This packet does not appear to be changed by Carnoy's fluid either.
4	Max. width 91µ Max. depth 104µ Edge of packet is smooth, and base is scalloped	di-ethyl ether	25 mins.	Edge is rough, and the scallops have disappeared.			Outer coating has been dissolved, exposing the tangle of fungal threads.
5	The base has three scallops	di-ethyl ether	18 hrs.	A few discoloured fragments lay on the bottom of the excavated block.			All the waxy coat and the waxy matrix has been dissolved away, leaving fungal fragments in the glass block.
6	Max. width 90µ	Xylene	6 mins.	One corner disappeared leaving a tangle of exposed threads.			The waxy coat dissolved in xylene, exposing the discoloured fungal threads.

Appendix I contd.

Packet	Shape and Measurements	Wax Solvent	Time	Result	Stain	Result	Interpretation
7		Carnoy's Fluid	10 mins.		Phloxine B	After dehydrating and clearing, there was a pink tangle of threads.	If there was a waxy coat, it must have been sufficiently thin to have been dissolved, exposing the fungus which stained pink.
8		Carnoy's Fluid	10 mins.		Ammoniacal Congo Red	A red bundle of threads could be seen.	As above.
9	Max width 78µ Max depth 117µ Edges are smooth.	di-ethyl ether	5 mins.	Slight alteration in outline and edges	Aniline Blue	A few patches were coloured blue.	Some of the wax had been dissolved away, exposing patches of fungus which stained blue.
10	Max width 85µ	Xylene	15 mins.	Change in shape and width evident.	Ammoniacal Congo Red	A tangle of red threads.	After waxy coat has been dissolved away, the threads of fungus are exposed.
11		Xylene	10 mins.		Brilliant Crocoaine	No result.	There is no cuticular envelope beneath the waxy coat of the packets.
12		Xylene	5 mins.		Brilliant Crocoaine	Patches on the surface retained the red colour after being rinsed in water.	A few fragments of cuticle could have been adhering to the surface of the packet.
					Phloxine B	The tangle of threads stained pink.	The waxy coat dissolved, exposing the fungus which stained bright pink.