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Biological interrelationships between the ambrosia beetle Xyleborus dispar (F.) (Coleoptera:Scolytidae) with its symbiotic fungus, Ambrosiella hartigii Batra (Fungi Imperfecti) were investigated in western Oregon.

Postdiapause adults of X. dispar collected in March through June with rotary nets, and excised from overwintered and newly attacked host material, produced a single generation when the beetle was reared in vitro with A. hartigii. Diapause beetles excised from host materials in the fall failed to oviposit. This study is the first record of rearing a temperate zone scolytid in vitro and on a fungus in the genus Ambrosiella. Such in vitro rearing allowed controlled studies of ecological, behavioral, developmental and physiological aspects of this ectosymbiosis. Comparisons were made between

wild and in vitro populations.

Seasonal variations of the fungus within the female beetles mesonotal mycangium, and the synchronization of ovariole development were demonstrated.

Comparative concentrations of solube proteins and free amino acids suggested that the fungus in the mycangia was built up from free amino acids of the insects. At the period of emergence, flight and attack of new hosts, the females were found to have a concentration of soluble proteins more than double that found in the beetles during the remainder of the year. Whereas, the free amino acids were the lowest values recorded during this period (March-October). Ovariole development and oviposition only occurred after the post-diapause female had fed on the ambrosial form of A. hartigii. These beetles attacked a new host with empty intestinal tracts. The relationship between the number of progeny and the volume of the galleries was linear.

Experiments were conducted in an attempt to terminate maturation diapause in this univoltine species of ambrosia beetle using temperature, Juvenile Hormone Analogs (JHA) and an olfactometer.

Termination of the maturation diapause was not achieved with these procedures.

Qualitative and quantitative analyses of the major nitrogenous excretory products were made on the various life stages of X. dispar.

The main nitrogenous product found in excreta and hindguts of beetles, larvae and pupae, was uric acid (range 7.6-14.8 μg uric acid/beetle). No ninhydrin-positive compounds were located in excreta of the beetles. The concentration of ammonia-nitrogen in the various life stages averaged between 0.70-1.13 μg NH_3-N/beetle.

Total nitrogen determinations were made on sapwood samples of Malus sylvestris (0.34 ±0.005% N by dry weight), attacked wood, "pre-brood" (0.31 ±0.005% N by dry weight) and attacked wood-"post-brood" (0.17 +0.02% N). Similar determinations of the artificial medium (L-asparagine) indicated that a nitrogen requirement of about 0.08-0.1% N by dry weight was necessary before oviposition could occur.

Fixation of atmospheric nitrogen by individual X. dispar beetles in vitro was not indicated using the acetylene ethylene reductase method. In vivo situations may be different, but were not investigated.

Proteolytic enzyme activity was not found on examination of dispause beetles, their excreta, larval and pupal excreta, and the ambrosial and mycelial forms of A. hartigii.

Bioassays were used to study the interactions and effects of the various life stages of X. dispar on the induction of the ambrosial form of its symbiotic fungus, A. hartigii. Postdiapause adults and pupae of X. dispar were able to cause a change from the mycelial

to the ambrosial form of A. hartigii in culture.

Larvae fed on the mycelial form in vitro, but ambrosia is required by larvae to develop and pupate. One of the main factors inducing the ambrosial form of A. hartigii is probably a secretory product of X. dispar. Nitrogenous compounds are considered necessary for the cause of ambrosial induction, but not the primary factor alone for the continued growth of the ambrosia. Morphogenic compounds that caused ambrosial induction in vitro responded negatively to the Azocoll procedure, indicating no proteolytic activity.

The symbiotic association of X. dispar and its sole food fungus, A. hartigii is a reciprocal biochemical alliance based on carbon and nitrogen metabolic integration. The beetles contribute their free amino acids and nitrogenous excretions, and the fungus contributes its cellulose-degrading enzymic ability to the association, plus synthesizing proteins, sterols, vitamins and other growth factors. The beetle-fungus symbiosis has been depicted in the form of an abstract pattern.

Biological Interrelationships between the Ambrosia Beetle *Xyleborus dispar* and its Symbiotic Fungus *Ambrosiella hartigii*

by

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BIOLOGICAL INTERRELATIONSHIPS BETWEEN THE AMBROSIA BEETLE XYLEBORUS DISPAR AND ITS SYMBIOTIC FUNGUS AMBROSIELLA HARTIGII

I. INTRODUCTION

Ectosymbiotic associations between ambrosia beetles (Platypodidae and several Scolytidae) and their symbiotic fungi have recently been reviewed by Baker (1963), Batra (1963), Francke-Grosmann (1967), and Graham (1967), indicating that these relationships involved reciprocal nutrient contributions between partners.

The ambrosia beetle, Xyleborus dispar (F.) (Coleoptera: Scolytidae) lives ectosymbiotically with the microorganism Ambrosiella hartigii Batra (Fungi Imperfecti). The fungus is dependent on the beetle for its cultivation and transmission, and is protected during the flight and hibernation period within the mesonotal mycangium of the female beetle (Francke-Grosmann, 1956). The insect is xylomycetophagous (Schedl, 1958) and its larvae are true fungal feeders (Francke-Grosmann, 1967).

Accounts of X. dispar are numerous (Hartig, 1844; Eichhoff, 1879; Hubbard, 1897; Neger, 1911; Schneider-Orelli, 1913; Wilson, 1913; and Schvester, 1954), yet knowledge of many aspects of its biology and the mutualistic association with its primary symbiotic fungus, A. hartigii, are still scanty or lacking.

Preliminary studies by the author showed that the adult and pupa cause dimorphism of A. hartigii, with the mycelial form of the fungus being converted into the yeastlike or ambrosial form. In nature the ambrosial form is dominant in the immediate vicinity of the larvae, pupae, and adults (Batra and Downing Michie, 1963). Exactly how the adults and the pupae control this dimorphic phenomenon, if they do, is not yet known.

The present study was concerned with the ecological, developmental and physiological relationships between X. dispar and A. hartigii, and was conducted in the Corvallis-Salem area of the Willamette Valley in Oregon. The objectives of this study were:

1. To maintain X. dispar and its symbiont, A. hartigii, in a two member culture, on a defined medium in vitro.
2. To examine some behavioral, developmental, and physiological features of X. dispar to amplify aspects of its life cycle.
3. To determine qualitatively and quantitatively the main nitrogenous excretory products of the various life stages of X. dispar.
4. To examine induction of ambrosia in A. hartigii in symbiotic association with X. dispar.

This study represented a team approach, at the graduate level, in which an interedisciplinary investigation was carried out to examine ectosymbiotic relationships between an ambrosia beetle and its symbiotic fungus. The frequent citations to 'Roeper,

unpublished data', in Chapter VI, refer to the unpublished data in the doctoral thesis of my colleague Richard A. Roeper, of the Botany and Plant Pathology Department, Oregon State University, Corvallis.

II. REVIEW OF LITERATURE

General Biology of Ambrosia Beetles

The ambrosia or xylomycetophagous insects are considered by many to be a very highly evolved group of the Coleoptera, and belong to the superfamily Scolytoidea, which includes families, Scolytidae, and Platypodidae. The Scolytidae comprise species of Xyloterini, Corthylini, Xyleborini, and Webbini in the subfamily Ipinae, as well as all species of the tropical subfamily Scolytoplatypodinae. Considering their world-wide distribution and heterogenous nature, the habits and life histories of the ambrosia beetles are exceedingly diverse and no generalized account applicable to all types can be given (Baker, 1963). However, all the ambrosia beetles bore into sapwood, and some, the heartwood (e.g. Xyleborus saxesini, and Platypodids), and have a grass-free tunnel system. The adults bore into the woody tissues of the host plant and feed mainly on the ambrosial fungus that covers the walls of the tunnel system. They breed in all sizes of host material of both coniferous and deciduous forest and ornamental trees. Tunnel systems may be cave-like or have several breeding chambers. Eggs are laid randomly along the galleries and in niches, and the larvae feed on the ambrosial fungus. Teneral adults may remain in the gallery system overwinter, or

hibernate in the soil or duff, or successive generations may be produced in the same host plant as long as the moisture content remains favorable for growth and development of fungus. The beetles emerge from the parental entrance hole and seek new host plants. Mating may occur within or outside the gallery systems.

Reviews on the general biology of ambrosia beetles have been presented by Beeson (1941), Fisher et al. (1953-54), Bletchly (1961), Browne (1961), Chamberlin (1939, 1958), Kalshoven (1958-59, 1960), Rudinsky (1962), Schedl (1962), Baker (1963), Brader (1964), Francke-Grosmann (1967), Graham (1967) and Abrahamson (1969).

Ambrosia Fungi Associated with Ambrosia Beetles

Associations between ambrosia fungi and beetles were reviewed by Francke-Grosmann (1956, 1963), Baker (1963), Batra (1963, 1966), Graham (1967), and Abrahamson (1969). In 1967, Batra presented a taxonomic revision version and nutritional studies of some species. He mentioned that ambrosia fungi are mutualistic symbionts of Scolytidae, Platypodidae, and Lymexylidae (Coleoptera). The characteristic mass of sprout cells, called the ambrosia phase, in insect tunnels and in mycangia of the beetles is common to all species discussed. Batra (1967) has placed the primary ambrosial fungi in 4 genera of Tuberculariaceae, Ambrosiella Brader, Raffaelea v. Arx and Hennebert, Monacrosporium and

Phialophoropsis Batra and 2 genera of Endomycetales, Ascoidea Bref. and Lindau and Endomysopsis Dekker.

The ambrosia fungi, with their associated beetle species, occupy a biochemical niche in dead, dying, diseased, and recently cut trees. The fungi form a thin continuous palisade over the entire tunnel system. Most ambrosia fungi are dimorphic (Batra and Downing Michie, 1963). The ambrosial form (sprout cell) is produced in association with the insect and the mycelial form is produced in vitro without the insect. When isolated as the ambrosial form from beetles and gallery walls, and cultured on common laboratory medium such as potato dextrose agar (PDA), the fungus converts to the mycelial form (Batra, 1967).

Ambrosia fungi have no perfect stage (possess no ascus form) and have been shown to have three types of apparently similar reproductive structures (Batra, 1967): (a) The monilioid conidiospores or similar vegetative hyphae, both originating from ordinary, non-torulose hyphae, from sprout cells, or by the transformation of a germ tube. The individual cells of such chains in many species fall apart and usually do not germinate. (b) The conidia which are terminal, usually solitary, and borne on molilioid conidiophores described above. The conidia have somewhat thicker and darker walls than other cells. Their cytoplasm is homogeneous and they lack vacuoles until just after germination. (c) The sprout cells

usually originating from the conidia and either germinating in situ or from dislodged conidia. They are thin-walled, highly vacuolated, granular and appear to be short-lived. They represent the ambrosia phase in mycangia and in tunnels and constitute the bulk of the ambrosial colonies on agar media.

Ambrosia Fungi in Culture

Cultural methods of ambrosia fungi have been recently described by Batra (1963), Batra and Downing Michie (1963), Baker (1963), Batra (1967), and Abrahamson (1969).

As mentioned above, isolating the ambrosial form in nature and culturing on laboratory media resulted in growth of the mycelial form of the fungus. This phenomenon as Francke-Grosmann (1967) has pointed out, has been known since 1909, when Neger, Beauverie and Schneider-Orelli observed this dimorphic or pleomorphic growth form with the fungus of X. dispar.

Batra and Downing Michie (1963) remarked that the fungus associated with X. dispar was present in the immediate vicinity of adults, eggs, larvae and pupae in nature in the ambrosial form. Batra (1967) added that it was the active female beetle that is required for the controlled growth of the ambrosia in nature. How the adult encourages the ambrosial form at the expense of the mycelial form is not yet known. A complex of factors seems necessary to

promote ambrosial growth, some of which appear to be realized in the presence of yeast extract, malt extract, and neopeptone.

Francke-Grosmann (1967) mentioned that one of the characteristic features of ambrosia fungi in culture is the production of fruit esters, many of which exude substances that cause a dark discoloration of the nutrient and in some rare instances have hyphae turning black with age. Data on the enzymes produced by ambrosia fungi are lacking. Brader (1964) showed that glucose plays a prominent role as a nutrient. Other ambrosia fungi may use cellulose as a source of carbon; uric acid (Baker, 1963; Abrahamson, 1969), and urea (Abrahamson, 1969). Glutamic acid, asparagine, ariginine, ornithine, aspartic acid, alanine, proline, glutamine, and ammonium acetate were claimed to support the growth of "propagules" in A. ferrugineus associated with Trypodendron retusum (Abrahamson, 1969).

Ambrosia Beetles in Culture

The many attempts of culturing ambrosia beetles on artificial cultures of fungi isolated from their galleries have been described by Francke-Grosmann (1967). At Madison, Wisconsin, in 1967, Saunders and Knoke working with the tropical non-diapausing ambrosia beetle, Xyleborus ferrigineus (F.), successfully reared this insect through several generations on artificial media. Norris and Baker (1967), working in the same laboratory, showed that the fungus was

essential for insect reproduction. Further culturing by Norris and Baker (1968) produced a minimal nutritional substrate for the fungus, Fusarium solani to fulfill its mutualistic relationship with X. ferrigineus. Norris, Baker and Chu (1969) using aposymbiotic insects demonstrated the ability of X. ferrigineus to utilize ergosterol, but not cholesterol, as a source of sterol for pupation. They stated that this represented a fundamental nutritional interdependency between the components of this natural symbiotic complex. Also, they mentioned that ergosterol was the major sterol of fungi.

Norris and Chu (1970) developed a holidic diet for the continuous asceptic rearing of aposymbiotic X. ferrigineus. This beetle was found not to utilize cholesterol as a sole sterol source (Chu et al., 1970). However, this chemical met the sterol requirements for egg and larval development of this insect but they do not pupate. Kok et al. (1970) showed that the Δ^7 sterols ergosterol and 7-dehydrocholesterol, were adequate sole sterol sources for complete insect development. Because of these two distinct sterol requirements in this insect, an unusual block must exist in its sterol metabolism. The beetle apparently cannot oxidize the C7-8 bond in the 'B' ring of the sterol nucleus. Ecdysones have the Δ^7 bond, and so this beetle cannot make the necessary ecdysone(s) from cholesterol. Gilmour (1965) pointed out that micro-organisms have the ability to synthesize sterols from simple precursors. Insects are unable to

synthesize beyond squalene in the biosynthesis of terpenoids and steroids. However, it seems that the mutualistic fungus contains sufficient sterols (ergosterol) for the development of X. ferrigineus (Kok et al., 1970). Norris and Chu (1970) described how an aposymbiotic female X. ferrigineus transmitted, apparently transovarially, the sterol or sterol-dependent metabolites necessary for progeny pupation. Sterol for immature development was required in the progeny diet. Further, they showed that when maintained on a cholesterol-containing diet, maternal females lost their ability to pass required pupation factors to progeny. The ability of the insect to complete a first generation on diets lacking ergosterol probably can be attributed to adequate carry-over of sterol from the previous stock-culture generation fed diets with fungus. The inability of this insect to pupate in the second generation on diets void of fungus or ergosterol indicates that the insect requires a sterol other than cholesterol for pupation (Norris, Baker and Chu, 1969).

Xyleborus dispar (F.)

Synonymy and Classification

In 1792, Fabricius gave the first description of this insect, and placed it in the genus Apate with the species name of dispar. The species name seems to have been based on disparity of the males and females.

The original description is given in Entomologia Systematica (Fabricius), Volume I, part II, p. 363.

Synonyma for Xyleborus are given below, in part of an extract from page 1307-1308 in Canadian Entomologist, 100: 1288-1323, by D. E. Bright, Jr. 1968.

Bostrichus dispar: Herbst, 1793, p. 113.

Xyleborus dispar: Eichhoff, 1864, p. 38; Riley and Howard, 1890, p. 279; Schwarz, 1893, p. 63; Hubbard, 1897, p. 22; Swaine, 1909, p. 152 (additional references); Hagedorn, 1910a, p. 101 (additional references); Hagedorn, 1910b, p. 153; Hopkins, 1915, p. 67; Schedl, 1960, p. 11

Tomicus dispar: Thomson, 1865, p. 369.

Anisandrus dispar: Ferrari, 1867, p. 26.

Scolytus pyri Peck, 1817, p. 205 (type ♀, Massachusetts, destroyed ?); Schwarz, 1888, p. 138 (? = dispar); Riley and Howard, 1890, p. 279 (= dispar); Schedl, 1960, p. 11 (= dispar).

Tomicus pyri: Harris, 1852, p. 80.

Xyleborus pyri: Zimmerman, 1868, p. 144; Swaine, 1909, p. 153 (additional references).

Xyleborus dispar: Wilson, 1913, p. 97.

Anisandrus pyri: Hopkins, 1915, p. 69; Blatchley and Long, 1916, p. 626; Chamberlin, 1939, p. 444; Beal and Massey, 1945, p. 150; Chamberlin, 1958, p. 185.

Anisandrus swainei Drake, 1921, p. 203 (Holotype ♀, New York, USNM); Chamberlin, 1939, p. 444; Wood, 1957, p. 403 (= pyri).

Females. Length 3.2-3.7 mm, 2.0 times longer than wide. Frons minutely reticulate, opaque, punctures rather large but very shallow, more strongly punctured on lateral portions; surface very slightly impressed on epistoma on each side of a faintly elevated longitudinal carina. Antennal club 1.1 times longer than wide. Pronotum as wide; sides arcuate; anterior margin broadly rounded, with six to eight prominent asperites; asperites on anterior slope very large, prominent; posterior margin minutely reticulate, opaque, punctures very faint, small. Elytra 1.3 times longer than wide; sides parallel on basal three fourths, rather narrowly rounded behind; strial punctures rather large, closely placed, interspaces smooth, shining, becoming granulate near declivity, punctures less than half the size of strial punctures and not in regular rows on disc. Declivity convex; first and third interspaces slightly elevated; all interspaces faintly granulate; ridge of seventh interspaces acute, elevated, and unbroken.

Males. Length 1.8-2.1 mm, 1.6 times longer than wide. Frons broad, flat, minutely reticulate, shining, median longitudinal carina reduced to a low, broad tubercule. Pronotum slightly broader than long, strongly convex; narrowly rounded in front; asperites very low and faint; surface minutely reticulate, shining, faintly punctured.

Elytra strongly convex, widest at about middle, rather broadly rounded behind; striae and interstriae resembling female. Declivity strongly convex; unmodified.

Distribution and Habitat

Initially of Northern European and Asian origin, X. dispar has been spread by man into the greater part of the temperate world. The females of this ubiquitous beetle usually infests dying and diseased trees and recently cut timbers of deciduous species in its range and has been collected from conifers (Pinus, Tsuga) (Wilson, 1913; Mathers, 1940; Linsley and MacLeod, 1942; Downing et al., 1956 and Bright, 1968). The first report of this insect in the United States was by Peck (1816) in Massachusetts. In 1901, it was reported in Clarke County, Washington (Wilson, 1913). Since that period X. dispar specimens have been located and examined from eastern North America west to the Lake States and south to North Carolina; western Canada and the Pacific Northwest states (Bright, 1968).

The male and female adults pass the winter in their tunnels in the limbs and trunks of infested trees. Emergence of the females of this univoltine species (Schneider-Orelli, 1913; Schvester, 1954) begins in late March, April, and May (Wilson, 1913). The female adults fly to diseased and dying trees, and the entrance holes are usually made about a bud scar or in some roughened place. The

beetles select these diseased trees, whether by volatiles from the bacterial cankers (Cameron, 1970) or from the 'sour sap' (Linsley and MacLeod, 1942; and Graham, 1968), or both. The males are wingless and do not leave the burrows. Mating occurs within the galleries (Chararas, 1962) and near the tunnel entrances (Schneider-Orelli, 1913) and the females are fertilized when they emerge the following spring. Tunnel systems are entirely constructed by the female (Eichhoff, 1881). The galleries are not all constructed at the same time but are completed in sections, the female spending her time feeding on the growing ambrosia, ovipositing, and resting near the tunnel entrance (Doane et al., 1936).

Eggs are laid normally in small clutches randomly in the first branch chambers. Wilson (1913) reported that they may be found from the second week in April until the middle of June. When first deposited they are oblong in shape and pearly white in color. They measure about 1 mm in length by 0.06 mm diameter. The number laid is variable, with 10-40 eggs per female being the most frequently reported figure (Schneider-Orelli, 1913; Schvester, 1954). Wilson (1913) mentioned that over 45 eggs may be laid. Most authors (Eichhoff, 1881; Schneider-Orelli, 1913; Wilson, 1913; and Schvester, 1954) stated that under 'normal conditions' hatching takes from 2-3 weeks. Whereas, Hubbard (1897) said seven days, and Doane et al. (1936) considered 6-10 days to be the average period. Eggs removed

from the galleries and hatched in the insectary required 10-12 days (Wilson, 1913).

The larvae are pure white in color when hatched (Schneider-Orelli, 1913), but after feeding on the ambrosia the alimentary canal shows black through the body wall. They move freely throughout the gallery system and develop into pupae in 3-4 weeks (Eichhoff, 1881; Wilson, 1913; and Schvester, 1954). All larval instars can be found during this period.

Wilson (1913) found pupae from the second week in June until the first of August. About 3-4 weeks are required for the pupal stage, lasting from early June to early July. The pupae are easily distinguished, the males being much shorter.

The sex ratio of emerging adults was given by Mathers (1940) as 1:2.2 (males to females), Eichhoff (1881) as 1:4.2, Nüsslin (1913) as 1:4, Schneider-Orelli (1913) and Wilson (1913) as 1:4 or 5, Vasseur and Schvester (1948) as 1:3, to 1:10, and Doane et al. (1936) as 1:15 or 20.

The longevity of the adult was estimated to be a maximum of 11 months (Mathers, 1940). Eichhoff (1881) and Chamberlin (1939) considered X. dispar to have two generations a year, whereas Gillanders (1912), Schneider-Orelli (1913), Wilson (1913) Nüsslin and Rhumbler (1922), Doane et al. (1936), Vasseur and Schvester (1948) and Schvester (1954) reported a single generation.

Cleridae and Humenoptera as natural enemies of X. dispar were described by Eichhoff (1881) and Schneider-Orelli (1913). Schvester (1950) established the presence of the nematode Parasitylenchus dispar Fuchs parasitizing X. dispar male and female adults. Also, Schvester (1957) mentioned that predaceous mites, Pyemotes ventricosus Newport, and P. scolyti Oudemans, were found attacking the bark beetle, Ruguloscolytus rugulosus Muller, indicating that these mites were found in close association with X. dispar. However, no natural enemies of X. dispar have been reported from the Pacific Northwest (Wilson, 1913; Mathers, 1940).

Ambrosia Fungus of X. dispar

Descriptions of the ambrosia fungus of X. dispar were given by Hartig (1844), Goethe (1895), Smith (1896), Hubbard (1897), Neger (1909, 1911), Beaverie (1910), Schneider-Orelli (1911, 1913), Francke-Grosmann (1958), Batra and Downing Michie (1963), and Batra (1967).

The ambrosia fungus, Ambrosiella hartigii Batra, is the sole food source of this insect (Francke-Grosmann, 1967). Both the fungus and the insect are ectosymbiotically interrelated and associated. The fungus is protected in the mycangium of the X. dispar female adults during the hibernation and flight periods of the insect. Females disseminate the fungal spores (ambrosia) of the symbiont

into the excavated tunnels (Batra, 1967). The fungal hyphae ramify in all directions into the wood, often up to several centimeters. Seemingly utilizing its cellulose-degrading enzymes, the fungus provides the insect with a highly nitrogenous, low residue diet synthesized from nutrients inaccessible to the larvae and reutilizing the nitrogen excreted by the beetle for further growth (Batra and Downing Michie, 1963). Ambrosia supplies sterols (Norris et al., 1970), vitamins, and nutritious components for the adult and larval stages (Batra, 1963). In early spring, the fungus proliferates in the mycangium of the female adult, as was found with other ambrosia beetles (Schneider and Rudinsky, 1969a, b). The presence of the adults in one tunnel was one of the prerequisites for a controlled growth of ambrosia in nature (Batra and Downing Michie, 1963). How the beetles keep the fungus under control has not been determined. Francke-Grosmann (1967) considered that the ambrosia was being induced by secretions of beetles and larvae, and by antagonistic properties of the ambrosia fungi against the 'weed fungi'. Batra and Batra (1967) reported that saliva of workers and soldier termites showed indications of antimicrobial properties that inhibited the growth of alien fungi that do not normally proliferate. A study of the salivary glands of extracts of queen termites showed that caprylic acid revealed positive results for retarding the germination of alien micro-organisms (Sannasi, 1969). Wyss et al. (1945) showed that

long chain and unsaturated fatty acids possessed fungistatic and fungicidal activity. Koidsumi (1969) demonstrated that active antifungal constituents of cuticular lipids were free medium-chain saturated fatty acids, presumably caprylic acid and capric acid, which occur naturally in the cuticle. These studies, although not concerned with ambrosia beetle-fungus associations, may indicate a possible mechanism for control of the fungus by the beetle.

III. IN VITRO REARING OF XYLEBORUS DISPAR

Introduction

In the search for more effective methods of studying ectosymbiosis in wood boring beetles, adequate numbers of experimental insects are required. The xenic rearing (Gordon, 1959) of these beetles under laboratory conditions offers several advantages over field collections, e.g. (a) insects available during longer periods of the year, (b) increased brood survival, and (c) the selection of more homogenous populations than occur in nature (Ryan, 1959). But as Dougherty (1959) pointed out, the xenic rearing under laboratory controlled conditions poses the greatest potential difficulty for most invertebrate organsims; this has been particularly true for many scolytid beetles (Schmidt, 1966). The desired goal has been the development of a defined artificial medium which allows for a closer understanding of the nutritional requirements of the insects. Earlier efforts to rear scolytids through their entire life cycle on artificial media failed (Francke-Grosmann, 1967), although various media permitted larval development for certain bark beetles (Batra, 1963; Yearian and Wilkinson, 1963; 1965; Bedard, 1966; and Schmidt, 1966). The main problems in such artificial rearing were fungal contamination and the lack of ovipositional stimulations. However, Saunders and Knoke

(1967) first successfully reared a tropical ambrosia beetle, Xyleborus ferrugineus (F.) on several artificial media under laboratory conditions. Norris et al. (1967, 1968, 1970, and 1971) developed a chemically defined medium and studied the minimum substrate requirements for this same non-diapausing beetle both with and without its mutualistic fungus. Further reports by Norris and Chu (1970) indicated that aposymbiotic beetles of X. ferrugineus could be asceptically reared on an holidic diet. This successful continuous rearing in vitro of Xyleborus spp. for the choice in selecting Xyleborus dispar (F.) as the test insect in this present study. The main objective was to maintain X. dispar and its symbiont fungus, Ambrosiella hartigii Batra in a two member culture on a defined medium in vitro. The approach in developing the medium differed from other workers (Saunders and Knoke, 1967; Norris and Baker, 1967; and Norris and Chu, 1970) in that the substrate was first determined for the fungus and then it was considered that a beneficial diet for the fungus would also be satisfactory for the growth and development of the beetle.

Experimental Procedure

Collection of Beetles

Deciduous trees (Malus and Prunus spp.) infested with X. dispar were collected in Oregon (Figure 1) and Washington during September

Figure 1. Diseased Prunus sp. attacked by postdiapause female beetles of X. dispar in Salem, Oregon.

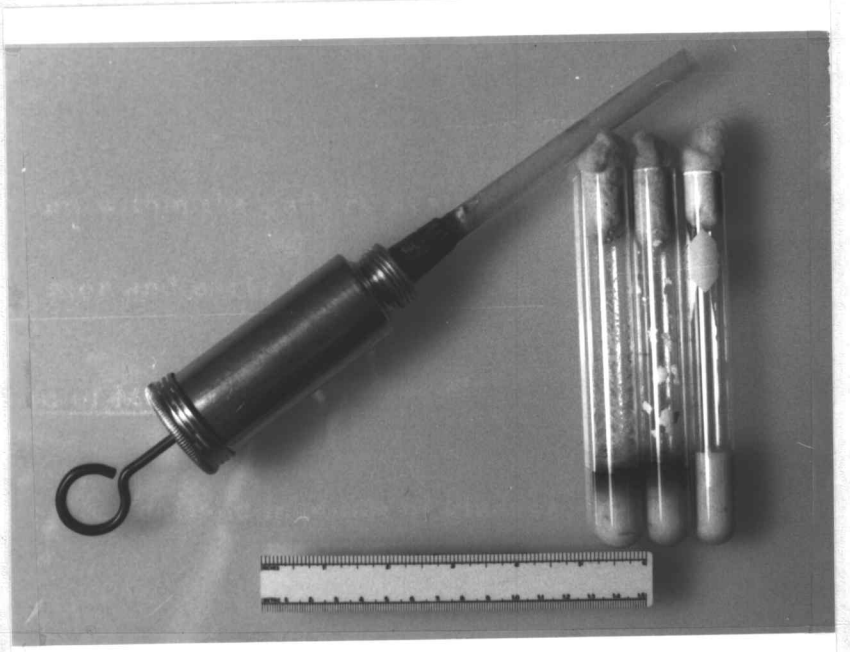

Figure 2. Modified cake decorator dispenser used for injecting the viscous artificial media into the culture tubes. At the right, 2 tubes with fungus pre-grown on the surface of the media.

most tubes were inoculated with the fungus, which had been previously isolated from the mycangium of postdiapause beetles and maintained on malt agar medium. Preinoculation with A. hartigii ensured that the beetle released in the tube was in the presence of the symbiotic fungus.

Cleaning and Transferring Beetles

In an attempt to reduce microbial contamination by the beetles, females were placed singly and alternately on wet and on dry sterile filter paper, each regime for 8-12 hr for 3 days prior to their introduction to the culture tubes (Batra, 1963). Beetles were transferred with a sterile vacuum tweezer system (Linscott, 1964). One female was put in each tube. The tubes were kept in the dark at about 22-24° C except when observations were made. Calculations of the sample data were made with suitable programs designed and operated through the computer center at Oregon State University.

Results and Discussion

Postdiapause beetles successfully reared their progeny in the medium regardless of source from which collected or if they were released in tubes that were or were not preinoculated with A. hartigii. In tubes that were not preinoculated, female beetles were able to establish A. hartigii (ambrosia) prior to oviposition. The differences in the number of tubes set up and the number in which the postdiapause beetles produced progeny were attributed to handling losses and the growth of nonambrosial microbes in the tubes. Common contaminants were species of Aspergillus, Penicillium and Trichoderma. These were considered nonambrosial fungi. The alternate exposure to wet and dry filter paper reduced the introduction of nonambrosial microbes by adult beetles but was not entirely successful. In cultures in which beetles produced progeny, A. hartigii was dominant in galleries but often the growth of other microbes was evident.

Prediapause or diapause beetles, either removed from culture tubes (Table 1, Population 5) or field collected (Table 1, Population 4), all failed to oviposit. Each merely tunnelled its length into the medium and remained inactive. Contamination by nonambrosial fungi also proved a problem when diapause beetles were introduced; however, in about 75% of the tubes of prediapause and diapause beetles, A. hartigii remained the sole or dominant fungus present.

Table 1. Summary of influence of physiological state, source and times of collection, and culture methods on X. dispar in vitro on A. hartigii (1970 and 1971).

Population[a]	Methods of collection	Months collected	No. cultures		
			Preinoculated	Not preinoculated	Producing progeny
1	Emergence in boxes from host trees collected in previous Sept.	Mar-Apr	311	78	168 51
2	Rotary nets; field beetles in flight	Apr-June	380	40	97 32
3	Excised from newly attacked trees prior to oviposition	Apr-June	110	40	79 27
4	Excised from galleries in host trees	July-Dec.	485	70	0 0
5	Progeny of postdiapause beetles cultured from Populations 1, 2, and 3	Aug-Jan	302	102	0 0

[a]Populations 1-3 were postdiapause beetles; 4 and 5 were prediapause and diapause beetles.

The observations summarized in Table 1 agree with the field observations of Schvester (1954) who stated that X. dispar had one generation per year and a maturation dispause in the adult stage.

The culture data for the 1970 and 1971 seasons were combined and the means calculated using 60 observations per life stage. The first eggs were laid 9.31 ±0.31 (SE) days after the postdiapause beetles were introduced in the tubes. In sampling 29 females, the mean number of eggs laid per female was 9.00 ±0.49 (range 2-14). Larvae were observed after 18.71 ±0.38 days and the larval-pupal transformations in 35.80 ±0.25 (SE) days. The mean developmental period from eggs to teneral adults was 41.75 ±0.44 days. These adults were fully pigmented within seven days, and remained inactive in their galleries. In several tubes, the location of the gallery in the medium did not permit direct observations. Only after careful dissections of the media were complete numbers of progeny recorded. A comparison of in vitro and wild populations of X. dispar showed that the developmental period from egg to adult was shorter using the artificial medium. Schvester (1954) observed that wild populations produced single generations in 58-60 days and Wilson (1913) stated 80-84 days.

The sex ratio of progeny produced in vitro was 1 ♂: 2 ♀, which was similar to observations based on field studies by Mathers (1940). Field data by Wilson (1913) showed a ratio of 1 ♂: 4 or 5 ♀.

Observations in this study indicated a 1 ♂: 1 ♀ ratio to 1 ♂: 3 ♀ ratio in the field.

IV. SOME BIOLOGICAL ASPECTS OF XYLEBORUS DISPAR

Introduction

Knowledge of nutritional requirements and physiology of insects may be gained in the development of artificial diets suitable for continuous rearing (Norris and Chu, 1970). Field observations and data from preliminary laboratory rearing experiments indicated that the insects cultured in artificial media compared to wild populations. The biology and reproductive behavior may be readily studied under controlled laboratory conditions.

Virtually every facet of the beetles behavior and life cycle depends on the fungus they culture. Abrahamson (1969) mentioned that obligatory interorganismal nitrogen metabolism was a nutritional basis for the mutualistic symbiosis which existed between ambrosia beetles and their associated fungi. A cyclic pattern of nitrogen utilization indicated uric acid was the primary excretory product of the ambrosia beetle, X. ferrugineus, and provided the ambrosial fungi with a nitrogen source in the beetle gallery which supported the ambrosial growth; however Abrahamson (1969) never confirmed the presence of uric acid in ambrosia beetle excreta. The insects in turn digested the fungus and the products included lipids, proteins, amino acids and vitamins required by the insect. Martin and Martin (1970a, b) studied the symbiosis between the attine ant, Atta colombica

tonsipes Santschi, and its food fungus and referred to a biochemical alliance predicated on metabolic integration. This mutualistic exchange of nutrients between symbiotic partners allows the fungus to exploit a substrate from which it would otherwise be excluded by other microorganisms better able to utilize the nutrient present in it. The beetles in return receive the use of the cellulose-degrading ability of the fungus, and gain access to the cellulose reserves of hardwood forests for use as a carbon source.

These following investigations examined the ecological importance of the behavioral, developmental and physiological aspects of X. dispar in relation to its mutualistic symbiotic association with A. hartigii. The physiological experiments were primarily designed to examine some of the patterns of nitrogen utilization in this mutualistic association. Although studies on the main excretory products of insects are numerous (Razet, 1966 and Bursell, 1967), data on ambrosia beetle excretory products are lacking. This 'nitrogen environment' was surveyed to obtain some parameters in this symbiosis. Also, the possibility of nitrogen fixation by X. dispar beetles was examined.

Ambrosia fungi are known to multiply in the mycangia of their respective symbiotic beetles (Schneider and Rudinsky, 1969). These microbes grow at the expense of the beetle and utilize chemicals in the insect as nutrients. The fungus can multiply on the forms of

nitrogen and carbon found in the mycangium and/or hemolymph of the beetles. The pool of amino acids are made available to the fungus and incorporated directly into proteins (Abrahamson, 1969). Analyses were made of extracts of X. dispar females during this present study, from March to October, to determine if this was the case with X. dispar.

Experimental Procedure

Behavioral Aspects

Field and laboratory reared beetles were used, with the adults 'cleaned up' by the methods of Retnakaran and French (1971) prior to transfer onto artificial media. The feeding of postdiapause and diapause beetles was verified by dissection under a binocular microscope. Tunnelling and dissemination of the ambrosia fungus was observed throughout the experiments. Culture tubes were incubated in the dark between 23-25° C.

Developmental Aspects

Beetles were examined from field and laboratory cultured materials under sterile conditiions. Dissections of beetle mycangia and ovaries were made using a fine jewellers forceps. The different structures were mounted in lactophenol blue on microscope slides.

Mycangial and ovariole development of postdiapause females

were examined anatomically. Six beetles were sequentially sampled from the culture tubes each day and microscopic and photographic records were made.

Experiments were designed to attempt to terminate the maturation diapause of X. dispar. This study was divided into 3 main parts: (a) effects of temperature and time on diapause; (b) effects of Juvenile Hormone Analogs (JHA) on diapause; and (c) effects of olfactometer treatments on diapause. Details of the experimental design are given in Table 2. The value for the alternating temperature experiment of 25° C for 16 hrs and 2° C for 8 hrs was selected to correspond with conditions that may be expected in the field at the period of greatest growth (spring) and during least growth (winter), in an attempt to increase the insect's metabolic rate. The varying periods of exposure of 7, 10, 20, 30, 40, 50, 90, and 360 days to the various low temperatures of 2 and 7° C were selected to correspond with conditions that may be expected in the field during winter months. Diapausing female beetles of X. dispar were set up in culture tubes preinoculated with A. hartigii in all regimes. No attempt was made to control humidity. Beetles were transferred to fresh tubes after exposure to various low temperatures and placed at room temperature to observe oviposition. After four weeks they were dissected to determine ovariole development and to check mycangial contents.

Effects of Juvenile Hormone Anaologs on Diapause. The objective of this treatment was to attempt to stimulate ovarian development in diapausing beetles using JHA and

Table 2. Temperature treatments and duration of exposure (days) experienced by X. dispar diapausing females in an attempt to terminate maturation diapause.

Temperature Regimes (°C)	Duration of Exposure (days)
Constant - 2°, 7°, 25°C	7, 10, 20, 30, 40, 50, 90, and 360
Alternating 2-25°C	

feeding stimulants. The experiments were conducted in two main sections, each using a different JHA and feeding stimulant. JHA were topically applied in acetone solvent, using a Hamilton syringe. Beetles were held at their posterior ends with the vacuum tweezer system, and the solutions applied onto the dorsal surface, between the thorax and abdomen.

Series I. Using JHA supplied by Calbiochem Co., California. Three concentrations of JHA were used: 25, 14, and 1.4 μg/μl. Ten beetles per treatment were tested. Applications of 2 μl were applied per beetle per treatment. Treated and untreated beetles were placed into culture tubes preinoculated with the mycelial form of A. hartigii. All tubes were kept at about 20-24° C. Daily observations were made for the appearance of ambrosia in the tunnels and evidence of oviposition. After four weeks, the insects were removed and dissected to observe feeding and ovariole development.

Sequential topical applications of JHA were made on diapause beetles which had been stored at three different regimes. These were from the out-of-door cage, from a constant light and temperature (21° C) room and from a cooler (2° C). Concentrations of JHA were similar to those used in section (a) above. Initially, 1 μl of the hormone was applied per beetle, and seven days later, a further 1 μl of the hormone was applied per beetle. The procedure which was adopted from the study by Bell and Barth (1970) in order to

encourage or stimulate ovariole development in X. dispar diapausing female beetles.

Series II. Using a JHA (ZR-512®) supplied by Zoecon, Palo Alto, California. Slama (personal communication) indicated that JHA from Calbiochem had no morphogenic effects on scolytids. He recommended the use of JHA (ZR-512®). Diapause beetles (10 per treatment concentration) were treated with 2 µl of the JHA, at a concentration of 20 µg/µl in the same manner as in Series I. This concentration was selected due to the limited availability of the JHA at the time of treating. Treated and untreated beetles were placed into tubes preinoculated with the ambrosial form of A. hartigii. Cultural studies had shown that the ambrosial form of A. hartigii could be maintained in vitro (see Chapter III, p. 25). Beetles were treated with the JHA (ZR-512®) and then released onto the ambrosia to feed. The combined effect of the JHA and ambrosial feeding was expected to stimulate ovariole development. Daily observations were made for the presence of ambrosia in the tunnels and evidence of oviposition. After four weeks the insects were removed and dissected to observe ovariole development.

Olfactometer Treatments. This experiment was designed to determine if ovipositional stimulation could be obtained using an olfactometer of the type used by Lu, Allen and Bollen (1957). Beetles were excised from infested material and transferred to

the central chamber of this two-choice-type of olfactometer. Individuals responding both positively and negatively to a combination of light and ethanol (95%) in one arm of the olfactometer were collected and placed in culture tubes preinoculated and uninoculated, with the mycelial form of A. hartigii. Daily observations were made for the presence of ambrosia in the tunnels and for evidence of oviposition. After four weeks, the insects were removed and dissected to observe ovariole development.

Physiological Aspects

Nitrogen Utilization

The various nitrogenous substances in faecal pellets, hemolymph or tissue extracts were identified by paper chromatography using Whatman No. 1 chromatographic paper. These nitrogenous components were extracted with 0.5% aqueous solution of lithium carbonate at 40° C for 15 minutes (Hoyt and Osborne, 1970). Unknown substances were identied by comparison of R_f values of standard substances run together in the varied solvent systems with the unknowns.

Uric acid, allantoin and urea were identified using either an ascending or descending single chromatogram run in 70% n-propanol (Berridge, 1965). They were visualized with the chlorine-potassium

iodide starch reaction (Rydon and Smith, 1952).

Allantoin and allantoic acid were identified in the various substances using an ascending single solvent system of butanol-ethanol-water (4:1:1, v/v/v) (Block, Durrum and Zweig, 1958). They were visualized with a spray of 1% diemthylaminobenzaldehyde in 2% alcoholic hydrochloric acid (Reddi and Kodicek, 1953). Measurements of uric acid in the body without pterine interference was achieved using a two dimensional development in iso-propanol--1% ammonia (7:3) followed by methanol--1% ammonia (7:3). Uric acid was located on the chromatogram using an ultraviolet lamp (254 mμ) and eluted off the paper with a 0.05 M solution of phosphate buffer (pH 8.0), eluate was made up to 5 ml and its absorption was determined with aBeckman grating spectrophotometer (Berridge, 1965). Uric acid values were recorded from a standard curve (Bhattacharya and Waldbauer, 1971; and Liddle et al., 1959).

Ammonia was extracted from whole animals and artificial diets by the method described by Staddon (1955, 1959) and isolated by the Conway (1963) microdiffusion procedure. Using the phenol-hypochlorite reaction (Russell, 1944) the concentrations of ammonia were read off from a standard curve of ammonium sulphate (Stegemann, 1958).

Total nitrogen determinations of attacked and unattacked wood (Malus sylvestris Mill) and artificial diets were measured on the

Technicon apparatus (Ferrari, 1960; Uhl et al., 1971). The analysis was conducted by Mr. F. Dickson, Department of Horticulture, Oregon State University.

A study was made to determine the minimal nitrogen (N) requirements of postdiapause adults for oviposition in artificial medium. The defined medium contained varying amounts of L-asparagine (1.0, 0.5, 0.25, 0.12, and 0.06% w/w) plus the following ingredients per liter: alphacellulose, 180.0 g; purified Bactoagar®, 45.0 g; and glucose, 18.0 g. Controls contained no L-asparagine. Ten tubes were prepared for each level of L-asparagine. Beetles were surface sterilized by the method of Retnakaran and French (1971) prior to release into the tubes.

Fixation of free or atmospheric nitrogen was investigated using an acetylene reductase procedure (Koch and Evans, 1966; and Koch, Evans, and Russell, 1967). The analysis was conducted by Mr. D. Israel, Department of Botany and Plant Pathology, Oregon State University. Postdiapause females (one per tube) were released onto (a) medium (L-asparagine), (b) medium (L-asparagine) preinoculated for four days with mycelia of A. hartigii, (c) nitrogen free medium; similar to medium in (a) and (b) with L-asparagine and Wessons Salt Mixture omitted, viz., - alphacellulose, 18.0 g; glucose, 1.8 g; Bactoagar®, 4.5 g; ethanol (95%), 1.0 ml; and distilled water, 100 ml. The gas volume (ca.22 mm^3) of acetylene in each reaction tube

was 0.1 atm. At the times indicated (day 1 and day 7 after beetles were released onto media), 1 ml samples of gas were removed through the serum cap of each tube and analyzed for ethylene by the gas chromatographic procedure (Koch and Evans, 1966). The serum caps replaced the absorbent cotton caps that were initially used with the preparation of the tubes.

Soluble protein contents of female X. dispar beetles collected throughout nine months of theyear were extracted and determined as follows: Beetles were homogenized in 1.0 ml of extraction solvent (0.01 N NaOH + 0.4 N NaCL). Spun for 10 minutes at 5,000 g. Decanted, and the supernatant retained. The pellets were rehomogenized. The homogenate (ca. 2 ml) was added to previous supernatant, made up to 10 ml with extraction solvent, stirred magnetically for 10 minutes and centrifuged at 10,000 g. for 10 minutes. The supernatant contained soluble proteins and total free amino acids.

Fractions (5 ml) of the supernatant were cooled in an ice bath prior to adding an equal volume of 20% trichloroacetic acid (TCA) to each tube. The mixtures were stirred, to initiate precipitation of the proteins, then spun at 10,000 g. for 10 minutes. The precipitate contained the soluble proteins, the supernatant contained the free amino acids. Protein analysis was then determined by the Lowry method (1951).

The modified ninhydrin colorimetric analysis for amino acids

as outlined by Rosen (1957) was adapted to measure the free amino acids in the extracts. A standard curve using Edamin® Type-S Enzymatic digest of lactoalbumin (Sheffield Chemicals, a Division of Natural Dairy Products Corp., Norwich, N. Y.) was prepared using approximately 120 g. digest per liter (= 1 M solution). To 1 ml samples of beetle homogenate extracts, 0. 5 ml cyanide-acetate buffer and 0. 5 ml 4% ninhydrin solution in Methyl Cellusolve were introduced. Heated for 15 minutes in a 100° C water bath for the color to develop. Immediately after removing from the water bath, 5 ml of isopropyl alcohol-water diluent was added. Then, vigorously shaken, and allowed to cool to room temperature, and read in a colorimeter at 570 mμ (proline and hydroxyproline at 440 mμ). If the color density in a given series of tubes was too high to determine accurately, further 5 ml portions of diluent were added, until the optical densities were below 0. 8.

Proteolytic enzyme activity of diapause beetles was investigated with the Azocoll procedure (Martin and Martin, 1970a). This included beetles that responded positively and negatively to light and ethanol in the olfactometer. The ambrosial and mycelial forms of A. hartigii were also investigated for proteolytic enzyme activity by this method.

Free amino acids in the excreta of diapause females were qualitatively examined by paper chromatographic methods of Roberts and Smith (1971).

Calculations of mean errors and analysis of variances were made with suitable programs designed and operated through the computer center at Oregon State University.

Results

Gallery Construction and Dissemination of Ambrosia

Field Observations. Postdiapause beetles caught in the rotary nets and from newly attacked host materials had empty intestinal tracts. In-flight female X. dispar beetles initiated their attacks on susceptible hosts in lenticular tissue (Figure 3) and on the underside of branches. These trees were dead, dying or diseased, and the majority were infested with the bacteria, Pseudomonas syringae van Hall (Cameron, 1962; 1970). Possibly the ethanol given off by bacterial metabolism was the primary cause of attraction for the pioneer or attacking beetles. Other workers (Person, 1931; Graham, 1969; Cade, Hrutfiord and Gara, 1970; and Samaniego and Gara, 1970) have demonstrated that ambrosia beetles are attracted to ethanolic odors. Rudinsky (1966) and Moeck (1971) caught X. dispar postdiapause beetles using ethanol. On splitting the infested host material, beetles were seen to have excavated simple branched gallery systems (Figure 4), and disseminated their fungus over the walls of the tunnels. The exact time of the developmental period

Figure 3. Showing infested Prunus sp., the entrance tunnels made by postdiapause X. dispar beetles are mostly in lenticular tissues.

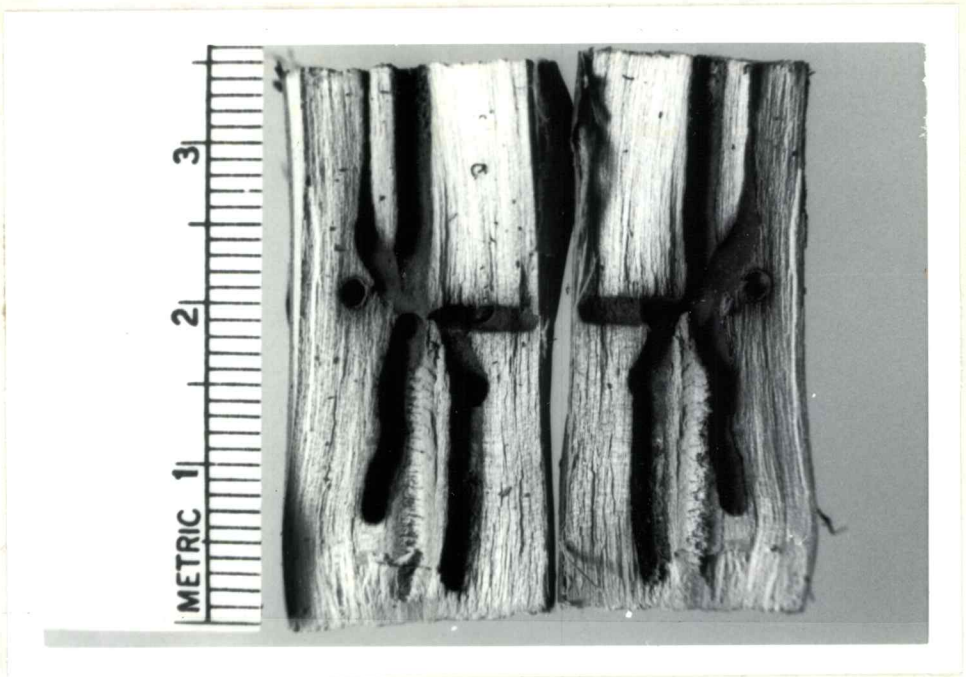

Figure 4. The gallery system of postdiapause beetles of X. dispar.

encompassing the initial attack, deposition of fungus, adult feeding on ambrosia, and subsequent ovariole development and oviposition was not obtained in this study. However, data (Table 4, p. 45) from in vitro studies indicated from 2-4 days tunnelling of the postdiapause beetle prior to visible ambrosia growth and ovariole development.

Table 3 shows the effect of probing attacks by postdiapause beetles. There was no deposition of ambrosia, and no beetles or progeny were found.

Laboratory Observations. Both postdiapause and diapause female beetles tunnelled into the artificial media within 10 minutes after being transferred into the culture tubes, regardless of whether or not the tubes were preinoculated with A. hartigii. Postdiapause individuals fed on the ambrosia fungus, which was apparent on the walls of the tunnels in the artificial media, within 2 days of the beetle transfer, Table 4 indicated the rate at which the ambrosia develops within the gallery system. Most of the ambrosia was laid down within 6 days. Hindgut dissections of the postdiapause beetles showed that feeding had taken place within 8-12 hours after the females were introduced into the medium. Prediapause and diapause beetles dissected during the months July through March had empty intestinal tracts.

In the successful culture tubes, i.e., in which progeny were produced, no chewings or frass were apparent in the tunnels. But in tunnel systems that did not produce ambrosia or progeny, frass

Table 3. Probing attacks by postdiapause X. dispar beetles. No deposition of ambrosia, and no oviposition was observed.

Gallery No.	Diam. host mm	Tunnel dimensions	
		Length mm	Volume mm^3
1	21	12	30.48
2	24	15	38.1
3	20	2	5.1
4	19	8	20.3
5	21	9	22.8
6	22	11	27.9
7	27	17	43.1
8	20	12	30.4

Average volume per female adult = 7.6 mm^3; per male adult = 3.8 mm^3.

was evident in much of the system. Beetles that did not produce ambrosia on the gallery walls within 10 days failed to oviposit. These either died in the tunnel system, or were found inactive, or dead, on the surface of the medium.

Table 4. Rate of development of ambrosia of A. hartigii forming in gallery systems of X. dispar postdiapause females cultures in vitro (133 tubes sampled).

Number of days beetle in tube	1	2	3	4	5	6	7	8	9	10
Cumulative percent of tubes showing ambrosia	0	33	59	74	83	85	91	96	98	100%

The addition of ethanol, which acted as a tunnelling (feeding) stimulant for X. ferrugineus (Norris and Baker, 1969) had no apparent effect on the postdiapause beetles of X. dispar. Beetles placed in ethanol treated tubes (40) and untreated tubes (40) showed no marked difference in tunnelling activity.

In approximately 50% of the tubes set up with active postdiapause beetles the tunnels were excavated adjacent to the side of the tube.

Tending of Brood

On several occasions the postdiapause female (mother) beetles were seen to assist or tend the newly emerging host instar larvae from the eggs. The mother 'pushed' the larvae with her pronotum onto the ambrosial lining of the gallery wall. This tending behavior

was also observed at later periods in the early larval stages. The mothers, after oviposition was completed, backed up the tunnel to the entrance and were seen to frequently return down into the gallery system to tend the larvae. Although the mother was seen to move among the pupae, no particular tending behavior could be discerned.

The question as to whether or not broods in the galleries could remain viable with the removal, or death, of the mother was not attempted due to the practical difficulties involved and the risk of fungal contamination. But studies were conducted in vitro.

Mating

Postdiapause beetles (20) collected in the rotary nets and 20 postdiapause beetles excised from host materials were dissected and shown to have sperm in their spermathecae (Figure 5). This suggested that they had been mated the previous summer within the old gallery. Males were seen to copulate with their mothers and sisters. They also mated with more than one female over the period of test. In an experiment 10 males mated with (a) their mother, (b) sister, and (c) several females from another gallery system that were born the same season.

Even after cold storage (5° C) for periods of several hours to three months, males and females quickly recovered and copulated within minutes of being mobile. The average period for copulation,

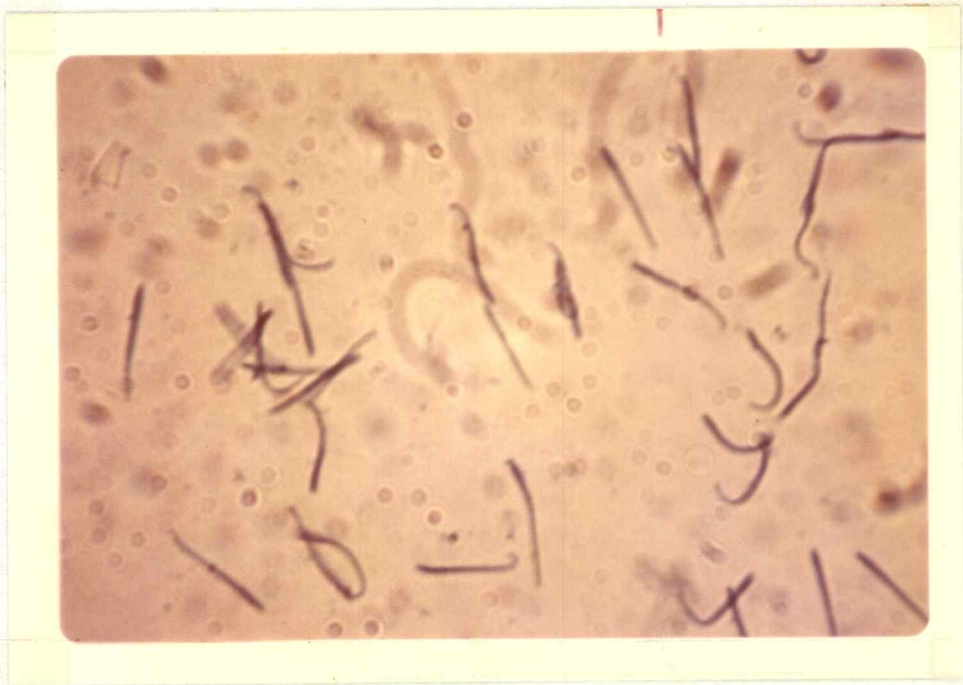

Figure 5. Presence of sperm in the spermatheca of a postdiapause X. dispar female beetle. X160.

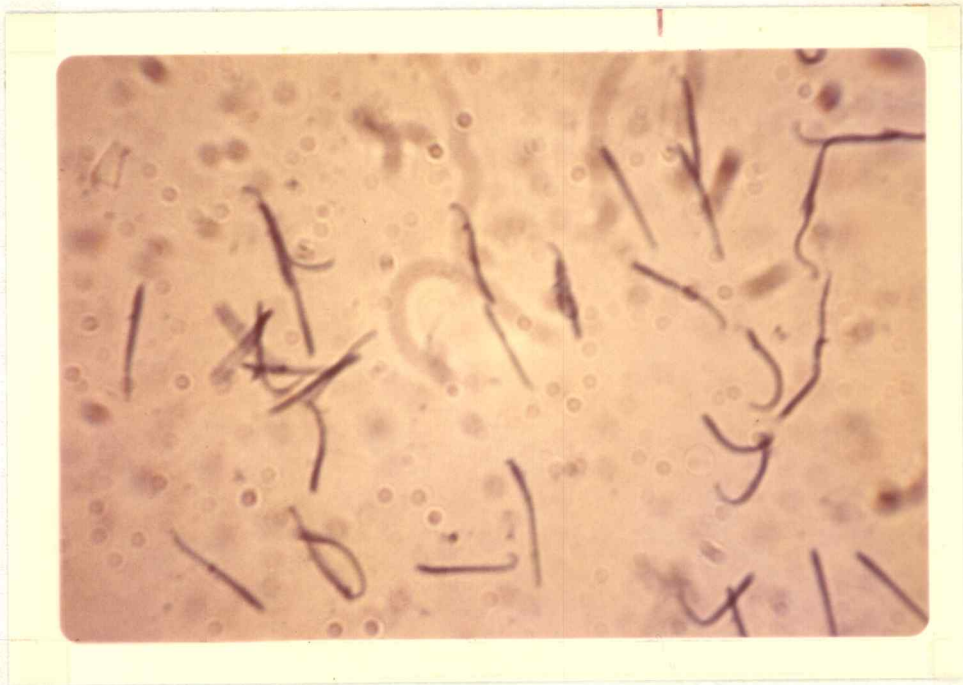

Figure 5. Presence of sperm in the spermatheca of a postdiapause X. dispar female beetle. X160.

whether from active galleries or from cold storage situations, was from 10 to 20 minutes. Male beetles were always sexually active when taken from their overwintering galleries and placed with females in petri dishes. Connin and Hoopingarner (1971) reported similar behavior in adult males of the cereal leaf beetles, Oulema melanopus (L.) (Coleoptera:Chrysomelidae). The males were always sexually mature and needed only to be with a receptive female to become active and disseminate viable sperm. In the species O. melanopus and X. dispar male activity appeared to be governed by the activity of the female.

It appears that persistent inbreeding and multiple brood fecundity is common in X. dispar. But, whether or not this insect depicts arrhenotokous reproduction as Norris and Chu (1970) stated was the case with X. ferrugineus, was not shown. They suggested that this genus includes nearly 1500 described species, and each has apparently most of the biological capabilities of X. ferrugineus.

Natural Enemies

Data from field collected material and the in vitro cultures did not show or indicate any natural enemies of this insect in Oregon. Wilson (1913) and Mathers (1940) reported similarly, and mentioned that this was probably due to the insects recent importation to North America.

On virus-diseased apple trees, collected at Odell, Oregon, in May (1971), many grape twig borers, Poa quadrisignata (LeC.) (Coleoptera:Bostrychidae) were seen actively moving over the trees. But they did not appear to attack X. dispar that were on the surface of the same trees.

Dr. J. Wernz, Department of Entomology, Oregon State University, identified several mites that were collected from within X. dispar galleries of these infested apple trees. The timber had been stored in the cooler (2° C) for about three months. The mites were from the family Anoetidae, or slime mites, and were in the hypopus or immature stage which is considered to be phoretic on beetles.

Fungal Contamination

In nature, few fungi, other than a Ceratocystis sp. and Schizophyllum sp. were found with A. hartigii in the galleries within infested trees. Roeper (personal communication) isolated a Ceratocystis sp. from postdiapause and diapause X. dispar females. The in vitro studies (cf. Chap. III, p. 25) indicated that the common contaminants were species of Aspergillus, Penicillium and Trichoderma.

Developmental Aspects

Mycangial Development

Beetles stored in the out-of-doors cages contained small amounts of fungus in their mycangia by the 1st week of February, 1971. Other beetles from these cages were removed indoors on 11 March and placed in galvanized emergence boxes Sampling the first emerging females on 17 March showed that all these postdiapause beetles had their mycangial cavities packed with ambrosia fungus (Figure 6). It was at this stage that the 280 strain of A. hartigii was isolated and cultured, which was used as the fungal source in this present study.

Schneider and Rudinsky (1969b) mentioned that the situation in the ambrosia beetle, Gnathotrichus spp. was similar in that the gland cells around the mycangium increased, and the first fungus hyphae were found in this structure in March. These authors reported no cell divisions at the end of the flight period; and in burrowing males the gland cells disintegrated. At the same time, most of the fungus mass had left the mycangia; the remaining fungus cells were very small globules of irregular shape.

X. dispar female adults had fungus in their mycangia during the flight and tending period; which was depleted by the last larval instar. The meteorological data for the periods of flight in 1968 and 1971

Figure 6. The mycangium of an X. dispar postdiapause female beetle packed with ambrosial form of A. hartigii. X 160.

showed that the mean temperatures range from 47.1 to 48.1° F (1968) and from 41.0 to 48.0° F (1971) respectively.

Measurements of 30 mycangia of X. dispar females showed that the mean width was 0.43 ± 0.032 (SE) mm and the mean length was 0.65 ± 0.04 (SE) mm. The mycangial contents of prediapause and diapause beetles contained little or no fungal cells (Figure 7). When these mycangia were smashed and plated on 0.6% malt agar at 25° C, the mycelial phase of the fungus eventually developed. This, however, took several days suggesting that a small number of fungal cells were present.

Ovariole Development

Dissections of 10 postdiapause female beetles trapped in flight showed that there was no food in the intestinal tracts, and no ovariole development (=ovaries were immature). However, the mycangial cavities were packed with ambrosia.

The result of ovariole development of beetles which were set up on the artificial medium are seen in Table 5. Sequential sampling of six females each day was carried out.

Tunnelling commenced within minutes after the beetles were introduced into the culture tubes. By the fourth day, the average tunnel per tube per female was about 6-7 mm long, twice the body length of the female adult. Ambrosia appeared on the tunnel walls

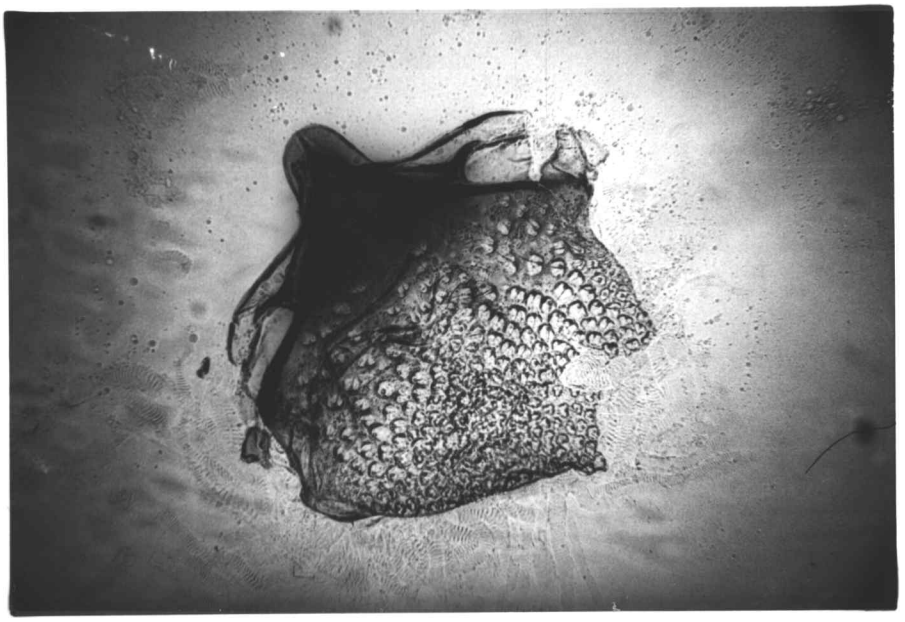

Figure 7. The mycangium of a X. dispar diapause female with no fungal cells visible. X 240.

Table 5. The result of the sequential sampling of X. dispar postdiapause females from artificial media, and extent of ovariole development as seen by dissections.

Day beetles removed after	Average tunnelling per beetle per tube (mm)	No. tubes ambrosia present	No. beetles with oocytes present	Mean no. oocytes per beetle	Mean no. eggs laid per beetle	Beetle feeding
2	3	0	0	0	0	0
3	6	0	0	0	0	0
4	6	2	1	1	1	+
5	6	5	3	2	1	+
6	9	6	6	2	3	+
7	9	5	2	2	2	+
8	12	5	4	3	5	+
9	12	5	5	2	7	+
10	15	4	4	2	3	+
11	15	4	4	2	3	+
12	15	3	3	1	5	+
13	18	4	4	1	5	+
14	18	2	2	2	6	+

by the fourth day. At the same time, feeding and ovariole development was first observed (Figure 8). In tubes in which no ambrosia had formed by the 6-10 day, the insect had not fed and no ovariole development was seen.

Beetles (6) which were set up in tubes with filter paper at the same time as the above beetles did not lay eggs. These beetles were dissected after 14 days and had immature ovaries and empty intestinal tracts. Abundant ambrosia was removed from their mycangia.

During these investigations a postdiapause field collected female was found with an abnormal reproductive system. The beetle had five ovarioles with five corresponding ovariole pedicels; three on one side and two on the other. These converged to a lateral oviduct on their respective sides and then into a common oviduct. Normally, the female has four ovarioles with two pedicels on each side. The lengths of the ovarioles (ca. 0.2 mm) and pedicels (0.8 mm) were average when compared with the mean lengths of 10 normally developed females collected at the same time. Due to the method of viewing this phenomenon, it was not possible to analyse egg production and persistence in subsequent generations. Hower (1971) found the same condition in two female alfalfa weevils; and the suggestion presented by him was that this was a random teratological condition. This could be the case with X. dispar; also, extreme environmental stresses such as overcrowding may have induced this malformation.

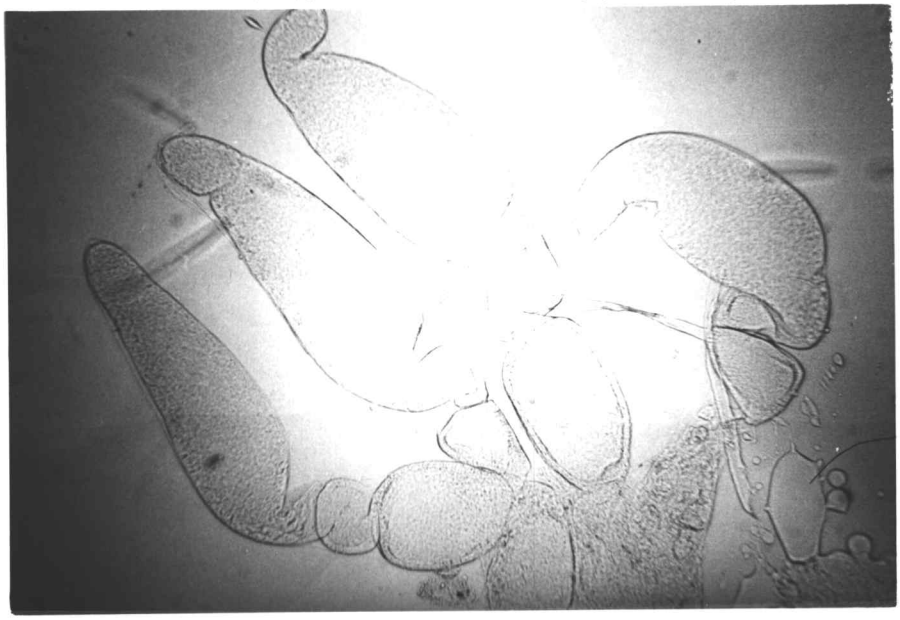

Figure 8. The presence of two oocytes in the ovary of X. dispar postdiapause female beetle after the insect was put on an artificial diet for four days.

Number of Progeny

The number of galleries observed, eggs laid, and the amount of ambrosial growth found on the walls of tunnels in infested field collected materials were recorded (Table 6). No eggs were found in galleries in which the ambrosia had not developed, and only three eggs were seen when the ambrosia was slightly developed. In galleries with full or complete ambrosial development the number of the eggs laid ranged from 6-20 per gallery.

Table 7 shows the number of eggs laid and the volume of the gallery systems. Comparisons of the number of eggs laid and the volume of galleries were not made as oviposition was incomplete at the time the field infested material was sampled. But in every gallery the ambrosia was fully developed like a fungal mat over the entire tunnel surface. Color of the ambrosia was pale-buff to creamy-white. The postdiapause beetles expelled their mycangial contents by the period of early larval instars. Head capsule measurements were not recorded in this study. However, Gadd (1947) reported that three stadia could be distinguished with X. fornicatus Eich.; and Brader (1964) distinguished the same number of stadia with X. compactus Eichh. so X. dispar may have a similar number of stadia.

Larval-pupal transformations in the host were marked by a noticeable depletion of ambrosia on the tunnel walls. Table 8 shows

Table 6. The number of galleries observed, eggs laid by X. dispar postdiapause females and the amount of ambrosia on the walls of the galleries in field collected host materials.

No. of galleries observed	No. of eggs per gallery	No. of eggs found in galleries as related to the amount of ambrosia on the tunnel walls			
		Full ambrosia	Medium ambrosia	Slight ambrosia	No ambrosia
117	1-5	69	26	3	
34	6-10	232	24		
9	11-15	113			
6	16-20	104			
1	22	22			
1	30	30			
1	34	34			

Table 7. Number of eggs laid by X. dispar postdiapause females in field collected host material, amount of ambrosia present, and diameters (mm) of host, lengths (mm) and volumes (mm^3) of the galleries.

Gallery No.	Eggs found	Walls covered fully by ambrosia	Gallery Data		
			Diam. mm Host	Length mm	Volume mm^3
1	4	+	22	20	50.8
2	10	+	20	18	45.7
3	2	+	19	32	81.3
4	3	+	22	25	63.5
5	8	+	16	25	63.5
6	4	+	40	26	66.0
7	7	+	28	24	60.9
8	6	+	30	21	53.3
9	15	+	36	16	40.6
10	9	+	37	19	48.2
11	4	+	22	20	50.8
12	10	+	20	18	45.7
13	2	+	19	32	81.2
14	3	+	22	20	50.8
15	8	+	16	25	63.5
16	4	+	40	24	60.9
17	7	+	28	25	63.5
18	6	+	28	24	60.9
Total	112.0			414.0	1051.5
Mean	6.2			23.0	58.4

Table 8. The number of larvae, pupae, teneral adults of X. dispar and amount of ambrosia on the gallery walls in field collected host materials.

Ref observations	No. larvae	No. pupae ♂♂	No. pupae ♀♀	No. teneral adults ♂♂	No. teneral adults ♀♀	Ambrosial growth: Full	Medium	Slight	Nil
1	61	-	2				+		
2	4	1	3					+	
3	3	1	4					+	
4	3	1	5					+	
5	4	1	8					+	
6	10	2	12					+	
7	2	1	7						+
8	10	-	-	3	19			-	+
9	2	-	2	-	-			-	+
10	-	-	-	1	2			-	+
11	18	3	19					+	
12	19	5	32					+	
13	13	4	26					+	
14	8	3	11					+	
15	8	4	15				+		
16	17	4	7					+	
17	9	4	18					+	
18	31							+	
19	21							+	
20	3	1	12				+	+	
21	14	1	3					+	
22	17							+	
23	15	1	20					+	
24	14	1	12					+	
25	15	1	7					+	
26	16	2	25					+	
27	10	2	12					+	
28	4	1	4					+	
29	3	1	4					+	
30	3	1	5					+	

the number of larvae, pupae and teneral adults in host material. Marked gradations of larval size were observed which were considered to be the result of intraspecific competition. Brader (1964) also observed this variation of larval size in X. compactus. Many of the X. dispar larvae had small larvae adhering to their body surfaces, as many as three larvae on one pupa were seen. These larvae usually died shortly after removal from the pupae. At the pupal stage the ambrosia was virtually depleted from all the tunnel surfaces, except for a small plug of fungus at the distal ends of the galleries (Figure 9). This appeared to be a source of food for the teneral adult, and probably the ambrosial spores were taken into the mesonotal mycangia at this time. During hibernation and maturation diapause the adults do not feed, as the ambrosia is absent in the tunnel systems.

The prediapause and diapause populations in the galleries of field collected material, diameters of hosts, lengths and volume of galleries were examined and the results shown in Table 9. The average number of progeny recorded was 16.8 per gallery system. The sex ratios varied from 1 ♂: 2 ♀ to 1 ♂ : 8 ♀, with the mean at 1 ♂ : 2.2 ♀. Every gallery examined during 1970 and 1971 contained at least 1 male and 1 female. In Figure 10 the number of progeny per female were plotted against the volume of the galleries. The relationship was linear and the correlation for the 26 observations was 0.69.

Figure 9. A pupa of X. dispar at the distal end of a gallery. Note the plug of fungus at the end of the tunnel.

Table 9. The number of progeny of X. dispar produced and the diameters of the host materials, and the lengths and volumes of the gallery systems.

Ref. No.	No. Progeny	Male	Female	Sex Ratio ♂♂ : ♀♀	Diam. Host mm	Gallery Data Length mm	Gallery Data Volume mm^3
1	64	44	20	2: 1	40	153	338.6
2	4	2	2	1: 1	40	25	63.5
3	18	2	16	1:16	41	90	228.6
4	16	1	15	1:15	41	90	228.6
5	43	27	16	1.7: 1	40	66	167.6
6	8	1	7	1: 7	40	20	50.8
7	24	3	21	1: 7	40	92	233.7
8	20	5	15	1: 3	38	88	223.5
9	10	1	9	1: 9	40	91	231.1
10	5	1	4	1: 4	34	40	101.6
11	4	1	3	1: 3	42	57	144.8
12	3	1	2	1: 2	40	40	101.6
13	4	3	1	3: 1	42	44	111.7
14	4	2	2	1: 1	37	70	177.8
15	15	4	11	1:2.7	40	71	180.3
16	12	1	11	1:11	48	43	109.2
17	20	2	18	1: 9	48	94	238.7
18	24	1	23	1:23	48	83	210.8
19	27	2	25	1:12	48	71	180.3
20	16	2	14	1: 7	50	63	160.0
21	28	4	24	1: 6	65	143	363.2
22	11	1	10	1:10	65	90	228.6
23	13	2	11	1:5.5	66	67	170.2
24	15	1	14	1:14	66	91	231.4
25	17	2	15	1:7.5	75	105	266.7
26	12	3	9	1: 3	75	68	172.7
Total	419	142	318	1:2.2		1183	4,965.3
Mean	16.81	5.46	12.2			45.5	190.97

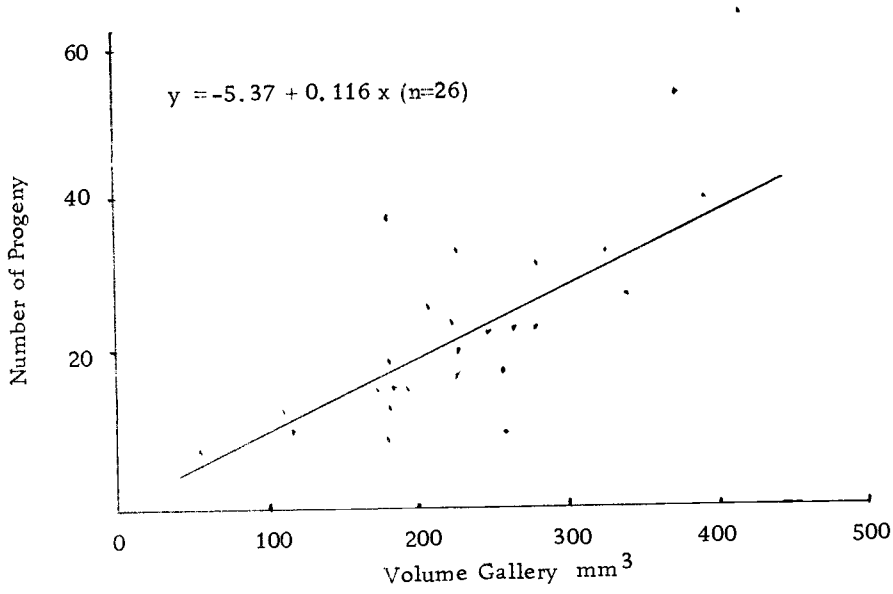

Figure 10. The relation between the number of progeny per female X. dispar and the volume of the gallery.

Figure 10. The relation between the number of progeny per female X. dispar and the volume of the gallery.

Cessation of Diapause

Effects of Temperature on Diapause. All the diapausing beetles tunnelled into the media in the tubes prior to the temperature treatments. Details of the experimental plan, number of days exposed to the various constant and alternating temperatures and number of adults alive at the end of this test are shown in Table 10. It appears that few beetles survived the alternating temperatures. The highest mortality was recorded in this regime. No beetles survived at any temperature after 90 days exposure. On transferring the females after the various periods of exposure, no oviposition was recorded. After 14 days at room temperature there were no eggs laid nor was

Table 10. Number of X. dispar diapause females surviving the constant and alternating temperature regimes. Twenty-four beetles were used per treatment.

Duration of exposure (days)	Temperature Regimes (°C)			
	2° C *	7° C *	25° C*	2-25° C
7	24	24	24	20
10	24	24	23	19
20	20	24	23	17
30	21	10	19	9
40	18	19	4	0
50	8	7	0	0
90	0	0	0	0
360	0	0	0	0

*Constant temperatures.

ovariole development apparent in any female dissected. Beetles that were exposed to 2° and 7° C for over 50 days all died. The media in the tubes after three months was dehydrated and no signs of beetle activity were observed. The mycelial form of A. hartigii persisted over the period, but was not viable when re-inoculated on fresh malt agar plates after 60 days. No ambrosial form of the symbiont was seen in any of the tubes.

The beetles in the alternating temperature regime were all dead by the 30th day. No eggs were laid, and no ovariole development occurred. The media was extremely dehydrated and the symbiotic fungus was not viable when subcultured.

Clearly, these temperature and time regimes were not successful in terminating diapause in X. dispar adults. It would seem that a combination of interacting factors are required to effect cessation of diapause in this insect.

Juvenile Hormone Analog JHA Treatments.

Series I. Using JHA from Calbiochem. Co., California and the mycelial form of A. hartigii. Details of the effects of the JHA topically applied to diapausing X. dispar female beetles in October 1970 are shown in Table 11. Beetles bored into the media in all tubes. After four weeks, high mortality was noted; no feeding or ovariole development had occurred. The cultural form of A. hartigii was mycelial at the end of the test. Contamination by

Aspergillus sp. was found in two solvent control tubes, and in one tube in which the beetle had been treated with 1.4 µg/µl of JHA.

Table 11. Effects of topically applying JHA to X. dispar diapausing beetles released onto the mycelia of A. hartigii in culture tubes.

Treatment conc.(µg/µl)	No. beetles used per treatment	Dose per beetle (µl)	% Mortality after 4 weeks
25	10	2	50
14	10	2	60
1.4	10	2	60
(Acetone) solvent control	10	2	40
Untreated control	10	-	20

Sequential Topical Application of JHA. The effect of the sequential topical application of JHA to diapausing beetles in January 1971 indicated that diapause was not terminated. As with the Series I experiments, 50 µg of JHA produced no desired morphogenic effect on this scolytid. Irrespective of storage regimes prior to treatment, there were no eggs produced by any beetle. After four weeks, beetles were removed, dissected and found to have no ovariole development. Fungal contamination, mainly Penicillium sp. was prevalent in most tubes. This suggests contamination at the time of JHA application, as the JHA was not filter sterilized when applied. Only two tubes had traces of ambrosia present as verified by microscopic examination. These tubes had beetles that were treated with 2 µl of 14.0

and 1.4 μg/μl of JHA per beetle respectively. As the test terminated about 3-4 weeks before the 1971 flight period, it seems that any morphogenic changes at this stage could be attributed to the altering environmental factors, which would naturally cause cessation of diapause, rather than the applied JHA. Future experiments could consider this possibility!

Series II. Using JHA (ZR-512®) from Zoecon, California.

Results of this experiment are detailed in Table 12. Ambrosia was observed in the galleries in the $(NH_4)_2SO_4$ media within three days of releasing the treated females into the tubes. Of the three tubes with developing ambrosia, one of the tubes was a solvent control. The ambrosia in this tube was by far the most luxuriant. Eight days after the commencement of the test, ambrosia was apparent in all the tubes, even in tubes which contained untreated beetles. At the same time, two tubes with the casitone media were seen to have ambrosia in the galleries. But this ambrosia diminished five days later, and was overgrown by a yeast sp. and the tube discarded. Dissection of the beetle showed no change from its original diapausing condition. Beetles in the casitone media had no developed ovarioles and feeding was not observed. The beetles in the $(NH_4)_2SO_4$ media fed and excreted uric acid but after four weeks no eggs were produced and no ovariole development was seen. The ambrosia in these tubes diminished by the 20th day of test, and by the end of the

Table 12. The effect of topically applying JHA (ZR-512®) to X. dispar diapausing beetles. Dose per beetle was 2 µl of 20 µg/µl of JHA.

Treatments	No. beetles treated	Media used*	No. tubes with ambrosia	No. beetles feeding
Treated control	6	Casitone	2	0
Solvent control	2			
Untreated control	2			
Treated control	6	$(NH_4)_2SO_4$	6	6
Solvent control	2		1	
Untreated control	2		1	
Treated control	6	L-asparagine	0	0
Solvent control	2			
Untreated control	2			

*The minimal media was used throughout this study unless otherwise stated. Casitone and $(NH_4)_2SO_4$ media indicated that L-asparagine in the minimal media had been replaced by each of these two components respectively.

experiment it was barely visible. All beetles, after initial tunnelling, were inactive during the remainder of the test. In the minimal media no ambrosia was observed and dissections of the beetles showed no feeding or ovariole development had taken place.

Olfactometer Treatments. Table 13 summarizes the data obtained using the olfactometer (Lu, Allen and Bollen, 1957). The majority of diapause beetles responded negatively to the light and ethanol. The response in culture media showed little differences between treatment regimes. Most beetles tunnelled into the medium and even in those that had not, the ambrosia form of A. hartigii was seen on the surface of the mycelial form. Fungal contaminants, mainly Penicillium spp., overgrew about a quarter of the cultures set up. No oviposition was found in any tube; and on dissection no ovariole development was recorded. Only two beetles had fed on the ambrosia, both had responded differently to light and ethanol. The beetle responding positively had the mycangium full of ambrosial cells.

Regardless of how the beetles responded in the olfactometer, none were induced to terminate diapause.

Nitrogenous Excretory Products

The qualitative examination of the main nitrogenous excretory products of the various life stages of X. dispar are shown in Table 14.

Table 13. Numbers of X. dispar diapause females responding (+) and not responding (-) in the olfactometer and their cultural responses after four weeks.

Media	No. beetles tested initially	Olfactometer responses (+)	Olfactometer responses (-)	Cultural responses: No. beetles tunnelling	No. tubes with ambrosia	No. tubes showing oviposition	No. tubes in which ambrosia eaten	No. tubes with fungal contamination
L-asn	66	18	48	53	55*	0	2	11 (22%)
L-asn	26	Not tested		26	5	0	0	6 (24%)
$(NH_4)_2SO_4$	15	15	0	13	3	0	0	4 (26%)

*Dissections after 4 weeks indicated 2 beetles had eaten ambrosia.

Table 14. Qualitative examination of the nitrogenous excretory products of X. dispar based on a minimum of 20 individuals per life stage tested.

Substance	Postdiapause beetles	Diapause beetles			Larvae		Pupae
	Homogenates	Homogenates	Excreta	Hindgut	Homogenate	Excreta	Homogenates
Uric acid	+	+	+	+	+	+	+
Allantoin	-	-	-	-	-	-	-
Allantoic acid	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-
Ammonia N	+	+	+	+	+	+	+
Amino acids	+	+	-	NT*	NT	NT	NT

(+) Present (-) Absent

*NT = Not tested.

Uric acid was the most important nitrogenous excretory product produced by X. dispar. This is characteristic of the majority of terrestrial insects, and the degradation products of uric acid are minor constituents of the excreta of many insects (Gilmour, 1965), although such products were not detected in the present study. The uric acid was excreted in solid form by the diapause adults and larvae. The postdiapause beetles tested had been stored at 2° C for several months prior to testing homogenates of the whole bodies and indicated the presence of uric acid. Hindgut dissections were difficult to obtain due to the dehydrated state of the insects. Tests for allantoin, allantoic acid, and urea were negative with all the life stages of X. dispar. Although allantoin was found once in the frass from diapause beetles which had tunnelled into the L-asparagine medium, eight similar frass samples run at the same time gave a negative response to allantoin. Rechecking for allantoin in a further six samples of frass from other diapause females failed to show the presence of allantoin on the chromatograms.

Uricase was not investigated in excreta, frass or whole body homogenates of the insect, in view of the repeated negative tests for allantoin.

Quantitative analyses of the uric acid in the hindguts of diapausing X. dispar females indicated a range of 7.8-14.8 μg uric acid/beetle (Table 15).

Table 15. Quantitative uric acid determinations from the hindguts of X. dispar diapausing females.

Absorbance reading at 290 mμ	Concentration (μg uric acid/ beetle
.188	14.8
.187	14.7
.186	14.6
.180	14.3
.178	14.2
.172	13.7
.169	13.3
.168	13.2
.159	12.5
.155	12.4
.147	11.7
.111	8.8
.102	8.3
.101	8.2
.099	7.9
.098	7.8

Table 16 showed the values for ammonia extracted from whole individuals in various life stages of X. dispar. The ammonia values for postdiapause females (1.13 μg·NH_3·N/beetle was higher than for the diapause females (1.08 μg NH_3·N/beetle. The total ammonia content of the postdiapause and diapause females averaged about 0.30 μg N/mg. wet weight of tissue. Pupal concentrations of ammonia-N were double those values of the larvae. The total ammonia content of the larvae and pupae averaged 0.09 and 0.16 μg N/mg. wet weight of tissue respectively. The ammonia-N content of fecal pellets (ca. 0.0025 gm. dry weight/female) of ten diapause females averaged 0.60 μg N/mg. dry weight of fecal matter.

Analyses of the excreta of diapausing females for free amino acids were negative. After spraying Ehrlich's reagent over the inhydrin on the chromatograms, no purine derivatives were visualized. This suggested that allantoin and urea were not present. Uric acid was not detected by this method as it does not react with Ehrlich's reagent.

Total Nitrogen Determinations

Table 17 shows the total nitrogen (N) determinations of the samples of attacked and unattacked sapwood of Malus sylvestris Mill. The unattacked sapwood contained the highest percentage of N. The attacked sapwood was sampled at two distinct life stages of X. dispar.

Table 16. Quantitative total ammonia-N determinations on the various life stages of X. dispar.

Life stage examined*	Average wet weight / individual (mg.)	Range $\mu g\ NH_3 \cdot N$ /individual	Average value $\mu g\ NH_3 \cdot N$ /individual
Postdiapause	3.73	0.70 - 2.40	1.13
Diapause	3.70	0.65 - 1.33	1.08
Larvae	3.71	0.15 - 0.75	0.37
Pupae	4.30	0.20 - 1.00	0.70

*Minum of 10 individuals per test.

The 'pre-brood' (i.e., prior to oviposition and hatching) and 'post-brood' sapwood samples showed that the latter samples were about 50% lower in total N than the former samples.

Table 17. Total nitrogen content of unattacked and attacked samples of M. sylvestris sapwood. Attacked samples had contained postdiapause ('pre-brood') and diapause ('post-brood') females of X. dispar.

Component	N content (% dry weight)	
	Run 1	Run 2
Unattacked wood	0.34 ± 0.05	0.37 ± 0.05
Attacked wood, postdiapause beetles, (pre-brood)**	0.31 ± 0.05	0.31 ± 0.04
Attacked wood, diapause beetles (post-brood)**	0.17 ± 0.02	0.18 ± 0.02

*Mean of 6 observations per component per run. (± S.E.)
**Beetles were excluded in the total N determinations.

Minimal Nitrogen Requirement for Oviposition in vitro

Table 18 details the results of an investigation to determine the minimal N requirements of postdiapause females for oviposition in vitro. Only in the 1.0% L-asparagine cultures (0.08% N dry weight) was oviposition observed. It seemed that the optimum level for the ambrosia to grow and stimulate ovariole development, and subsequent oviposition, was between 0.5-1.0% L-asparagine (0.04-0.08% N dry weight). Other in vitro studies had shown that viable broods were produced after the postdiapause females had fed on the ambrosial form of A. hartigii, so this experiment was not continued after the

Table 18. Minimal total nitrogen requirement in the artificial media by X. dispar postdiapause females for oviposition and hatching in vitro. Ten tubes were used per treatment.

L-asparagine (%) in media	N equiv. (%) dry weight	Observations in tubes after 4 weeks			
		No. tubes with eggs	No. eggs (total)	No. larvae observed	No. tubes with fungal contamination
Nil	0	0	0	0	2
1.0	0.1	9	68	64	1
0.5	0.08	0	0	0	0
0.25	0.04	0	0	0	0
0.12	0.02	0	0	0	0
0.06	0.02	0	0	0	0

fourth week.

Nitrogen Fixation by X. dispar

Results of the acetylene reductase procedure to investigate nitrogen fixation by postdiapause females are shown in Table 19. The amounts of ethylene in the tubes indicated no fixation of atmospheric nitrogen. Dr. H. J. Evans[1] (personal communication) observed that for a reaction suggesting positive nitrogen fixation, at least a ten-fold increase in the highest amount of the acetylene reduction values would be anticipated. He suggested that the level of ethylene in the controls represented the background level of ethylene in the laboratory atmosphere.

Soluble Proteins

Results of the Lowry method to determine the soluble proteins of X. dispar females sampled from March through October 1971 are shown in Table 20. The highest protein concentration was found in the postdiapause females sampled in March. This concentration was significant at the 0.05 level of probability compared to the concentrations of the other months. At this period the mycangia were packed with plugs of ambrosial fungus prior to the females initiating attacks

[1] Dr. H. J. Evans, Botany and Plant Pathology Department, Oregon State University, Corvallis.

Table 19. Amount of acetylene reduction in tubes containing X. dispar postdiapause females with and without their symbiotic fungus and on media, containing nitrogen (L-asparagine) and nitrogen free ingredients. Two tubes per component were sampled as indicated.

Components of culture tubes	Ethylene produced (n moles/reaction/hr) in tubes sampled on day 1 and day 7 after beetles released on media	
	Day 1	Day 7
Beetle	0.08*	0.07*
Beetle + Media (L-asn.)	0.10	0.91
Beetle + Media (N free)	0.14	0.11
Beetle + Media (L-asn.) + Fungus	0.88	0.95
Media (N free) + Fungus	0.16	0.16
Media (L-asn.)	0.14	0.08
Media (N free)	0.11	0.89
Empty tubes	0.08	0.06

*Mean of 2 observations.

Table 20. Concentration of soluble proteins of whole body homogenates of X. dispar females sampled from March - October 1971 (μg/mg dry weight).

Month samples	No. beetles used per sample	Soluble proteins ** (μg/mg dry weight) Mean (± SE)
March	14	128.0 ± 0.37
April	30	46.0 ± 0.22
May	30	44.0 ± 0.58
June	20	30.0 ± 0.36
July	30	51.0 ± 0.43
August	20	54.3 ± 0.43
September	30	51.8 ± 0.13
October*	30	60.0 ± 0.39
October	30	61.5 ± 0.31

*Beetles collected in 1970 and stored at 2° C until analyzed.

**Mean of 6 observations.

on new trees and the dissemination of the spores. Whereas beetles sampled from the galleries after March showed a marked decrease in protein concentration. Beetles sampled between April to September remained approximately constant in their protein concentrations. The beetles collected in October 1970, and stored for 12 months at 2° C prior to analysis, and those collected in October 1971, gave values of 60.0 and 61.5 μg protein/mg dry weight of beetle respectively. This seemed to indicate that the protein concentration had varied little with cold storage.

Attacked and unattacked wood was sampled, and the average of four samples per wood-type recorded were 0.69 and 0.28 μg/ml respectively. The attacked wood, with the ambrosial form of A. hartigii lining the gallery system, gave an expected higher value than the unattacked samples.

Free Amino Acids

The data of the ninhydrin colorimetric analysis of the free amino acids of X. dispar females sampled from March through October 1971 are summarized in Table 21. Variations in the concentrations of the free amino acids of X. dispar females sampled ranged from 60-410 μmoles/g dry weight. The postdiapause female fractions were the lowest at 60-170 μmoles/g dry weight. In May, the highest concentrations of free amino acids were found. This concentration was

Table 21. Concentration of free amino acids of whole body homogenates of X. dispar females sampled from March - October 1971 (μmoles/g dry weight).

Month sampled	No. beetles used per sample	Free amino acids** (μmoles/g dry weight) Mean (SE)
March	14	60 ± 0.44
April	30	170 ± 0.31
May	30	410 ± 0.26
June	20	132 ± 0.81
July	30	238 ± 0.59
August	20	270 ± 0.18
September	30	138 ± 0.21
October*	30	177 ± 0.28
October	30	125 ± 0.27

*Beetles collected in 1970 and stored at 2° C until analyzed.

**Mean of 6 observations.

significant at the 0.05 level of probability compared to the concentrations of the other months. It seemed that with the onset of winter, the free amino acids were depleted.

Determinations of the free amino acids for the four samples of attacked and unattacked woods gave values of 0.60 and 0.30 μmoles/g dry weight respectively.

Proteolytic Enzyme Activity

Examinations of the hindguts (10) of diapause females, their excreta, larval and pupal excreta, and ambrosial and mycelial forms of *A. hartigii* gave negative responses in the Azocoll procedure. This indicated that there was no proteolytic enzyme activity in these components tested at this particular time.

Discussion

Fungus-Insect Relationships

The behavioral, developmental, and physiological aspects of *X. dispar* were separated for purposes of amplification, but in reality the insects are vibrant composites of all these activities. Present patterns of behavior and development of *X. dispar* have evolved by their association with the symbiotic fungus, *A. hartigii*. The selection of a suitable substrate by the postdiapause females ensured the

continuation of this ectosymbiotic association. The interdependency was shown in the field and laboratory experiments. The basic biological process of interspecific microbial competition was illustrated. Outside of the mycangia and tunnel systems the beetles' fungus was a poor competitor and excluded from otherwise suitable substrates by better competitors. Whereas in the mycangia and gallery, it was the dominant species. So the fungus-culture activity of the beetle had reversed the interspecific fungal competition. Activities such as tending, cropping, secretion, and excretion by the postdiapause females enabled the ambrosial form of A. hartigii to remain the dominant fungal species. Also, A. hartigii was the single crop of X. dispar, confirming the studies of Francke-Grosmann (1956, 1957).

Seasonal variations of the fungus in the mycangia of X. dispar were confirmed, and seen to coincide with environmental stimuli. Barras and Perry (1971) and Happ[2] (1972, personal communication) have shown that the activity of the gland cells around the mycangia selectively influence their fungal existence during the period between emergence and attack on new hosts. Further histochemical and electron-microscopy investigations will probably determine the biochemical factors affecting this seasonal variation in X. dispar, and other scolytids.

Ovariole development and oviposition only occurred after the

[2]Dr. G. Happ, Department of Biology, University of New York, New York.

postdiapause female had fed on the ambrosial form of A. hartigii. Francke-Grosmann (1967) reported that several other workers had found a similar phenomenon with other scolytids. Also, these beetles attacked a new host with empty intestinal tracts, which was observed with X. dispar.

Roberts (1968) working on the platypodid Trachyostus ghanaensis Schedl found that if the fungus failed to grow on the walls of the tunnels the adults eventually died. He observed no incidence of beetles initiating galleries, then moving to another site to construct a tunnel, if the ambrosia fungus failed to grow. He suggested that the beetles utilised their food reserves and that degeneration of the flight muscles occurred. This does not appear to be the case with X. dispar as galleries 15-17 mm in length were excavated into the hosts 15-17 mm, with no sign of beetles or fungus. It appears that females of X. dispar are able to excavate at least one probing gallery, in order to discern whether or not the fungus will be able to develop. If it fails after probing just over its own length, the female selects other areas in the host materials.

The relationship between the number of progeny and the volume of the galleries was linear. This was comparable to the results of Brader (1964) who explained the main factors involved, namely (a) the female excavates her gallery in proportion to her reproductive capacity, and (b) the quality of the tissues in which the females

extend their galleries determines the volume of the gallery, and consequently the numbers of eggs laid. Brader considered that the most important factor was the quality of the tissues, a conclusion that is more tenable judging from the results of the in vitro rearing (See Chap. III, p. 19).

As the insect completes its life cycle and hibernates within the same gallery system, studies of population dynamics of this beetle may be readily conducted. Especially as no predators or parasites of X. dispar were recorded and mortality factors were considered to be due to intraspecific competition. Such studies may also provide evidence that relate climatic effects and bacterial canker (Cameron, 1970) to the density of the beetle populations in a study area.

Termination of Maturation Diapause

The attempts at terminating maturation diapause in X. dispar were not successful. Although Novak (1963) reported that temperature was the most important factor for inducing or interrupting maturation diapause in the ambrosia beetle Trypodendron lineatum 01. it did not stimulate ovariole development in X. dispar after cold treatments of 90 days and more.

The absence of ovariole development associated with adult diapause in some Coleoptera is due to lack of the corpus allatum hormone (Sahota, Chapman, and Nijholt, 1970). However, the results in the

present study suggest that JHA tested were not able to stimulate ovariole development in diapause beetles. Although some beetles fed on the ambrosia, still no eggs were produced. It seems that other factors besides just a high titer of a JHA and the presence of the ambrosial form of A. hartigii are required for ovariole stimulation and development. Light and ethanol stimuli were unable to start ovariole development in this insect. There may be inhibition of the neurosecretory system due to a lack of trophic stimulation or through active inhibition by some unknown factor. Whatever the contributing factor(s), and in what combination(s), maturation diapause remains the biggest obstacle in rearing such insects in vitro.

Physiological Aspects

The uric acid concentration found in the hindguts of diapausing beetles indicated only the proportionate composition among the substances assayed. In some cases it is possible that unidentified substances, or substances not quantitatively estimated may exceed uric acid in importance. So, these uric acid values should be regarded as no more than generally indicative of the proportionate composition in the diapausing adult phase in the life cycle of X. dispar.

It seemed that the A. hartigii was not able to degrade uric acid to allantoin, allantoic acid, urea or ammonia, suggesting that allantoicase and urease were not present. These enzymes have not yet

been found in insects. Also, there is no evidence, as yet, to suggest that ammonotelic insects produce ammonia by the degradation of uric acid (Staddon, 1955; and Stobbart and Shaw, 1964). In vitro cultural studies of A. hartigii indicated that ammonia and NH_4^+ caused ambrosial induction (Roeper, unpublished data) though not ambrosial growth and differentiation.

Although Hopkins and Logfren (1968) explained that uric acid stored in the fat body of insects is strongly bound to proteins, peptides and amino acids at the physiological pH, the evidence to date indicated that uric acid was the major excretory product of purine and protein metabolism in X. dispar. It seemed that this insect did not show a pronounced shift in excretory metabolism during the course of its life cycle. Bursell (1967) mentioned that where this is the case an insight may be gained relating the observed changes in the proportion of different end products to the level and type of nutrition and to the availability of water. However, a more intensive study of excretory metabolism in X. dispar may be undertaken when a complete analysis is made in terms of protein, nucleoprotein, carbohydrate and lipid composition of the ambrosial form of A. hartigii. Then one may be able to resolve these aspects of nitrogenous metabolism as it may be able to rigidly control insect nutrition, N intake, culturing aposymbiotic beetles in holidic diets and asceptic rearing conditions (Norris and Chu, 1970).

Reviews by Gilmour (1965) and Bursell (1967) indicated that insects and birds synthesize uric acid in the same way, although the complete pathway of uric acid synthesis in insects has not been fully documented. Barrett and Friend (1970), using isotopically labelled precursors, determined the origin of all five carbon atoms of uric acid. Further, they reported that uric acid with its low solubility and low toxicity, is an extremely efficient molecule to use as an end product of nitrogen, metabolism in animals where water conservation is a problem. Considering this, the similarities of uric acid metabolism found in such diverse groups as insects, birds, reptiles, and land snails become less surprising. Lamb (1959) mentioned that when proteins or amino acids are digested by animals, ammonia is usually converted into various nitrogenous compounds (uric acid, urea) before excretion, but it is sometimes excreted unchanged, and he suggested that this is correlated with ecological habitats. Levenbook *et al.* (1971) stated that at eclosion nearly all the uric acid is accumulated in the meconium; the transport mechanism by which this is achieved is entirely unknown, even though its rapidity would indicate a highly active system. Further experiments are required on the pupae of *X. dispar*, particularly the meconium, to investigate all the metabolic products including meconial amino acids. Examination of the excreta of diapause females indicated a negative ninhydrin reaction; and the excreta of postdiapause females is yet to be analyzed.

Martin and Martin (1970a) demonstrated that all 21 of the common natural amino acids were present in the rectal fluid of the attine and A. colombica tonsipes. They considered that when the ants defecated on their fungus gardens they were supplementing the substrate with nitrogeneous substances which would be very beneficial to the growth of the fungus. However, Schildknecht (1971) showed that attine ants produce substances from the metathoracic gland. These substances ensure that only the desired variety of nutrient fungi grows in the nest. Indolyl acetic acid which controls growth of mycelia, myrmiacin (the laevo-rotatory-β-hydroxy-decanoic acid) prevents growth of undesirable fungal spores, and phenyl acetic acid keeps the colony free from bacteria.

These examples of ectosymbiotic associations point up the reciprocal contributions between symbiotic partners, making identification of causative factors difficult.

Bletchly and Taylor (1964) showed that increases in the weight of Anobium punctatum (Coleoptera:Anobiidae) larvae growing in wood was directly correlated with the N content of the material. Merrill and Cowling (1965) and Levi and Cowling (1968, 1969) demonstrated that the rate of decay of wood by fungi was also related directly to its N content. In the present study, the total N determinations of sapwood (M. sylvestris) showed a reduction of N content caused by the growth of A. hartigii and the feeding life stages of X. dispar. The

results showed (see Table 17) a reduction of 50% in the total N between the unattacked and attacked (post-brood) sapwood. Examination of total N determinations of artificial and natural (sapwood) substrates indicated that the ambrosial form of A. hartigii, essential for insect growth and development, grew within the range of 0.08-0.34% N dry weight.

The total N determinations of artificial media (L-asparagine) with the mycelial form of A. hartigii was 0.08 ± 0.002% N dry weight for two consecutive analyses. The same media, after activity by postdiapause females and the removal of these insects prior to analysis was 0.09±0.01 % N dry weight. Using the same media components, less L-asparagine, values of 0.02±0.002% N and 0.05±0.005% N dry weight respectively were recorded. In the latter tubes no ambrosia formed, and no oviposition occurred.

It is considered that the reduced N and moisture content of the attacked sapwood, containing diapause beetles, probably accounts for the rare presence of fungal contamination in the galleries of infested trees. Observations of in vitro cultures suggested a similar pattern. Once brood development ceased, the teneral adults merely remained in the ambrosial-free tunnels and were rarely overgrown by fungal contaminants. Results shown in Table 18 indicated that fungal contamination was negligible in cultures with total N values below 0.08 % N dry weight.

Norris and Baker (1968) reported that the symbiotic fungus, Fusarium solani, isolated from the ambrosia beetle X. ferrugineus, grew moderately well on a diet with cellulose as the sole source of carbohydrates, and with only 0.027% N by weight. They stated that this quantity of N compared favorably with the amount in various woods; frequently 0.01-0.03%, seldom greater than 0.3% (Merrill and Cowling, 1966a). However, Norris and Baker (1968) presented the N % as wet weight, whereas Merrill and Cowling (1966a) gave dry weight values. Calculated dry weight values from the data of Norris and Baker (1968) indicated that 0.21-0.22% N were utilized in their media. At the 0.21% N level the X. ferrugineus females oviposited and produced progeny. In the present study, X. dispar postdiapause females oviposited and produced progeny in a media with 0.08-0.1% N dry weight. This may suggest that the media of Norris and Baker (1968) was not the least minimal substrate for the insect-fungal symbiotic association.

Molds and soft-rot fungi have been shown to cause much more rapid deterioration of wood to which N has been added artificially (Allison et al., 1963). This may be the case with A. hartigii, in the xylem cylinder of tree stems, receiving further N from the excretory products of the life stages of X. dispar.

Fixation of atmospheric nitrogen by an individual X. dispar beetles in vitro was not indicated by the acetylene ethylene reductase

method. However, it must be considered that the situation in vivo, never investigated by this method, may prove to be different to the in vitro condition. Reports of nitrogen enrichment in culture by symbiotic organisms of insects (Peklo, 1946; Peklo and Satava, 1949, 1950; and Toth, 1951) have never been unequivocally demonstrated. Baker, Laidlaw and Smith (1970) mentioned that further work on this point, including a critical study with N15 labelled atmospheres, was needed. Also, Dr. H. J. Evans (personal communication) considered that unless N15 or acetylene ethylene reductase procedures were employed on intact organisms, the results would be questionable.

Cowling and Merrill (1966) observed that fixation of atmospheric N has often been suggested, but never yet been proved to occur in fungi. The possibility that A. hartigii may fix atmospheric nitrogen is therefore somewhat questionable. Maybe retesting in vitro cultures of ambrosial form of A. hartigii and of newly emerged postdiapause females of X. dispar, or their endosymbionts, could determine if such fixation were possible.

Cochrane (1958) and Nicholas (1965) stated that from existing knowledge most fungi do not degrade amino acids to ammonia; the fungi incorporated the acids directly into proteins. Abrahamson (1969) considered that the amino acids present in insects could be N sources for the ambrosial fungus in the mycangia of the beetle.

The concentrations of soluble proteins and free amino acids in X. dispar females sampled during March - October 1971 indicated that this theoretical mechanism may be involved. Concentrations of soluble proteins were relatively high in the whole body homogenates of postdiapause beetles, and low in diapause individuals. The free amino acids were found in reverse proportions to the soluble proteins, indicating their incorporation into proteins by the proliferating fungus in the mycangium. Evidence from Schneider and Rudinsky (1969a) and observations in the present study on mycangial development, indicated that the fungus proliferated in the mycangia about February each year. The comparative concentrations of soluble proteins and free amino acids suggested that the fungus in the mycangia was built up from the free amino acids of the insects. There seemed to be no other mechanism that could explain this phenomenon, particularly as the insects do not feed on fungus during the period of July through March. Tables 20 and 21 lacked data for the months of January, February, November, and December due to the practical problem of identifying and collecting infested materials in the field at these times. Despite the differences between the concentrations of soluble proteins of postdiapause and diapause beetles, there was no pronounced difference in the average wet weights of these individuals. After establishing the gallery and disseminating its fungus from its mycangium, the maternal females browsed on the ambrosia, as did

the subsequent larvae and teneral adults. The gut contents of these individuals showed the presence of ambrosia. Baker (1963) reported a similar observation with Platypus cylindrus Fab.

Potty (1969) mentioned that conventional methods of protein estimations gave highly erroneous values for preparations obtained from sources in which phenolic compounds and pectins are found. He mentioned that in preparations of citrus fruits these compounds were shown to be the major interfering contaminants in these methods. However, these substances were not considered to affect the protein estimations of X. dispar in this present study (Dr. W. D. Loomis,[3] personal communication).

Many living cells are present in the sapwood, and the N sources available to microorganisms that inhabit wood include free amino acids, peptides, proteins, and nucleic acids (Merrill and Cowling, 1966b). Although proteolytic enzymes have been demonstrated in wood-destroying fungi, Merrill and Cowling (1966a) concluded that the comparative suitability of proteins, peptides, and nucleic acids as sources of N for growth of wood-destroying fungi has yet to be determined.

It seems that the action of enzyme systems that modify the cellulose, hemicelluloses, or lignin may be essential before much

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[3]Dr. W. D. Loomis, Biochemistry Department, Oregon State University, Corvallis.

of the N in wood becomes accessible to microbial proteases or other enzymes that act on the nitrogenous constituents of the cell walls of the wood. The Azocoll tests indicated that A. hartigii showed no proteolytic enzyme activity. Similar results were obtained using the hindguts of X. dispar beetles. These experiments were in no way exhaustive and the presence of proteolytic enzymes occurring in this insect-fungal association remains a possibility.

V. INTERACTIONS OF X. DISPAR WITH ITS SYMBIOTIC FUNGUS A. HARTIGII

Introduction

The symbiotic fungus Ambrosiella hartigii Batra of the ambrosia beetle Xyleborus dispar (F.) grows as a continuous palisade of yeast-like sprout cells within the galleries of the active brood (Batra, 1967). Schmidberger (1836) called this material 'ambrosia', but the fungal nature of this material was not recognized until Hartig's study of 1844. A. hartigii has two growth forms. The ambrosial form (sprout cell) is produced in association with the insect and the mycelial form is produced in vitro without the insect. When isolated as the ambrosial form from beetles and gallery walls, and cultured on common laboratory media such as potato dextrose agar (PDA), A. hartigii converts to the mycelial form (Batra, 1967).

The study describes experiments and bioassays showing the interactions and effects of various life stages of X. dispar on A. hartigii, with particular emphasis on morphogenesis of the fungi.

Experimental Procedure

The life stages of X. dispar were obtained from field collections and from in vitro cultures. Bioassays were designed to examine the

ability of X. dispar to induce the mycelial form of A. hartigii to convert to the ambrosial form. In the bioassays all stages of X. dispar were released onto the mycelia using a sterile vacuum tweezer system (Linscott, 1964). The organism used in this study, A. hartigii, isolate RAR-280, was maintained at the Oregon State University. Growth of ambrosia (sprout) cells was determined by direct microscopic examination and by streaking the ambrosial mass on 3% malt extract agar (Difco) to verify that A. hartigii was the only microbe present. A positive response was recorded when the brownish-olive-green mycelia lost pigmentation and became a mass of white monilioid ambrosia (sprout) cells. Cultures that remained in the mycelial form for 3-4 weeks after the release of a particular life stage or extracts of X. dispar were considered to exhibit a negative response.

Bioassay I

(a) Postdiapause and diapause female adults, eggs, larvae and pupae were placed individually and in groups (minimum of 4 individuals per plate), on the surface of the mycelial form of A. hartigii, preinoculated (7 days) on culture plates of corn meal agar medium (Difco). Adults (two per dish) were also attached to steel staples with silicone grease and suspended over the surface of the mycelial form of A. hartigii on the same medium. Eggs and pupae were

separated into two groups, half were untreated and the other half were surface sterilized (Retnakaran and French, 1971), prior to placement on the mycelial form.

(b) Using postdiapause and diapause females and pupae (minimum of 10 individuals per sample), distilled water extracts (50 ml per sample) were obtained from (i) whole adults, (ii) whole body homogenates, and (iii) homogenates of mycangia. These extracts were concentrated and serially diluted (x10, x100, x 1000); and 5-10 µl droplets of concentrated extracts and diluted extracts were injected onto the surface of the mycelial form of A. hartigii, growing on corn meal agar medium, using a milli-pore filter system.

Bioassay II

(a) The postdiapause and diapause female adults used in this bioassay in 1970 and early 1971 were 'cleaned up' with the wet and dry technique of Francke-Grosmann (Batra, 1963), and adults used later in 1971 were surface sterilized similarly to Bioassay I. The latter method was quicker, yet as efficient as the wet and dry technique. To prevent microbial contamination, only surface sterilized beetles were released into the culture tubes. The adults were then placed individually in tubes, preinoculated with A. hartigii and growing on the mycelial form in artificial media in culture tubes. Briefly, the ingredients of the sterile defined

medium included: alphacellulose, Bactoagar®, glucose, L-asparagine, Wessons Salt Mixture and distilled water.

(b) Eggs and larvae were transferred onto the ambrosial form of A. hartigii to determine whether or not these life stages could be reared through to the teneral adult stage without the presence of the tending (mother) beetles. A liquid medium or 'broth' consisting of Czapek Dox Broth (Difco), casamino acids, enzymatic digest (4%) (Difco) and distilled water was made up (Roeper, unpublished data) and inoculated with several plugs of A. hartigii (strain RAR-280), and placed on a shaker for seven days at 25° C. At the same time, these ingredients, plus Bactoagar®, were autoclaved, and slants made. After seven days, the 'broth', about 1 ml, was poured over the slant surface. After 48 hrs, eggs and larvae were surface sterilized (Retnakaran and French, 1971), and placed onto the developing ambrosial mat or 'ambro-turf'.

Diapause female adults (30) were surface sterilized and placed on the ambro-turf to encourage feeding and oviposition.

Bioassay III

A bioassay was developed in an attempt to examine morphogenic substances, i.e., substances able to induce the ambrosial form of A. hartigii, without the presence of active postdiapause female X. dispar, and the following procedure was adopted.

The mycelial form of A. hartigii was grown on 3% malt extract agar medium in plates for about one week prior to placing the following components on the fungal surface: (a) Czapek Dox Broth (35 g), Bactoagar® (25 g), Bacto-Casitone, Difco (1, 2, 3, and 4 %), and distilled water (1000 ml) were prepared using varying buffered (phosphate) solutions (pH 5.7, 7.0, and 8.0). These various solutions were poured into petri dishes and allowed to stand for 24-36 hrs. After this period, plugs were cut from these various buffered plates, and placed onto the mycelial mat. (b) droplets (2-4 µl) of Czapek Dox Broth, Casitone and water, and (c) sterilized filter paper discs, with 2-4 µl droplets of the same solution as in (b) above.

Experiments were conducted under laboratory conditions of temperature (ca. 20-24° C) and light. Controls consisted of A. hartigii cultures without the life stages of X. dispar. Determinations of pH were made using a Corning (Scientific Instruments), Model 12, Research pH meter, with an expanding range.

Results and Discussion

Bioassay I

Ambrosia Induction by Insect. Table 22 summarizes data obtained using Bioassay Ia. Postdiapause females, both untreated (30) and surface sterilized (28) induced ambrosia formation in A.

Table 22. Numbers of cultures of A. hartigii mycelium producing ambrosia (+), or failing to respond (-) to specific life stages of X. dispar using Bioassay Ia.

Treatment	Eggs		Larvae hatched from eggs		Larvae direct from wood		Pupae		Postdiapause adults		Diapause adults	
	+	-	+	-	+	-	+	-	+	-	+	-
Untreated	0	200	0	33	0	100	31	69	30	0	0	30
Surface sterilized	0	200	0	42	0	100	29	71	28	0	0	30

hartigii. These beetles walked over the surface of the mycelium (Figure 11) and tunnelled into the medium within an hour after release. Within two days ambrosia (sprout) cells were observed where the beetles had walked and at burrow entrances. By the fourth day ambrosia was seen in all petri dishes with postdiapause adults.

All the postdiapause adults (30) suspended over the mycelial form failed to induce ambrosia. Most of these beetles were dead after the fourth week of test.

Diapause females (60) also burrowed into the corn meal agar within the hour but none induced any morphological change in A. hartigii mycelia. The controls (10) without life stages remained in the mycelia form. Regardless of the sterilization method, no eggs induced ambrosia in the mycelial form of A. hartigii.

None of the mycelia in any of the larval cultures changed to the ambrosial form regardless of the larval source and treatment. Mycelia at the sites of larval feeding were suppressed, which was also observed by Batra (1967). Fecal 'tailings' were deposited over the surface of the colonies, but no change in the growth form of A. hartigii was seen beside or beneath these feces.

Both untreated and surface sterilized pupae induced ambrosia formation. Figure 12 shows a mass of white ambrosia (sprout) cells forming at the posterior of a female pupa on the mycelial form of A. hartigii. A positive response did not occur with every pupa tested. Of 100 pupae bioassayed in each treatment, only 30%

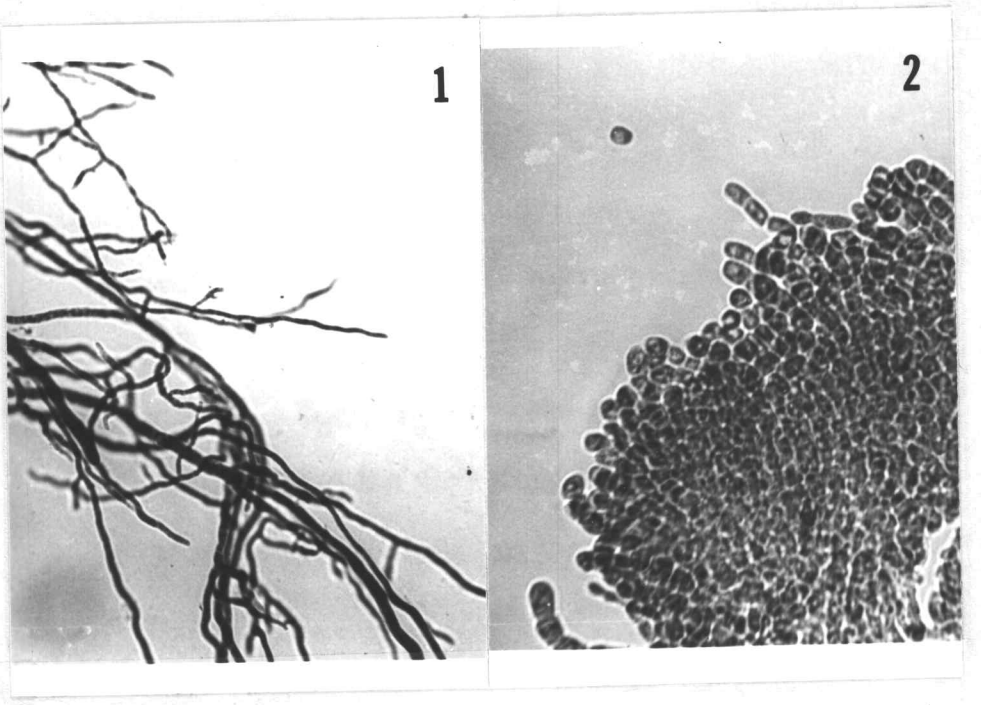

Figure 11. Ambrosiella hartigii. The growth of the mycelial and ambrosial forms cultured on corn meal agar medium. 1, brownish-olive-green hyphal tips of mycelia. X286. 2, ambrosia (sprout) cells formed at posterior of female pupa of X. dispar and stained with lactophenol, X286. 1 mm = 32 μ.

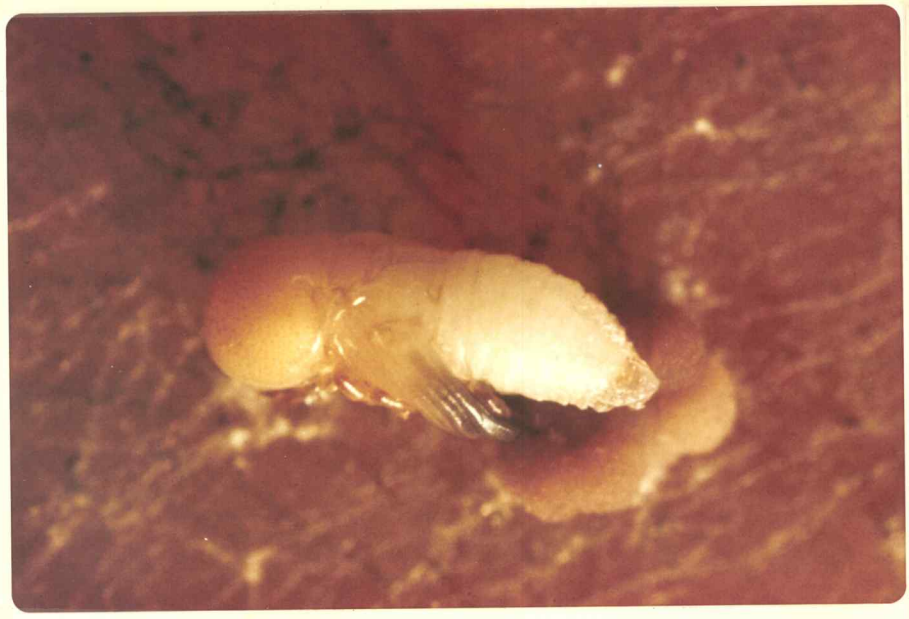

Figure 12. Induction of the ambrosial form of A. hartigii at the posterior of a female pupa of X. dispar. The pupa was initially placed on a mycelial culture of the fungus growing on corn meal agar, X17.

induced ambrosia formation. Ambrosia induction appears to occur at the pupal stage in which wing tips, eyes and appendages are pigmenting.

(b) The water extracts of whole insects, homogenized insects and mycangia did not induce ambrosia in the mycelial form of A. hartigii.

Insect Responses to Fungal Form. Both untreated and surface sterilized postdiapause females failed to oviposit in the corn meal agar. No beetles fed on the mycelia of A. hartigii.

Over 70% of the eggs (400) in both treatments were overgrown by mycelia before hatching. First instar larvae hatched more readily from clumps of eggs than from single eggs on the surface of the mycelia. These early instars died within 2-7 days in the petri dishes. Larvae (200) obtained from infested wood were later instars, and fed on the hyphal tips of the mycelia on the surface of the colonies. Pigmented hyphae were readily seen in their intestinal tracts. These larvae persisted for over two weeks before dying, and then were overgrown by the mycelial form.

Of the larvae that were produced from eggs (75) in vitro and those excised from infested wood, none developed to pupae.

Some pupae (22) were overgrown by mycelia of A. hartigii. These pupae were either injured or dying. Apart from frequent flexing of their abdomens, pupae remained immobile on the mycelial

surface. These pupae pigmented, shed their exuviae, developed to callow adults, and walked over the surface of the mycelia before tunnelling into the media to become inactive. The ambrosia in these cultures diminished slowly, gradually pigmented and changed to the mycelial form.

Flexing or rocking motions of pupal abdomens has been reported in scolytids in association with fungi and yeasts present during the brood development. Whitney (1971) mentioned that most puape of Dendroctonus ponderosae Hopk., examined in situ, exerted a rocking motion of their abdomens and brushed against the fungi growing on the walls of the pupal chamber. He suggested that this abdominal flexing prevented suffocation and entanglement in the fungal growth during pupal metamorphosis. Whitney also suggested that the activity of the pupae of D. ponderosae produced a fungal reaction, similar to the ambrosia of the ambrosia beetles, and that this response allowed the ambrosia to lodge in and fill the mycangia of the teneral adults. My observations of the pupal activity of X. dispar in nature also indicated the presence of ambrosia at the critical period when the mycangia presumably receive their fungal symbiont. Within galleries of infested wood, small regrowths of ambrosia were seen around the pronotum of the pupae. Later examination of galleries in which the diapause adults were present, showed the tunnel walls to be darkly pigmented with no trace of ambrosia. This is in

agreement with the field observations of Wilson (1913).

Untreated postdiapause and diapause female adults were overgrown by fungal contaminants (mainly Aspergillus spp. and Penicillium spp.) and about 50% died by the third week. Contamination was much less with surface sterilized beetles and all adults were alive at the end of three weeks, showing that the mycelial form of A. hartigii, although not suitable for ovariole development, seemingly caused no pathogenic effects. Sampling wild populations of X. dispar showed that fungal contamination was not observed in active broods. Reasons for contaminants in the in vitro cultures appears complex and as yet not understood.

Bioassay II

Ambrosia Induction by Insect. Within minutes of release the postdiapause females (344) commenced to tunnel into the media regardless of whether the tubes were or were not, preinoculated with the mycelial form of A. hartigii. After 12 hours the females had burrowed about 10-12 mm into the media. Frass was expelled from the tunnels and deposited around the entrance. By day 2-4, ambrosia was observed in both preinoculated and non-preinoculated tubes. Ambrosia was first seen near the tunnel entrances as whitish patches of monilioid cells (Fig. 13), which gradually developed as a continuous palisade over the entire tunnel system where the females were active and feeding. Tubes with and without preinoculation of

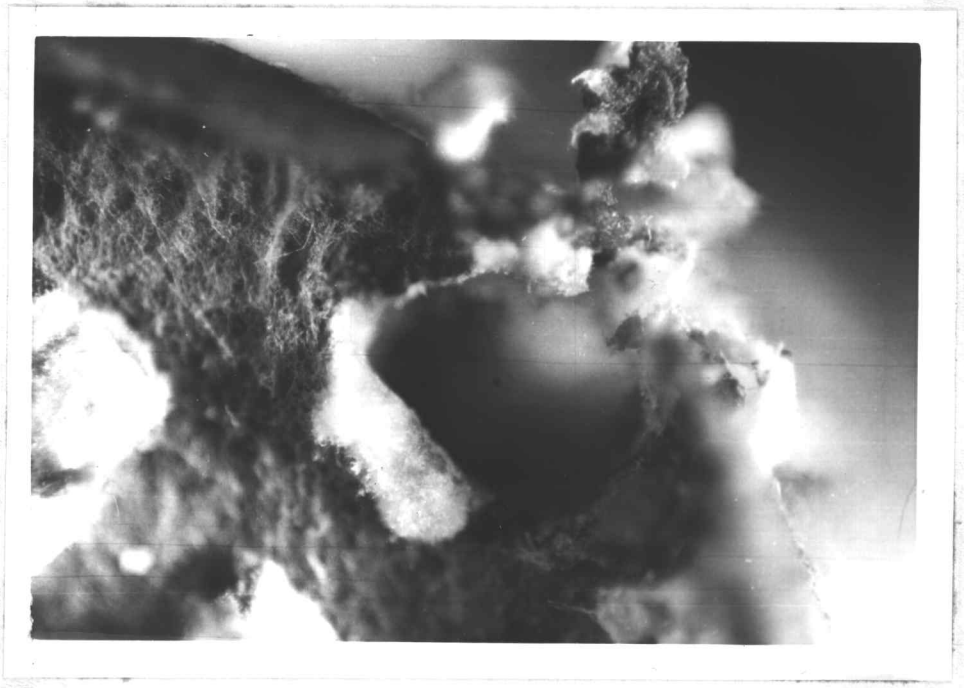

Figure 13. Ambrosial (white) and mycelial (dark) forms of A. hartigii around the tunnel entrance (0.12 mm diam.) of a postdiapause X. dispar female on the artificial medium.

mycelia had ambrosia present in the galleries. The source of the ambrosia in the inoculated tubes was initially from the mesonotal mycangia of the post-diapause females. In the preinoculated tubes the source of the ambrosia could have been from mycangia, or from the induction of pre-grown mycelia, or from both. In both types of cultures, the mycelial form grew over almost the entire surface of the media except at the tunnel entrances and galleries. About 5-6 days after ambrosia was seen the first eggs were observed, laid seemingly at random in the tunnels. There was no frass on the ambrosia. Only fecal deposits were found on the ambrosia, and these gradually disappeared, overgrown and seemingly utilized by the ambrosial form of A. hartigii. Ambrosia was prominent in broods during all egg and larval stages, diminishing to a few isolated specks by the pupal stage.

In the summer and fall of 1970, 485 diapause beetles were cultured with no evidence of ambrosia induction. The following year at the same time, a further 302 diapause beetles failed to induce ambrosia. These beetles, as in the previous year, merely tunnelled their body length (3 mm) into the medium and remained inactive.

Insect Responses to Fungal Form. (a) Beetles were seen feeding on the ambrosia 3-4 days prior to egg laying. On several occasions the postdiapause females were seen assisting or tending the newly emerging larvae. The mother pushed the larvae, with her

pronotum, onto the ambrosia lining the gallery wall. This tending behavior was also observed at later periods in the early larval stages. The females, after ovipositing, backed up the tunnel to the entrance and frequently returned down into the gallery system to tend the feeding larvae. Although the females were observed moving among pupae, no particular tending behavior could be discerned.

With time, the media dehydrated and shrank from the walls of the tubes. Of the larvae that wandered out from the galleries most fed on the mycelial form growing on the surface of the medium and some stuck to the glass walls of the tubes. These individuals all perished as larvae. However, larvae remaining within the gallery systems fed on ambrosia and, about 25 days after oviposition, the larval-pupal transformation occurred, with the teneral adults developing several days later. Often late larval instars and early pupae were seen on the uppermost portion of the medium on the surface of the mycelia and not on or near the ambrosial form around the tunnel entrance. Thereafter, these individuals, although ceasing to feed, developed into teneral adults and returned into the tunnels, the walls of which had little or no ambrosia visible at this stage.

(b) Mortality of the eggs (60) and larvae (40) placed on the 'ambro-turf' was high. No hatching was observed in any of the culture tubes. Many of the larvae fed on the ambrosia but only

eight larvae pupated and developed into teneral adults (five females, three males). These adults tunnelled into the medium a short distance (3-5 mm) and remained immobile. Dissecting two of these female beetles showed that no feeding had occurred, ovaries were immature, and mycangia had few fungal cells.

pH Measurements. Determinations of the pH of various substrates and of the ambrosial and mycelial forms of A. hartigii are shown in Table 23. Of the natural substrates, unattacked sapwood registered the highest pH, whereas the attacked sapwood (post-brood) was more acidic. Values of ambrosial and mycelial forms of A. hartigii on the artificial media indicated that the ambrosia (sprout) cells favoured a more alkaline environment than the mycelia. This was found with both the medium (L-asparagine) and corn meal agar medium.

Bioassay III

No induction of ambrosia occurred in any of the various regimes tested. Good growth of mycelia was found on all the plates after four weeks.

Conclusions

Batra and Downing Michie (1963) remarked that the ambrosial form of the fungus associated with X. dispar is dominant in the

Table 23. Measurements of pH of natural and artificial substrates and of ambrosial and mycelial forms of A. hartigii. Minimum of 6 samples per component.

Component measured	Range of pH values	Average pH value
Unattacked wood (Malus)	5.57 - 6.48	6.40
Attacked wood (Pre-Brood)	5.20 - 5.60	5.39
Attacked wood (Post-Brood)	5.06 - 5.60	5.08
Ambrosia on corn meal agar	5.57 - 6.10	5.75
Mycelia on corn meal agar	5.48 - 5.64	5.56
Ambrosia on medium (L-asparagine)	7.31 - 8.34	8.02
Mycelia on medium (L-asparagine)	7.58 - 8.06	7.78
Medium (L-asparagine)	5.28 - 5.63	5.38

immediate vicinity of adults, eggs, larvae and pupae in nature. But the evidence from Batra (1967) and the present study suggested that it required the presence of an active (= postdiapause) female beetle for the controlled growth of the ambrosia in nature. In addition it was confirmed that the postdiapause females and pupae of X. dispar were capable of inducing ambrosia formation in A. hartigii as was shown in Bioassay Ia, causing a conversion from the mycelial to the ambrosial form. But, the insects had to be in direct contact with the mycelial mat or surface. The bioassays demonstrated not only the dimorphic nature of A. hartigii, but also the importance of the ambrosial form of the symbiont for the growth and development of X. dispar postdiapause adults and larvae. Without the ambrosial form, oviposition and pupation did not occur. This agrees with observations by Brader (1964) on the tropical ambrosia beetle, X. compactus, which showed that egg-laying occurred only when the ambrosia fungus had begun to grow and the beetle had started to feed on it.

Although only a few larvae (8) pupated and developed into teneral adults in Bioassay IIb, the dependency of X. dispar for the ambrosial form of A. hartigii was most apparent. From Bioassays Ia and IIb it may be suggested that the absence of tending female (mother) beetles probably accounted for the low percentage of hatching in these cultural conditions. Bioassay III failed to

demonstrate any induction of ambrosia, even though these same compounds have been shown to cause ambrosial induction in vitro (Roeper, unpublished data). This suggests that such bioassays are indeed feasible, but the procedure adopted in Bioassay III needs modification to illustrate dimorphism in vitro of A. hartigii.

Measurements of pH indicated that the ambrosial form grew in a more alkaline environment than the mycelial form. This was seen in both the natural and artificial substrates. Dry weight measurements of the two forms of A. hartigii showed that the ambrosia contained about 52% moisture content, whereas the mycelia contained about 26%.

The water extracts from whole adults, homogenized adults, and mycangia failed to induce ambrosia. That these organisms may well have secreted components capable of inducing ambrosia is still tenable. Several possibilities could be suggested, namely, that the water extraction methods diluted the component(s) too much, they were volatile, solubility or pH phenomena inhibited or masked a positive response in the bioassays.

Hubbard (1897) considered that mechanical cropping of the ambrosia by the insect kept the ambrosial form viable. Batra (1967) demonstrated that mechanically cutting the hyphal tips of the mycelial form of A. hartigii with a sterile scalpel produced a change from the mycelial to the ambrosial form. Physical or mechanical contact

contact may be a contributing factor, but it seemed that one of the main mechanisms in ambrosia induction and control involves a secretion of the insect. This conclusion was based primarily on the observations of negative response by inactive diapause beetles and feeding larvae, and the positive effects caused by active and excreting postdiapause beetles and non-feeding pupae. This mechanism for the control of its symbiotic fungus by this temperate zone scolytid ensures the continued presence of the ambrosial form necessary for the growth and development of the various life stages of X. dispar. The filling of the mycangia of the teneral adults with ambrosia previously stimulated by the female beetle and pupae, protects this fungal form during the periods of the insects hibernation and flight, and provides both symbiotic partners with an elegant survival and dispersal arrangement.

VI. GENERAL DISCUSSION

Comparison of Wild and in vitro Populations

The development of wild populations of X. dispar in the present work agreed well with the records in the literature (Schneider-Orelli et al., 1913). The pattern of adult emergence was similar to that reported by Wilson (1913) and Mathers (1940).

In vitro culture data indicated that the period (42 days) for X. dispar to complete its life cycle was slightly shorter than in the wild populations. Schvester (1954) mentioned 58-60 days and Wilson (1913) reported 80-84 days. Observations in vitro rearing agree with field observations of other authors (Schneider-Orelli, 1913; Wilson, 1913; and Schvester, 1954) who reported that X. dispar was a univoltine species and had a maturation diapause in the adult stage. Tending or nursing behavior of the female was readily seen in vitro. The beetles were observed assisting the first instar larvae from the eggs, and 'pushing' them onto the ambrosia on the walls of the tunnels with their pronotums. The number of progeny produced in the different populations were shown to be comparable and were found to be linear compared to the volume of the gallery.

The ambrosial form of A. hartigii as grown in vitro was morphologically the same as that isolated from mycangia and galleries.

Growth characteristics of the ambrosia from both situations also compared favorably. The observations of the activity of X. dispar in nature and in vitro culture indicated that ambrosia was associated with active (=postdiapause) females and pupae. Also, such observations confirmed the presence of ambrosia at the critical period when the mycangia presumably receive their fungal symbiont. Small regrowths of ambrosia were seen within the galleries in wood and in vitro cultures, which suggested that the beetles in both situations behaved similarly.

Sampling wild populations of X. dispar showed that fungal contamination was rarely observed in active broods. And when found, the main species collected were Ceratocystis and Schizophyllum, whereas, the main contaminants within the in vitro cultures were species of Aspergillus, Penicillium and Trichoderma.

Diapause in X. dispar

The presence or absence of a diapause has considerable ecological significance. Absence of a diapause would allow X. dispar to breed continuously throughout its temperature range. This would be significant in equable climatic conditions and would favor its establishment and spread when the species is introduced into warmer parts of the world. However, even South of the Equator, acclimatization of an indigenous northern species is nearly always restricted to

regions with an annual temperature range similar to its original home (Danilevskii, 1965).

Mansingh and Smallman (1967) stated that all diapause in natural populations may be potentially under the influence of environmental factors and should be regarded as faculative. Mansingh (1971) mentioned that the return of favorable conditions does not terminate diapause immediately, it depends on the nature of stimuli and the activated condition of the individuals. In addition, he stated, that generally, almost all overwintered insects show visible initiation of developmental activity within two weeks at room temperatures. This was not quite the case with X. dispar females in the experiments undertaken in this present study attempting cessation of diapause. However, beetles within infested wood were stored at 2° C on 8 October 1971, removed on 10 January 1972 and placed in a greenhouse at 25° C, emerged about 12 days later. The period of flight in the field is usually about March-April. This observation seems to agree with Mansingh (1971) who suggested that appropriate ecological stimuli gradually stimulate the endocrines of diapausing individuals which have been in activated phase. This in turn triggers DNA synthesis and mitotic activity. Until this is achieved, physiological diapause exists. RNA synthesis precedes protein synthesis and gradually all physiological and biochemical developmental processes are restored.

Induction of Ambrosia

The induction of ambrosia of A. hartigii was found in three situations: (a) in association with postdiapause females, (b) in association with female pupae, and (c) in vitro conditions of high nitrogen content, e.g., casamino acids, uric acid, allantoin, ammonium sulphate (Roeper, unpublished data).

Induction in (a) and (b) above, involved a secretion of the insect. This indicated two phases of A. hartigii, the ambrosial and mycelial form. And experiments and literature data suggested the dependency of the insect for the ambrosial form.

Although a high nitrogen content was found to be necessary for differentiation in A. hartigii, it appeared that there is a two-fold mechanism. Namely, a definite differentiation stage, and a distinct growth stage of the fungus. Nitrogenous excretory products (e.g., uric acid, NH_4^+ compounds) cause induction, but do not satisfy fully the continued growth of the ambrosial form. Growth factors, which included high nitrogen content constituents, a particular range of pH (6.8-7.2), of the buffered solutions were found in vitro studies (Roeper, unpublished data). Other factors are considered contributory, but remain unknown at this stage. However, it may appear that unsaturated and saturated fatty acids and vitamins have a possible role in the continued growth of the ambrosia.

In this study, mention has been made of two forms of A. hartigii. But, Roeper (unpublished data) has demonstrated that there are three growth phases of this fungus. Namely, ambrosial (sprout) cells, mycelial and aleureospores, the latter are seen as blastospores apically produced on the conidiophores. Nutritional variations caused the different morphological forms of the symbiont fungus of X. dispar.

Breeding of X. dispar

The presence of a maturation diapause in the females of X. dispar has led to new techniques in future handling. All life stages of the insect need to be surface sterilized (Retnakaran and French, 1971) prior to in vitro culture. Collections of flight female beetles with rotary nets and window-traps during March-June are enhanced by incorporating a 10% ethanol solution near the traps. Postdiapause insects excised from infested trees may be used if flight beetles are scarce, otherwise, the infested material must be placed in a cooler at about 4° C. To avoid high mortality insects should be removed before August of the same year. Infested material collected in the field after late July contains diapause beetles of X. dispar. This material should be transferred to a cooler (4° C) upon collection, and after 100-120 days, removed, and placed within an emergence box at a temperature of 20-25° C. Emerging beetles have their

mycangia packed with ambrosia at this stage, and on tunnelling into artificial media commence culturing ambrosia within 7-10 days, feeding on it, and subsequently, produce progeny.

Glucose is adequate as a carbon source in artificial media, and ammonium sulphate, casamino acids (enzymatic and vitamin free), L-asparagine, or mixtures of amino acids can supply the necessary nitrogen.

Progeny of the in vitro cultures may be transferred to tubes with agar media, stored at about 4° C for 100-120 days, and re-introduced to about 20-25° C for two weeks. On re-transferring these beetles to new culture tubes, egg laying may be expected within 10-20 days.

Basis of Symbiosis

The mutual association of the insect-fungus relationship calls for reciprocal nutritional contributions by both partners. Martin (1970) and Norris *et al.* (1969, 1970) discuss these associations in terms of the ecological and biochemical bases for such symbiotic interdependency. Figure 14 shows a model (Odum, 1971) of ecto-symbiosis between *X. dispar* and its symbiotic fungus *A. hartigii*.

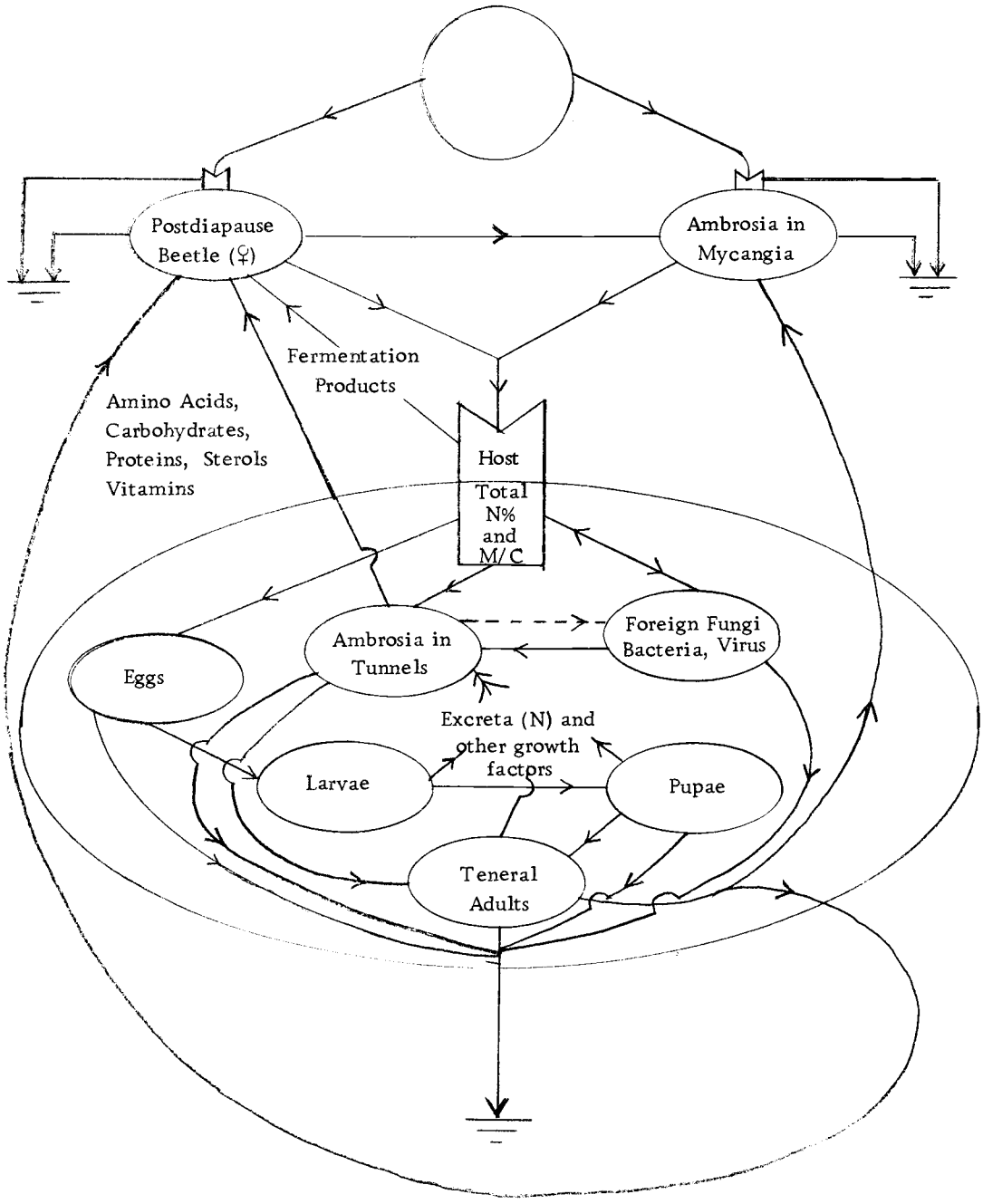

Figure 14. Model of ectosymbiosis between the ambrosia beetle X. dispar and its symbiotic fungus A. hartigii.

Contributions of Insect to Fungus

The fungus is protected during the periods of insect flight and hibernation within the female beetles mycangium. In this situation, the fungal inoculum incorporates the insects free amino acids to protein, and proliferates into a mass of ambrosial cells. The beetles open up the cellulosic substrate of the tree for the fungus. The entire repertoire of the insect's fungus culturing behavior can be interpreted in terms of the criteria for competitive ability of the fungus (Martin, 1970). Nitrogenous excretory products of the insect to the ambrosia enhances the competitive position of the fungus. Evidence from Schildknecht (1971) suggested that insects secreted chemicals to ensure this beneficial relationship. This could possibly be the case with X. dispar, though as yet unproven. As this present study has shown, it is the presence of active (=postdiapause) females and pupae that induced ambrosia. In vitro induction may be accomplished with high nitrogen inputs and specific cultural conditions (Roeper, unpublished data). Uric acid, the main excretory product of X. dispar, caused ambrosial induction of the mycelial form of A. hartigii in vitro (Roeper, unpublished data). The concentrations used of 10-25 µg uric acid/ml, were within the range of % N equivalents of uric acid found in the excreta of the beetles (7.8-14.8 µg uric acid/beetle.

Contribution of Fungus to Insect

It seems that the cellulose-degrading enzymes of the fungus allow the insects to gain access to the vast cellulose reserves of the temperate regions. The free nutrients available in the wood are utilized by the fungus. The fungus, the sole food source of X. dispar, is then utilized by the insect. Sterols, carbohydrates, vitamins, amino acids, proteins and other growth factors are obtained and required by the insect for its complete development and growth. Biochemically interpreted, the contribution of the fungus to the beetle is the enzymatic apparatus for degrading cellulose. And, as Martin (1970) has mentioned ectosymbiosis can be viewed as a metabolic alliance in which carbon and nitrogen metabolisms of the two organisms have been integrated.

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