AMER. ZOOL., 32:593–605 (1992)

The Evolution of Insect-Fungus Associations: From Contact to Stable Symbiosis[1]

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SYNOPSIS. Insect-fungus interactions range from agonistic to mutualistic, and include several spectacular examples of complex symbioses. A potential benefit of mycophagy (the ingestion of fungal tissue) is the augmentation of digestive capacity by the ingestion of fungal enzymes that remain active in the gut following ingestion. Cellulose digestion is mediated by ingested fungal enzymes in the wood-boring larvae of cerambycid beetles and siricid woodwasps, in detritus-feeding stonefly nymphs, and in the workers of fungus-growing termites. In this paper I discuss a plausible scenario for the evolution of stable symbiotic insect-fungus associations, in which the augmentation of digestive capacity through the ingestion of fungal enzymes is an important factor leading to the establishment of interdependence between the interacting partners in a mutualism. Ingested fungal enzymes play a different role in the mutualistic association of the attine ants and their symbiotic fungi.

Analyses of the associations of the siricid woodwasps, fungus-growing termites, and fungus-growing ants with their symbiotic fungi permit the testing of Law's (1985) predictions concerning the consequences of evolution in a mutualistic environment. As predicted, the rate of speciation has been slower in the protected partner than in the host partner, selection has favored asexual reproduction in the protected partner, and, at least in the attine ant-fungus symbiosis, the protected partner exhibits a low degree of specificity toward different host species.

Insect-fungus interactions provide rich material for the study of both mechanistic and theoretical aspects of mutualism.

INTRODUCTION

Since the late 1960s my laboratory has been engaged in research that explores the biochemical implications of invertebrate mycophagy (the consumption of fungal tissue). We have studied species that consume the fruiting bodies of higher fungi, species that feed on detritus that is colonized by microbes, wood-feeders that restrict their feeding to fungus-infected wood, and several species involved in elaborate symbiotic associations. The most important result to emerge from this research is the discovery that ingested fungal enzymes can remain active in the gut following ingestion and contribute to the digestion of the structural polysaccharides of plant tissue. Because we have observed this phenomenon in species from quite diverse taxa, we have proposed that the augmentation of digestive capacity through the ingestion of active fungal enzymes may be quite widespread among invertebrates, and may be a very general mechanism by which arthropods digest cellulose.

In this article I will describe some of the experiments that have implicated ingested fungal enzymes in cellulose digestion. A more comprehensive account of this work can be found in Martin (1987). Second, I will discuss a possible scenario for the evolution of stable symbiotic insect-fungus associations, in which the augmentation of digestive capacity through the ingestion of fungal enzymes is an important factor leading to the establishment of interdependence

[1] From the Workshop on *The Impact of Symbiosis on Invertebrate Physiology, Ecology, and Evolution* presented at the Centennial Meeting of the American Society of Zoologists, 27–30 December 1989, at Boston, Massachusetts.

between interacting partners. Finally, I will discuss characteristics of the hosts and symbionts in three stable insect-fungus associations that bear upon theoretical predictions that have been advanced concerning the consequences of evolution in a mutualistic environment.

In discussing ecological and evolutionary aspects of intracellular symbiosis, Taylor (1983) proposed that the evolution of an obligate mutualism from an initially harmful or neutral interaction proceeds through five stages: (1) consistent and extended contact; (2) avoidance of lethal harm during contact; (3) coadaptation, leading to reduced virulence and increased tolerance; (4) further coadaptation, leading to dependence and/or interdependence; and (5) still further coadaptation, leading to facilitative interactions that foster stability and permanence in the association. Although formulated to account for the evolution of intracellular symbioses and the origins of eukaryotic organelles, this scenario probably describes the stages through which any agonistic or antagonistic association evolves into a stable mutualism. Our work on insect-fungus associations illustrates the value of this scenario in understanding the evolution of insect-fungus mutualisms.

Despite its ecological importance and evolutionary interest, mutualism has received less attention from theoreticians than competition, predation, and parasitism. In an attempt to rectify this imbalance, Law (1985) provided an analysis of the consequences of evolution in a "mutualistic environment." He argues that the selective forces that operate on organisms that possess a mutualistic refuge (a place where they are protected from their biological antagonists and hostile environmental stresses) for a substantial part of their lives are very different from those experienced by organisms that live their entire lives in a hostile environment (one dominated by antagonistic interactions with predators, pathogens and competitors). Law makes three predictions concerning the evolution of organisms involved in symbiotic associations in which one organism (the inhabitant) lives for a major portion of its life within another organism (the exhabitant). (1) The rate of genetic change should be lower when mutualistic forces predominate. He suggests that a lower rate of genetic change would be reflected in a reduced rate of speciation, thus leading to the prediction that there will be many fewer species among the inhabitants than among the exhabitants in a mutualism. (2) There should be selection for asexual reproduction in environments where mutualistic forces predominate. Thus, sexual reproduction should be utilized less by the inhabitants than by the exhabitants in a symbiosis. (3) There should be less specialization between partners of mutualistic associations than between partners of antagonistic associations. Law contends that this prediction can be tested by determining whether a species or strain of the inhabitant isolated from one host is accepted by other hosts. Law formulated these predictions for endosymbiotic associations, whereas the insect-fungus associations discussed in this paper are ectosymbiotic, that is, the fungal symbionts occur entirely external to their insect hosts. Nonetheless, it is reasonable to suggest that the fungi in these insect-fungus associations are analogous to the inhabitants of endosymbiotic associations, since they are buffered from many environmental stresses and receive considerable protection from competitors.

INSECT-FUNGUS INTERACTIONS THAT HAVE EVOLVED INTO FACULTATIVE MUTUALISMS

Cerambycid beetles

The larvae of many species of cerambycid beetles can digest cellulose, with assimilation efficiencies that range from 12–57% (Martin, 1983, 1987). At various times it has been proposed that the actual agents of cellulolysis in these wood-boring beetles are endosymbiotic yeasts housed in larval mycetomes (Buchner, 1928; Uvarov, 1929) or enzymes produced by the beetles themselves (Schlottke, 1945). Work from my laboratory has demonstrated that cellulose digestion in five cerambycid species is mediated by ingested fungal enzymes (Kukor *et al.*, 1988). The most comprehensive effort

TABLE 1. *Approximate digestibility of balsam fir wood chips (BFWC) and cellulose in balsam fir wood chips by* Monochamus marmorator *larvae.*

Larval diet before experiment	Approximate digestibility (%)	
	BFWC	Cellulose in BFWC
Four weeks on BFWC permeated by live *Trichoderma harzianum*	52.2 ± 1.9 (4)	26.7 ± 4.0 (4)
Two weeks on artificial diet, followed by two weeks on autoclaved *Trichoderma harzianum*-infected BFWC	20.8 ± 5.4 (3)	3.7 ± 2.6 (2)

was directed toward the balsam fir sawyer, *Monochamus marmorator* (Lamiinae) (Kukor and Martin, 1986*a*).

Larvae of *M. marmorator* are common in stands of dead or damaged balsam fir. Females oviposit in slits chewed in the bark of recently felled logs or recently killed trees. After the eggs hatch, the larvae chew their way into the cambium and later into the sapwood and heartwood. At the time of oviposition the wood is free of fungal infection, but the larval galleries provide favorable conditions for fungal growth, and colonization by wind-born fungal spores generally follows. By the time the larvae have entered mid-to-late instars, their galleries pass through wood that has been extensively decayed by a variety of common wood-decay fungi. We have routinely isolated *Amylostereum chailletii, Hirschioporus abietinus, Stereum sanguinolentum,* and *Trichoderma harzianum* from wood adjacent to the larval galleries of *M. marmorator.*

Because of the ease with which *M. marmorator* larvae can be separated from the fungi they encounter in their galleries and reared on diets with and without live fungal tissue and active fungal enzymes, it has been possible to test the hypothesis that cellulose digestion is brought about by ingested fungal enzymes. Larvae maintained on a diet of balsam fir wood chips permeated by live mycelium of *T. harzianum* assimilated balsam fir wood 2.5 times as efficiently as larvae that had been fed diets that lacked any live fungal tissue or active fungal enzymes (Table 1). Larvae on the fungus-free diet digested virtually no cellulose, whereas the larvae on the fungus-containing diet assimilated more than 25% of the cellulose ingested. The dependence of cellulolytic capacity on the ingestion of live fungal tissue was confirmed in experiments in which cellulose digestion was monitored by measuring carbon-14 in carbon dioxide respired by larvae fed [U-^{14}C]cellulose suspended in an agar-based artificial diet. Labeled carbon dioxide was produced only by those larvae that had been feeding on fungus-infected wood chips prior to being fed the labeled cellulose. Larvae that had been maintained for two weeks on a fungus-free diet produced no labeled carbon dioxide. In a third series of experiments we showed that enzymatic activity toward microcrystalline cellulose can be detected in a midgut extract from *M. marmorator* larvae collected from their natural galleries or cultured on *T. harzianum*-infected wood chips, but not in a midgut extract from larvae maintained on a fungus-free artificial diet or a diet of autoclaved wood chips (Table 2). Thus, three independent sets of experiments demonstrated that cellulose digestion in *M. marmorator* larvae is due entirely to fungal enzymes that originate in the food.

The four fungi routinely isolated from wood through which the larval galleries pass are cellulose digesters. *T. harzianum,* when grown axenically either in a defined liquid medium or on balsam fir wood chips, secretes a soluble cellulase complex (Table 2). We demonstrated that the cellulase complex present in the midgut fluid of *M. marmorator* larvae feeding on *T. harzianum*-infected wood chips is identical to the cellulase complex secreted by *T. harzianum* growing in an axenic culture. We isolated the cellulase complexes from larval midguts and from an axenic culture and compared them with respect to chromatographic behavior on a high resolution anion exchange column using a fast protein liquid chromatography system, molecular weight

TABLE 2. *Enzymatic activity toward microcrystalline cellulose of extracts of midguts of* Monochamus marmorator *larvae, balsam fir wood chips (BFWC) and* Trichoderma harzianum.

Source of extract	Activity toward microcrystalline cellulose (Units/mg dissolved solids)
Larvae	
Collected from natural galleries in balsam fir	0.55 ± 0.04 (5)
Cultured for 2 weeks on BFWC permeated by *T. harzianum*	0.45 ± 0.08 (10)
Cultured for 2 weeks on artificial diet	0.01 ± 0.005 (6)
Cultured for 2 weeks on artificial diet, followed by 2 weeks on autoclaved *T. harzianum*-infected BFWC	0.01 ± 0.005 (5)
BFWC	
Sterile	0.00 (1)
Permeated by *T. harzianum*	0.40 ± 0.01 (3)
Culture fluid from 2-week-old axenic culture of *T. harzianum*	2.3 ± 0.7 (3)

as determined by discontinuous-SDS-gradient electrophoresis on polyacrylamide gels, and isoelectric point as determined by analytical isoelectric focusing on ultrathin polyacrylamide gels. In no case was it possible to distinguish the cellulases of fungal and insect origin.

The association of the *M. marmorator* larvae with fungi is one with "consistent and extended contact," but it is non-specific and non-obligate. The beetles are able to derive nourishment from wood, even without the cellulolytic capacity derived from ingested fungal enzymes, because they are able to digest non-cellulosic polysaccharides. Beetles on a fungus-free diet digest 21% of the dry matter of wood, even though they fail to digest cellulose (Table 1). The ability of early instars to survive in wood that has not yet been colonized by fungus is further testimony to the ability of larvae to exploit the nutritive value of wood without the acquisition of cellulolytic capacity by the ingestion of fungal enzymes. Moreover, the beetles do not seem to possess any traits that can be identified clearly as adaptive mechanisms that facilitate the establishment, stability, or continuity of the association. Gallery construction by the larvae benefits the fungi by providing additional suitable habitat for colonization, but there is no suggestion that the larvae adjust their behavior in ways that enhance the quality of the galleries as a fungal habitat or that the adult females are vectors of the fungi associated with larval galleries. Thus, this casual association of *M. marmorator* and various common wood-decay fungi is a facultative mutualism, albeit a rather asymmetric one with the benefits skewed in the direction of the beetles, and also one in which there is little or no coadaptation. When considered in terms of the five stages involved in the evolution of a stable symbiosis proposed by Taylor, it is evident that the association of cerambycid larvae with wood decay fungi has progressed beyond the first, but no further than the fourth, stage.

For ingested fungal enzymes to augment an organism's digestive capabilities, the enzymes must be stable and active under the conditions encountered in the digestive tract. The pH of the gut fluids of cerambycid larvae is close to neutrality, generally ranging from 6.8 to 7.2 (Kukor *et al.*, 1988). Although this range is slightly above the pH at which most fungal cellulases are maximally active, it is, nonetheless, a range in which such enzymes are stable and exhibit significant activity. Schlottke (1945) detected protease activity in the gut fluids of several species and found that activity is quite low in the anterior portion of the midgut where activity toward polysaccharides is highest. He also noted that the anterior portion of the midgut is slightly acidic while the posterior portion is slightly alkaline, and that the proteases are more active under alkaline than acidic conditions. Thus, under the conditions that prevail in the midgut, especially in the anterior portion, ingested fungal enzymes would not be subject to rapid digestion or denaturation.

Our studies of the aspen borer, *Saperda*

calcarata (Lamiinae) provide further insights into the evolution of mutualistic associations between cerambycid larvae and wood-decay fungi (Kukor and Martin, 1986*b*). Females oviposit and larvae develop only in live trees. Thus, *Saperda* larvae do not encounter fungus-infected wood and must rely upon their own enzymes for the digestion of dietary polysaccharides. As expected, extracts of the midguts of *S. calcarata* larvae collected from their natural galleries in live aspen branches are not active against microcrystalline cellulose. Enzymes active against other polysaccharides, including xylan, starch and pectin, are present in the midgut fluid, however. *S. calcarata* is in the evolutionarily advanced subfamily Lamiinae. The use of live wood by *S. calcarata* has come about by a diet shift away from fungus-decayed wood, which is the larval food of species in the more primitive subfamily Parandrinae. This emphasizes the evolutionary lability of the associations of cerambycid beetles with wood-decay fungi. There has been no evolutionarily irreversible commitment on the part of either partner to mutual codependency.

The conditions that prevail in the digestive tract of *S. calcarata* larvae are quite compatible with the use of ingested fungal enzymes for cellulose digestion. If *S. calcarata* larvae are fed aspen wood shavings impregnated with a solution of the soluble cellulase complex from the fungus *Penicillium funiculosum*, they are transformed into cellulose digesters. A midgut extract of *S. calcarata* larvae that had been feeding on cellulase-impregnated aspen wood is active against microcrystalline cellulose, and the larvae are able to digest labeled cellulose. If adult *S. calcarata* females were to alter their oviposition behavior to include wood that was, or soon would be, infected with fungi, and if the larvae were able to survive their early instars in such wood, an association between *S. calcarata* and wood-decay fungi, very similar to the one involving *M. marmorator*, could evolve.

Thus, in wood-feeders it is possible to envision an evolutionary progression in which an association with fungi is first established by a diet shift from live or sound wood to fungus-decayed wood, after which selection, acting on acquired cellulolytic capacity, results in the evolution of greater dependence by the wood-feeder on the associated fungi, and a progression from stage one (consistent and extended contact) to stage four (dependence or interdependence). Of course, an association between wood-boring larvae and wood-decay fungi need not evolve inevitably toward mutualism. Evolution from stage one to stage four could be prevented by adverse consequences of association, such as the pathogenicity or toxicity of the fungi. However, there is little reason to expect that fungi specialized as saprophytes would also exhibit virulent pathogenicity toward insects. The extent to which chemical toxins in the fungi would be a barrier to the evolution of tolerance is unclear, since the allelochemicals produced by the mycelia of wood-decay fungi and the detoxification capacities of wood-boring beetles have received scant attention.

ARTHROPOD-FUNGUS INTERACTIONS THAT HAVE NOT EVOLVED INTO FACULTATIVE MUTUALISMS

In this section I discuss arthropods that regularly ingest a mixture of plant tissue and fungi but have not evolved mutualistic associations with fungi. These systems help to clarify the conditions that must be fulfilled for the ingestion of fungal enzymes to be a factor that favors the evolution of an arthropod-fungus interaction from one of extended and consistent contact into a facultative mutualism.

Crane fly larvae

Detritivores might be expected to augment digestive capacity by the ingestion of enzymes produced by members of the decomposer community normally associated with decaying plant debris. This possibility has been explored in several species of aquatic shredders (macroinvertebrates that feed on large particulate matter, especially leaves). Larvae of the crane fly *Tipula abdominalis* (Diptera) are common shredders in small woodland streams in the eastern and northern parts of the United States. Larvae prefer leaves that have been "conditioned" by several weeks' residence in a stream, and maximal larval growth rates

occur when the fungal content of the detrital food reaches its maximum (Lawson *et al.*, 1984). Although *T. abdominalis* larvae assimilate 18–19% of the cellulose they ingest, ingested microbial enzymes do not contribute to cellulose digestion in this species (Sinsabaugh *et al.*, 1985). Rather, cellulose degradation is mediated by bacteria in the rectal caecum.

Ingested fungal enzymes fail to augment the digestive capacity of *T. abdominalis* larvae because ingested enzymes are not stable in the midgut of this species (Martin *et al.*, 1980; Sharma *et al.*, 1984). The midgut is highly alkaline, with a maximum pH of 11.0–11.6 near the midpoint. Proteolytic activity in the midgut is dependent upon pH, increasing sharply above pH 9 and reaching a maximum at pH 11 or above. The level of proteolytic activity in the midgut is extraordinarily high. Unpurified gut material, assayed at pH 11.5, possesses activity within an order of magnitude of that in some commercial preparations of partially purified protreolytic enzymes (such as Pronase), assayed at their pH optima. Ingested microbial enzymes would not survive long under such harsh conditions. Even in the unlikely event that an ingested microbial enzymes were resistant to proteolysis, it is doubtful that it would manifest normal catalytic activity under these conditions, since most microbial cellulases and hemicellulases are inactive under highly alkaline conditions.

A reliance on ingested microbial enzymes for the digestion of plant structural polysaccharides is, therefore, precluded in species that have digestive systems selected for rapid or efficient protein digestion. Other insects with highly alkaline and highly proteolytic midgut fluids include mosquito larvae, blackfly larvae, lepidopteran larvae and scarab beetle larvae. Members of these taxa are, therefore, unlikely to established commensalistic or mutualistic relationships with fungi based upon the augmentation of digestive capacity through the ingestion of fungal enzymes.

Isopods

The common woodlouse, *Tracheoniscus rathkei* (Isopoda), is an abundant litter-feeder in the deciduous woodlands of the United States. Reyes and Tiedje (1976) established that this detritivore is able to digest hemicellulose, but not cellulose. We have shown that the gut fluid of *T. rathkei* contains enzymes active against a variety of substrates representative of major classes of polysaccharides of vascular plants, algae, fungi, and animals, but that it does not contain the complete cellulase complex required to degrade native lignocellulose (Kukor and Martin, 1986*c*). Highest activity is toward amylose and xylan (a hemicellulose). An extract of the leaf litter from which the isopods were collected exhibited activity toward all of the same substrates as the gut fluid. The highest activity in the litter extract was also toward xylan, and there was no activity toward cellulose. Xylanase activity in the gut fluid of *T. rathkei* declined to about 50% of its normal level when the isopods were cultured for four weeks on enzyme- and microbe-free substrates, indicating that some of the activity toward xylan in the gut fluid might be of dietary origin. The conditions in the gut fluid of *T. rathkei* are quite compatible with a role for ingested microbial enzymes. The pH lies in the range 6.5–7.1, and proteolytic activity is not so high as in the gut fluid of crane fly larvae. We conducted experiments, similar to the ones described for *M. marmorator,* to test the hypothesis that ingested microbial enzymes are responsible, at least in part, for the capacity of this isopod to digest hemicellulose. Our results forced us to reject that hypothesis.

We showed that one of the gut fluid xylanases, which contributed 8% of the total xylanase activity of the midgut, was identical to a xylanase present in the litter. Another gut fluid xylanase, accounting for an additional 7% of the total activity of the gut fluid, was also very probably derived from the litter. Ingested microbial xylanases are, therefore, present, stable and active in the midgut fluids of *T. rathkei,* but they account for only a fraction of the total xylanase activity. To assess the importance of the ingested xylanases to digestion in *T. rathkei,* we compared the approximate digestibility of autoclaved leaf litter in animals reared on an enzyme-free diet of rabbit chow and

in animals reared on normal enzyme-containing leaf litter. Animals reared on the enzyme-free diet actually assimilated a higher fraction of dry matter from autoclaved leaf litter than animals reared on enzyme-containing natural litter, a result clearly demonstrating that the ingestion of microbial enzymes did not make the isopods more efficient at digesting their natural food.

Ingested microbial xylanases fail to enhance the digestive capacity of *T. rathke* because the ingested enzymes are redundant enzymes already present in the isopod's gut. The level of xylanase activity due to enzymes produced by the isopods, or their gut symbionts, is sufficiently high that merely adding more of the same kind of enzyme does not enhance digestive efficiency. For an ingested enzymes to augment an arthropod's digestive capacity, it must be of a type not produced in significant amounts by the arthropod or its endosymbionts. Since the ability to digest cellulose is less common in arthropods than the ability to digest other plant polysaccharides, the ingestion of microbial cellulases is more likely to expand the digestive capacity of an arthropod than the ingestion of other microbial polysaccharidases.

Stonefly nymphs and caddisfly larvae

Stonefly nymphs (Plecoptera) and caddisfly larvae (Trichoptera), like crane fly larvae, are common aquatic shredders in small woodland streams. Using dual-labeled cellulose, Sinsabaugh *et al.* (1985) demonstrated that nymphs of the stonefly *Pteronarcys proteus* and larvae of the caddisfly *Pycnopsyche luculenta* digested 11% and 12%, respectively, of the cellulose they ingested. Since the digestive tracts of stoneflies and caddisflies possess no enlarged segments that might serve as fermentation chambers and are not thought to harbor permanent gut floras, the authors concluded that cellulose digestion in these species cannot be attributed to gut microbes. They proposed instead that cellulolytic capacity is acquired by the ingestion of microbial enzymes present in the detrital food.

The associations of stoneflies and caddisflies with aquatic hyphomycetes, like the associations of cerambycid beetles with wood-decay fungi, are casual non-specific associations. However, these associations differ from those between wood-feeders and fungi in the potential they possess to evolve into mutualistic associations. In the associations of wood-feeding beetles with wood-decay fungi, the fungus does, or can, benefit from its association with the beetles. Larval galleries provide new colonization sites for the fungi, and the fungus is able to escape into portions of the tree where it is not consumed by the beetles. By contrast, aquatic hyphomycetes do not benefit from their interactions with aquatic shredders. The shredders do not provide new substrate or habitat for the fungi, and their feeding activities reduce both the amount of available substrate and the standing crop of the fungus. Thus, from the perspective of the fungus, the interaction is entirely antagonistic, and the association would seem to have little potential to evolve toward mutualism.

INSECT-FUNGUS INTERACTIONS THAT HAVE EVOLVED INTO STABLE OLIBGATE SYMBIOSES

Ingested enzymes play an important role in the associations of fungi with siricid wood wasps, fungus-growing termites, and fungus-growing ants. These associations have evolved into stable symbioses. What distinguishes stable symbioses (stage 5) from beneficial but facultative associations (stage 4) are the numerous adaptations of each partner that facilitate the perpetuation of the association and promote interdependence.

The siricid woodwasps

Woodwasp larvae (Siricidae) are wood borers whose galleries are colonized by wood-decay fungi. The larval diets of *Sirex* and *Urocerus* are predominantly wood, but fragments of fungal mycelium always occur among the wood fragments in the gut. The fungal associates of siricid larvae are cellulose digesters (Francke-Grosmann, 1939). Cellulose and hemicellulose are digested during the passage of wood through a larva's alimentary canal. In a series of experiments similar to the those conducted on the cerambycid beetles, Kukor and Martin (1983) established that cellulose and hemicellulose

digestion in the midgut of *S. cyaneus* larvae is brought about entirely by ingested fungal enzymes. The gut fluid of larvae collected from their natural galleries or maintained on balsam fir wood chips infected with their symbiotic fungus, *Amylostereum chailletii,* is active against microcrystalline cellulose and xylan, whereas the gut fluid from larvae maintained on sterile autoclaved balsam fir wood chips is not active against either of these polysaccharides. We also demonstrated that the C_x-cellulases and xylanases isolated from the larval gut fluid are indistinguishable from the C_x-cellulases and xylanases produced by an axenic culture of *A. chailletii,* when compared with respect to chromatographic behavior on an anion exchange resin, molecular weight, and isoelectric point.

The siricid-fungus interaction is distinguished from the cerambycid-fungus interaction by the degree of interdependence of the two partners, the stability of the association, and numerous adaptations of the woodwasps that perpetuate and sustain it. Buchner (1928, 1965) was the first to draw attention to the mechanism by which ovipositing females ensure that the fungus is introduced into larval galleries. He observed that two pear-shaped structures, the intersegmental pockets, at the base of the egg-laying apparatus are filled with fragments of fungal mycelium (oidia or arthrospores) suspended in a mucus, and suggested that these mycelial fragments are implanted in the wood during oviposition. This hypothesis was verified by careful studies of oviposition in several species of *Sirex, Urocerus* and *Tremex* (for reference, see Martin, 1987). Following implantation of the arthrospores into a suitable tree, the fungus produces vegetative hyphae that colonize the wood surrounding the oviposition hole and, subsequently, the larval galleries. The fungal hyphae cover the surface of the tunnels and penetrate into the surrounding wood. Before emergence, the intersegmental pockets of the newly ecdysed females are reinoculated with the fungal symbiont (Francke-Grosmann, 1957).

Stillwell (1964) first demonstrated the dependence of siricid larvae on their fungal symbionts. He interrupted the reinoculation of the intersegmental pockets of the adult females with oidia, thus producing aposymbiotic females. Normal and aposymbiotic females were allowed to oviposit in logs of natural host wood. Normal larval development occurred only in those logs in which the fungal symbiont had been introduced during oviposition by females having oidia in their intersegmental pockets. The eggs laid by the aposymbiotic females hatched, but the larvae died during the first instar.

The fungal symbionts of the siricid woodwasps are Basidiomycetes which are easily cultured from arthrospores isolated from the intersegmental pockets of adult females (Francke-Grosmann, 1939). The fungal symbiont of *Tremex columba,* which attacks hardwoods, has been identified as *Cerrena* (=*Daedalea*) *unicolor* (Stillwell, 1964). Woodwasps that attack conifers are associated with fungi in the genus *Amylostereum* (Gilbertson, 1984). *A. chailletii* has been identified as the symbiont of four species of *Sirex* and five of *Urocerus,* whereas *A. aerolatum* has been identified as the symbiont of three species of *Sirex.* Thus, the siricid-fungus symbiosis conforms to Law's first prediction that in an evolving symbiotic association the more protected symbiont should be less species rich than the partner that is more exposed to hostile selective forces.

The genus *Amylostereum* (Thelophoraceae) includes many free-living species that produce fruiting bodies. The species or strains associated with *Sirex,* however, do not form fruiting bodies readily, and woodwasps are the major, or in some cases probably the exclusive, agents of dispersal. For example, propagation of *A. chailletii* in Nova Scotia and New Brunswick (Stillwell, 1964) and of *A. aerolatum* in Australia (Gilmour, 1965) is believed to be due entirely to woodwasps. The adoption of an asexual mode of reproduction by the *Amylostereum* species that are symbionts of woodwasps is in accord with the second prediction of Law (1985) that there should be selection for asexual reproduction in protected mutualists.

The fungus-growing termites

The fungus-growing termites are often dominant components of the termite fauna of Africa and Indomalaya. The workers for-

age on structural timber, dead wood, dead grass and the dung of herbivorous mammals, and often play a major role in litter removal. The association of the fungus-growing termites (Macrotermitinae) and their symbionts exemplifies a stable symbiosis with an even higher degree of interdependence and coadaptation than is evident in the woodwasp-fungus association. Not only do the termites disperse the fungus, but they also foster its vegetative growth in their nests. They collect substrate, chew it into very small fragments, maintain constant conditions in the nest which are favorable for growth, and possibly restrict the growth of potential competitors by mixing the comminuted substrate with their saliva and other secretions. For recent reviews of this symbiosis, see Martin (1987) and Wood and Thomas (1989).

The termites culture their symbionts on structures known as fungus combs. The mature combs of *Macrotermes* are firm highly convoluted structures which are covered with a sparse growth of mycelium and numerous small white spheres or nodules, called mycotêtes. Mycotêtes, which are aggregations of conidiophores and conidia, are the asexual reproductive stage of the symbiotic fungus. The combs are constructed of plant material that has been chewed into very small particles and passed rapidly through the workers' guts. The presence of intact cells and cell walls, the high cellulose content of the comb, and the similarity of the cellulose-to-lignin ratio in the comb to that in the forage material indicate that very little digestion of cell wall polysaccharides occurs during the rapid passage of the comminuted plant material through the digestive tract.

Both the fungus comb and the mycotêtes are consumed by the workers, and both are necessary components of the termites' diet (Sands, 1956; Ausat *et al.*, 1962). Termites survive no longer on a diet of wood chips or filter paper, in the absence of any fungus comb, than they do when they are deprived of all food. The fungal symbiont makes at least two crucial contributions to the nutrition of the termites. First, the fungus comb is a richer source of nitrogen than the substrate on which it grows (Martin, 1987; Wood and Thomas, 1989). The nitrogen contents of the mycotêtes and the fungus comb range from 5.7 to 7.9% and 0.81 to 2.1%, respectively, whereas the normal range for wood is 0.02 to 0.3%. The second contribution is cellulolytic capacity. Fungal enzymes obtained when the workers ingest the mycotêtes, working in combination with enzymes produced by the termites, are responsible for cellulose digestion, and possibly hemicellulose digestion, in three species of *Macrotermes* (Abo-Khatwa, 1978; Martin and Martin, 1978, 1979; Rouland *et al.*, 1988*a*, *b*). For an alternative interpretation of cellulose digestion in *Macrotermes* consult Veivers *et al.* (1991).

The fungi associated with the fungus-growing termites belong to the Basidiomycete genus *Termitomyces* (Amanitaceae), which is known only in association with Macrotermitinae. Although the taxonomy of *Termitomyces* has been difficult (Heim, 1977), it is clear that there are fewer species of fungus than there are species of host termite. Wood and Thomas (1989) list 14 species of *Termitomyces* that have been identified as symbionts of 27 species of termites from the genera *Odontotermes, Macrotermes, Microtermes, Pseudoacanthotermes,* and *Acanthotermes.* Thus, Law's first prediction that there should be fewer species of the protected symbiont than host in an evolving symbiosis is upheld.

Termitomyces inoculum is introduced into newly constructed sterile combs by two different mechanisms (Johnson *et al.*, 1981; Sieber, 1983). In *Microtermes* and at least one species of *Macrotermes,* whose associated *Termitomyces* symbionts do not form fruiting bodies (basidiocarps), sterile combs are inoculated by conidia (asexual spores) carried to the nest in the guts of the founding alates. In *Odontotermes* and most species of *Macrotermes,* sterile combs are inoculated by basidiospores (sexual spores) dispersed by basidiocarps and collected by foraging workers. The *Termitomyces* species that produce fruiting bodies are associated with termite species in the more primitive termite genera, such as *Odontotermes* and *Macrotermes,* whereas those that do not form fruiting bodies are, with the exception of the symbiont of *Macrotermes bellicosus,* associated with *Microtermes,* which is a more advanced genus. This shift from sex-

ual to asexual reproduction in *Termitomyces* during the evolution of the symbiosis is in accord with Law's second prediction that selection should favor asexual reproduction in a mutualistic environment.

The degree of specificity of the associations between the fungi and their termite hosts has not been clarified sufficiently to test Law's third prediction that specificity should be low. The development of a selective medium for the isolation of *Termitomyces* from fungus comb (Thomas, 1985) should greatly facilitate the experimental study of specificity between the partners.

The fungus-growing ants

The fungus-growing ants (subfamily Myrmicinae, tribe Attini) are familiar insects in the New World Tropics. The symbiosis between the attine ants and the fungus they culture in their nests is probably the most elaborate of any insect-fungus association (Martin, 1987; Cherrett *et al.*, 1989). Primitive genera cultivate their symbionts on insect feces, insect carcasses and dead plant matter, whereas the advanced genera use leaf material cut from live plants. The fungus gardens of *Atta* are fragile sponge-like structures, 15–30 cm in diameter, consisting of many small pieces of substrate held together by a dense growth of mycelium. The symbionts of the attine ants produce peculiar hyphae with swollen tips, which form clusters, 1–2 millimeters in diameter, called staphylae. The staphylae are plucked by the adult workers, and the liquid contents are squeezed into the infrabuccal chamber (a small cuticle-lined cavity underneath the oesophagus). The fluid is then pumped into the crop and is subsequently admitted to the midgut. The workers meet 5% of their energy requirement by the ingestion of the fluid contents of the staphylae, the balance coming from plant sap ingested while cutting leaves (Quinlan and Cherrett, 1979). The larvae meet all of their nutritional requirements from the fluid contents of the staphylae.

The fungus-culturing activities of the attine ants are quite elaborate (Weber, 1972). Fragments of leaves brought into the nest are antennated by workers, and acceptable pieces are subjected to a rasping action of the glossae, after which the ants bite into the edges of the leaf fragment and remove small pieces which are chewed to a pulpy mass. This process of substrate preparation removes the protective wax layer, which serves as both a chemical and physical barrier to fungal infection, and eliminates a significant fraction of the microbial contaminants originally present on the leaf surface (Quinlan and Cherrett, 1977). The chewing process destroys cuticle and barricade cells and liberates cell contents, making nutrients available for rapid uptake by the fungal hyphae. In the final step of fungus garden construction, the workers apply a drop of liquid fecal material to the fragment of substrate, which is inserted into the garden and inoculated with a few tufts of mycelium.

Ingested fungal enzymes play an important role in the attine-fungus symbiosis, albeit a very different one from the role played in the other insect-fungus associations discussed in this paper. The fluid from the staphylae that is ingested by both workers and larvae contains proteases, amylases, pectinases, hemicellulases, cellulases (Martin, 1987), polyphenol oxidases, and "tannases" (Cherrett *et al.*, 1989). All of these enzymes remain active in the gut following ingestion. However, the absence of structural polysaccharides from the diets of both adult workers and larvae makes it unlikely that the ants derive any benefit from the expanded digestive capacity conferred upon them by the active fungal enzymes present in their digestive fluids.

The importance of the ingested fungal enzymes in this symbiosis depends upon their survival during passage through the alimentary tract and their presence in the drop of fecal material added to substrate prior to its incorporation into the garden. The fecal droplet is a concentrated solution of fungal enzymes (Martin *et al.*, 1975; Boyd and Martin, 1975*a*, *b*; Martin, 1987). The addition of enzymes to newly inoculated substrate increases the initial growth of the fungal inoculum by catalyzing the digestion of proteins and polysaccharides in the substrate and augmenting the supply of nutrients that might limit fungal growth (Martin and Martin, 1970; Boyd and Martin, 1975*a*). The fecal enzymes, especially the pectinases

and proteases, also contribute to the initial growth of the fungus by bringing about the maceration of the plant tissue, thereby facilitating hyphal invasion and subsequent ramification within the tissue. Rapid initial growth rate is an important component of competitive ability in fungi. The polyphenol oxidases and "tannases" may also facilitate the growth of the inoculum by detoxifying fungicidal substances in the leaves. Thus, in this symbiosis, the feeding and defecation behavior of the ants augment the secretory activities of the fungus. By feeding in a mature portion of a fungus garden, where enzymes are produced in abundance, and defecating at a site of newly implanted inoculum, where digestive enzymes are in short supply, the ants achieve a redistribution of fungal enzymes that allows more rapid initial growth of the inoculum than would occur if direct secretion by the fungus were the only source of enzymes at the inoculation site.

Broad host ranges are atypical of both tropical herbivorous insects and fungi that invade live plant tissue, yet ants from the genera *Atta* and *Acromyrmex* cultivate their fungi on foliage cut from dozens of different plant species. *Atta cephalotes* and *A. colombica* cut foliage from 50–75% of the plants in their nest area (Cherrett, 1968; Rockwood, 1976). Cherrett *et al.* (1989) argue that the basis for this mutualism lies in the advantages of polyphagy in a diverse tropical environment, and that the mutualism probably evolved in response to selection on both the fungus and the ants to expand host range by exploiting each other's detoxification capacities.

The fungal associates of the attine ants do not normally produce either sexual or asexual reproductive structures within the ants' nests. Propagation is entirely vegetative. When a female reproductive embarks on her mating flight, her infrabuccal pocket contains a compressed pellet of live hyphae. Upon founding a new colony, the mated female expels this pellet and sustains it by the addition of her own feces and trophic eggs until workers have been produced which will forage for additional substrate.

Attempts to characterize the fungal symbionts of the attine ants have been frustrated by the difficulty of obtaining sporophores. Two strains of fungi have been isolated consistently from the gardens, one a species of *Lepiota* that can sometimes be induced to form fruiting bodies in the laboratory (Hervey *et al.*, 1977), the other a vegetative mycelium that has never produced sporophores and which has been assigned the name *Attamyces bromatificus* (Kreisel, 1972). *Lepiota* is a well-known Basidiomycete genus with many free-living forms. This strain is isolated from the gardens of attine species from the more primitive genera, whereas *A. bromatificus* has been isolated from the gardens of species in one primitive genus, *Cyphomyrmex*, and from species in the intermediate and advanced genera. Isolates from different ant species are indistinguishable on the basis of hyphal structure (Cherrett *et al.*, 1989). On the basis of compatibility studies using paired culture tests, Stradling and Powell (1986) concluded that the same strain of fungus is cultured by many different species of ants. Thus, all three of Law's predictions about evolution in a mutualistic environment are upheld in the attine ant-fungus symbiosis. There are many fewer species of fungi than of ants, sexual reproduction has been lost in the fungal symbionts, and the host specificity of the fungus is low.

SUMMARY

Arthropod species differ in the extent to which they depend upon ingested fungal enzymes for the digestion of cellulose and other structural polysaccharides. In some species, such as the stone fly *Pteronarcys pictetii*, ingested fungal enzymes increase the efficiency of food utilization only slightly, but in others, such as the cerambycid beetle *Monochamus marmorator*, the contribution of ingested fungal enzymes to digestive efficiency is quite significant. A reliance on ingested fungal enzymes for cellulose digestion has probably been a major factor leading to the evolution of the Siricidae-*Amylostereum* and Macrotermitinae-*Termitomyces* mutualisms. A reliance on complementary detoxification systems may have been a major factor leading to the evolution of the Attini-*Lepiota/Attamyces* mutualism. Extensive interdependence and mutual

coadaptation are clearly evident in these elaborate obligate symbioses.

Analysis of the Siricidae-*Amylostereum*, Macrotermitinae-*Termitomyces*, and Attini-*Lepiota/Attamyces* symbioses have permitted a test of hypotheses concerning evolution in a mutualistic environment. As predicted, the rate of speciation in the protected partner has been slower than the rate of speciation in the host partner, selection has favored asexual reproduction in the protected partner, and, at least in the attine ant-fungus symbiosis, the protected partner exhibits a low degree of specificity toward different host species.

Insect-fungus interactions have provided rich material for the study of the mechanisms underlying mutualistic associations, the ecological significance of invertebrate-microbe interactions, and the evolution of symbiotic associations. This area of research remains an inviting one for investigators with interests that range from biochemistry and physiology to ecology and evolutionary theory.

ACKNOWLEDGMENTS

I thank the National Institutes of Health and the National Science Foundation for research grants (AI-07386, GB-30581, PCM78-22733, DEB80-22634, PCM82-03537) and a Faculty Fellowship in Science that supported the work summarized in this paper. I am particularly grateful to Drs. J. S. Martin, N. D. Boyd, and J. J. Kukor for their many contributions to this research.

REFERENCES

Abo-Khatwa, N. 1978. Cellulase of fungus-growing termites: A new hypothesis on its origin. Experientia 34:559–560.

Ausat, A., P. S. Cheema, T. Koskhi, S. L. Petri, and S. K. Ranganathan. 1962. Laboratory culturing of termites. *In Termites in the humid tropics: Proceedings of the New Delhi symposium*, 4–12 October 1960, pp. 121–125. UNESCO, Paris.

Boyd, N. D. and M. M. Martin. 1975*a*. Faecal proteinases of the fungus-growing ant *Atta texana*: Properties, significance, and possible origin. Insect Biochem. 5:619–635.

Boyd, N. D. and M. M. Martin. 1975*b*. Faecal proteinases of the fungus growing ant *Atta texana*: Their fungal origin and ecological significance. J. Insect Physiol. 21:1815–1820.

Buchner, P. 1928. *Holznahrung und Symbiose*. Springer Verlag, Berlin.

Buchner, P. 1965. *Endosymbiosis of animals with plant microorganisms*. Interscience Publishers, New York.

Cherrett, J. M. 1968. The foraging behaviour of *Atta cephalotes* L. (Hymenoptera, Formicidae). I. Foraging pattern and plant species attacked in tropical rain forest. J. Anim. Ecol. 37:387–403.

Cherrett, J. M., R. J. Powell, and D. J. Stradling. 1989. The mutualism between leaf-cutting ants and their fungus. *In* N. Wilding, N. M. Collins, P. M. Hammond, and J. F. Webber (eds.), *Insect-fungus interactions*, pp. 93–120. Academic Press, London.

Francke-Grosmann, H. 1939. Über das Zusammenleben von Holzwespen (Siricinae) mit Pilzen. Z. Angew. Ent. 25:647–680.

Francke-Grosmann, H. 1957. Über das Schicksal der Siricidenpilze während der Metamorphose. Wanderversamml. Dtsch. Ent. 8:37–43.

Gilbertson, R. L. 1984. Relationships between insects and wood-rotting Basidiomycetes. *In* Q. Wheeler and M. Blackwell (eds.), *Fungus-insect relationships*, pp. 130–165. Columbia University Press, New York.

Gilmour, J. W. 1965. The life cycle of the fungal symbiont of *Sirex noctilio*. N. Z. J. For. 10:80–89.

Heim, R. 1977. *Termites et champignons*. Société nouvelle des éditions Boubée, Paris.

Hervey, A., C. T. Rogerson, and I. Leong. 1977. Studies on fungi cultivated by ants. Brittonia 29:226–236.

Johnson, R. A., R. J. Thomas, T. G. Wood, and M. J. Swift. 1981. The inoculation of the fungus comb in newly founded colonies of some species of Macrotermitinae (Isoptera) from Nigeria. J. Nat. Hist. 15:751–756.

Kreisel, H. 1972. Pilze aus Pilzgärten von *Atta insularis* in Kuba. Z. Allg. Mikrobiol. 12:643–654.

Kukor, J. J. and M. M. Martin. 1983. Acquisition of digestive enzymes by siricid woodwasps from the fungal symbiont. Science 220:1161–1163.

Kukor, J. J. and M. M. Martin. 1986*a*. Cellulose digestion in *Monochamus marmorator* Kby. (Coleoptera: Cerambycidae): Role of acquired fungal enzymes. J. Chem. Ecol. 12:1057–1070.

Kukor, J. J. and M. M. Martin. 1986*b*. The transformation of *Saperda calcarata* (Coleoptera: Cerambycidae) into a cellulose digester through the inclusion of fungal enzymes in its diet. Oecologia 71:138–141.

Kukor, J. J. and M. M. Martin. 1986*c*. The effect of acquired microbial enzymes on assimilation efficiency in the common woodlouse. *Tracheoniscus rathkei*. Oecologia 69:360–366.

Kukor, J. J., D. P. Cowan, and M. M. Martin. 1988. The role of ingested fungal enzymes in cellulose digestion in the larvae of cerambycid beetles. Physiol. Zool. 61:364–371.

Law, R. 1985. Evolution in a mutualistic environment. *In* D. H. Boucher (ed.), *The biology of mutualism: Ecology and evolution*, pp. 145–170. Oxford University Press, New York.

Lawson, D. L., M. J. Klug, and R. W. Merritt. 1984. The influence of the physical, chemical, and microbiological characterstics of decomposing

leaves on the growth of the detritivore *Tipula abdominalis*. Can. J. Zool. 62:2339–2343.
Martin, M. M. 1983. Cellulose digestion in insects. Comp. Biochem. Physiol. 75A:313–324.
Martin, M. M. 1987. *Invertebrate-microbial interactions: Ingested fungal enzymes in arthropod biology*. Comstock Publishing Associates, Cornell University Press, Ithaca.
Martin, M. M. and J. S. Martin. 1970. The biochemical basis for the symbiosis between the ant *Atta colombica tonsipes* and its food fungus. J. Insect Physiol. 16:109–119.
Martin, M. M. and J. S. Martin. 1978. Cellulose digestion in the midgut of the fungus-growing termite *Macrotermes natalensis*: The role of acquired digestive enzymes. Science 199:1453–1455.
Martin, M. M. and J. S. Martin. 1979. The distribution and origins of the cellulolytic enzymes of the higher termite *Macrotermes natalensis*. Physiol. Zool. 52:11–21.
Martin, M. M., N. D. Boyd, M. J. Gieselmann, and R. G. Silver. 1975. Activity of faecal fluid of a leaf-cutting ant toward plant cell wall polysaccharides. J. Insect Physiol. 21:1887–1892.
Martin, M. M., J. S. Martin, J. J. Kukor, and R. W. Merritt. 1980. The digestion of protein and carbohydrate by the stream detritivore *Tipula abdominalis* (Diptera, Tipulidae). Oecologia 46: 360–364.
Quinlan, R. J. and J. M. Cherrett. 1977. The role of substrate preparation in the symbiosis between the leaf-cutting ant *Acromyrmex octospinosus* (Reich) and its food fungus. Ecol. Ent. 2:161–170.
Quinlan, R. J. and J. M. Cherrett. 1979. The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). Ecol. Ent. 4:151–160.
Reyes, V. G. and J. M. Tiedje. 1976. Metabolism of ^{14}C-labeled plant materials by wood lice (*Trachelioniscus rathkei* Brandt) and soil microorganisms. Soil Biol. Biochem. 8:103–108.
Rockwood, L. L. 1976. Plant selection and foraging patterns in two species of leaf-cutting ants (*Atta*). Ecology 57:48–61.
Rouland, C., A. Civas, J. Renoux, and F. Petek. 1988*a*. Synergistic activities of the enzymes involved in cellulose degradation, purified from *Macrotermes mülleri* and from its symbiotic fungus *Termitomyces* sp. Comp. Biochem. Physiol. 91B:459–465.
Rouland, C., J. Renoux, and F. Petek. 1988*b*. Purification and properties of two xylanases from *Macrotermes mülleri* (Termitidae, Macrotermitinae) and its symbiotic fungus *Termitomyces* sp. Insect Biochem. 18:709–715.
Sands, W. A. 1956. Some factors affecting the survival of *Odontotermes badius*. Insectes Soc. 3:531–536.
Schlottke, E. 1945. Über die Verdauungsfermente im holzfressender Käferlarven. Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere 61:88–140.
Sharma, B. R., M. M. Martin, and J. A. Shafer. 1984. Alkaline proteases from the gut fluids of detritus-feeding larvae of the crane fly *Tipula abdominalis* (Say) (Diptera, Tipulidae). Insect Biochem. 14:37–44.
Sieber, R. 1983. Establishment of fungus combs in laboratory colonies of *Macrotermes michaelseni* and *Odontotermes montanus* (Isoptera, Macrotermitinae). Insectes Soc. 30:204–209.
Sinsabaugh, R. L., A. E. Linkins, and E. F. Benfield. 1985. Cellulose digestion and assimilation by three leaf-shredding aquatic insects. Ecology 66:1464–1471.
Stillwell, M. A. 1964. The fungus associated with woodwasps occurring in beech in New Brunswick. Can. J. Bot. 42:495–496.
Stradling, D. J. and R. J. Powell. 1986. The cloning of more highly productive fungal strains: A factor in speciation of fungus growing ants. Experientia 42:962–964.
Taylor, F. J. R. 1983. Some eco-evolutionary aspects of intracellular symbiosis. *In* K. W. Jeon (ed.), *Intracellular symbiosis*, pp. 1–28. Academic Press, New York.
Thomas, R. J. 1985. Selective medium for isolation of *Termitomyces* from termite nests. Trans. Br. Mycol. Soc. 84:519–526.
Uvarov, B. P. 1929. Insect nutrition and metabolism. Trans. R. Ent. Soc. London 76:255–343.
Veivers, P. C., R. Mühlemann, M. Slaytor, R. H. Leuthold, and D. E. Bignell. 1991. Digestion, diet and polyethism in two fungus-growing termites: *Macrotermes subhyalinus* Rambur and *M. michaelseni* Sjøstedt. J. Insect Physiol. 37:675–682.
Weber, N. A. 1972. Gardening ants, the attine. Mem. Am. Phil. Soc. 92:1–146.
Wood, T. G. and R. J. Thomas. 1989. The mutualistic association between Macrotermitinae and *Termitomyces*. *In* N. Wilding, N. M. Collins, P. M. Hammond, and J. F. Webber (eds.), *Insect-fungus interactions*, pp. 69–92. Academic Press, London.