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
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# ACKNOWLEDGEMENTS

# 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 acces-
sory 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 mucopolysac-
charide-protein complex. The oily secretion contains five fatty
acids, one being a major component.

Pure cultures of Amylostereum were obtained from excised hypo-
pleural organs, from slivers of wood from larval tunnels, from the
walls of the pupal chamber and from wood fragments in the pupal cham-
ber and on the surface of the pupa. Negative results from all regions
of the larval gut indicate that if the fungus is eaten, it is digested
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

### (1)  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 _I. phantoms (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-seg ental 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, (1966) 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._ _sanguinolsn-_
_tum_ was invalid. He found that sub-cultures of the original iso-
lates 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 sug-
gestion that

> _S._ _noctilio_ in New Zealand
>
> _S._ _juvencus_ in Germany
>
> _S._ _cyaneus_ )
>                     in England
> _U._ _gigas_
>
> _U._ _gigas_ _flavicornis_ F.) (
>
> _U._ _albicornis_ F.        } Canada
>
> _S._ _cyaneus_              )

may be associated with the same fungus which is _S._ _chialletii_.

III.                        MATERIALS AND METHOD

A.  Anatomy

(1)  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) <u>Female Reproductive System.</u>

    (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. <u>Secretions of Accessory Glands</u>.

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

(1) <u>The Oil Gland</u>.

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 $\mu$l 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 accerding 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, L-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.