Graphania mutans (Walker) and *Acremonium lolii* (Latch)

The Relationship Between an Insect Herbivore and a Fungal Endophyte of Perennial Ryegrass.

A thesis presented in partial fulfilment
of the requirements for the degree of
Master of Science in Zoology
at Massey University

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1987

ABSTRACT

In examining the relationship between *Graphania mutans* (Walker) (Lepidoptera: Noctuidae) and perennial ryegrass infected with the fungal endophyte *Acremonium lolii* (Latch in press), the biology of *G. mutans* was investigated. Two types of larval development are identified: 'fast-track' larvae develop more rapidly through fewer instars and grow much larger than 'slow-track' larvae when reared on both artificial diet and perennial ryegrass. The complexity of *Graphania* speciation is discussed.

The presence of endophyte is shown to confer on perennial ryegrass resistance to *G. mutans* larvae in the laboratory, with strong antixenosis and possible antibiosis effects exhibited. Feeding preference tests show that neonate and sixth instar fast-track larvae significantly prefer excised endophyte-free ryegrass to endophyte-infected leaves. The effects of endophyte on the development of fast-track and slow-track larvae are to decrease larval weight, head capsule width, and the number of successful pupations.

The same methods were used to determine the effects of peramine (an antifeedant compound for Argentine stem weevil extracted from endophyte-infected perennial ryegrass) on fast-track *G. mutans* larvae. Incorporated into artificial diet at 10ppm, peramine has no affect on neonate and sixth instar larval feeding preference. Peramine does affect larval development, causing reduced larval weight, delayed pupation, and increased mortality.

The role of peramine in endophyte-induced resistance, and the possible adaptive significance for perennial ryegrass of endophyte infection is considered. The interactions between *G. mutans*, endophyte and perennial ryegrass within the pasture ecosystem are discussed, and suggestions and hypotheses presented for future investigation.

ACKNOWLEDGEMENTS

I am indebted to many people for the assistance and advice which helped make this thesis possible. Many thanks to my supervisor Professor Brian Springett, who provided constructive criticism and advice throughout the study, and to Dr. Peter Fenemore who critically appraised the draft of the thesis.

I am particularly grateful for the help, encouragement and facilities provided by the staff of the Entomology Division substation of the DSIR at Palmerston North. Peter McGregor was instrumental in guiding me into research on *Graphania mutans*, and his assistance in matters scientific, statistical, and photographic is greatly appreciated. I am also obliged to Dr. Jenny Dymock, Dr. Darryl Rowan and Steve Pilkington for much useful discussion.

John Dugdale confirmed the identity of my moths, and supplied helpful information about *Graphania* taxonomy. Advice on statistical analyses was provided by Dr. Ian Henderson, and Mike Moffat helped in the production of the figures. I am also most grateful for the interest shown by the staff and post-graduate students of the Botany and Zoology Department, Massey University.

Finally, I thank my family for their invaluable support and encouragement, and in particular I thank Keitha Eichstaedt for her assistance, understanding, and above all, tolerance.

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CHAPTER ONE

INTRODUCTION

The impact of insects on agricultural crops has been documented since the locust plagues of Egypt in biblical times. However, an understanding of the complexity of plant-insect interactions has been developed only recently. The most generally accepted theory for the evolution of insect-plant relationships suggests that angiosperms and insects have co-evolved (Ehrlich & Raven 1964), and over millions of years, have carried out a mutually counter-adaptive 'arms-race' (Edwards & Wratten 1980). The general assumption is made that greatest evolutionary fitness for the plant follows from a minimisation of herbivory. Thus any modification in plant development, chemistry, or ecological strategy that minimises nett loss to herbivores will confer a selective advantage. Phytophagous insects however, can evolve in response to these obstacles, and eventually develop different life cycle strategies and feeding mechanisms for the exploitation of their hosts. There is now a small but growing body of data that suggests that sometimes a third party may be involved, as interactions between many grasses and their herbivores are mediated by endophytic fungi. This study examines the relationship between an insect herbivore, *Graphania mutans*, and perennial ryegrass infected with a fungal endophyte, *Acremonium lolii*.

Graphania mutans (Walker) is an endemic noctuid moth found throughout New Zealand (Hudson 1928). Known as the grey brown cutworm moth, or the grey brown owlet moth (Forster & Forster 1970), it is a very common species, and may be observed on mild evenings all the year round.

Most information about the abundance of *G. mutans* has come from light-trapping records. Watt (1913), Hudson (1928), Cumber (1951), Gaskin (1964c & d), and White (1964) all showed that *G. mutans* was a dominant moth species. More recently, DSIR light-trapping records from Ballantrae, Woodville have shown that *G. mutans* was the third most abundant species, and by far the most common noctuid caught (McGregor *et al.* 1987). These records show that on average, more than 15 *G. mutans* adults/night/month/year were captured in the light-trap (P.J. Watts pers. comm.).

Light-trap records from Lincoln College (MacFarlane 1970) showed that adult flight reaches a peak during the first weeks of November, with a gradual decline until a second peak in early February. These two distinct peaks were thought to indicate two generations per year in the South Island. In the North Island, adult flight peaks are less distinct. At Paiaka, Cumber (1951) found a peak in *G. mutans* flight from late July to September, although D.E. Gaskin commented that this 'winter peak' may be due to the inclusion of a number of other species (MacFarlane 1970). Gaskin (1966a) found moths flying all year round and identified four peaks in February, March/April, October/November, and December. From these records he suggested that *G. mutans* has three or four generations in the North Island. Records of DSIR light-trapping from Ballantrae appear to confirm that *G. mutans* is multivoltine and has more generations per year than in the South Island (McGregor *et al.* 1987).

Several hosts have been described for *G. mutans* indicating that the larvae are probably polyphagous. Smith (1902) recorded the attacking of turnips at Omiki, although the identity of the larvae has subsequently been questioned (Gaskin 1966a). Thomson (1922) stated that *G. mutans* larvae seriously damage the leaves of young wheat plants, destroying considerable portions of the crops at various times, particularly in the Canterbury district. Muggeridge (1931) noted that young larvae perforated the foliage and buds and bored into the small fruit of apples, particularly Jonathons. He raised larvae to adults on a diet of apple leaves and very young apples, upon which they were "voracious feeders and thrived exceedingly well". Gaskin (1966a) listed groundsel, ragwort, plantain, petunia and pansy as host plants, and stated that this very common cutworm could possibly be a serious horticultural pest.

Although Gaskin (1966b) suggested that *G. mutans* larvae were probably responsible for significant local damage to fruit, vegetables, and ornamentals, several authors have implied its possible importance in pastures. *G. mutans* is one *Graphania* species that may eat pasture plants including grasses and clover (McGregor *et al.* 1987). Hudson (1928) writes that the sluggish larva feeds on low plants like plantain, and often "frequents the luxuriant growth surrounding logs and stones that have been undisturbed." Gaskin (1964a) noted that *G. mutans* was one of the dominant noctuid species collected in grassland samples netted during the night at Castlepoint. Quail (1901) found eggs and reared larvae on grass, and the cutworm has been recorded as a minor lucerne pest (Pottinger & MacFarlane 1967, MacFarlane 1970).

In contrast to the *G. mutans* literature, the body of information on plant-fungus interactions is rapidly expanding. For many years it had been recognised that fungi grow intercellularly in several species of grasses (McLennan 1920, Sampson 1935, Neill 1940). These fungi appeared to complete most, if not all of their life cycle within the plant, so they are commonly called endophytes. Many different grass species are now known to be affected, each with different endophytes (Clay *et al.* 1985a). The phenomenon of endophyte-mediated resistance to herbivores has been demonstrated only recently, and research has concentrated on two economically important grasses: tall fescue and perennial ryegrass. This study examines the relationship between perennial ryegrass (*Lolium perenne* L.) and its systemic fungal endophyte classified as *Acremonium loliae* (Latch, Christensen & Samuels 1984), and renamed *A. lolii* (Latch in press).

Perennial ryegrass is one of the most important and widely grown pasture plants in New Zealand. It is adaptable to a wide range of soil types and climate conditions, and generally has good nutritive forage value (Siegal *et al.* 1985). Its ability to withstand heavy grazing and pugging gives flexibility to a pasture management system, and its long-term yielding ability reduces resowing costs (Kerr 1987). Many seedlines of New Zealand perennial ryegrass cultivars contain a high incidence of endophyte mycelium (Neill 1940, Lloyd 1959, Latch & Christensen 1982, Scott 1983, Kerr 1987).

A. lolii completes its entire life cycle within the plant with no external signs of infection. Seed containing endophyte germinates to produce seedlings infected with the mycelia, and this is the only way in which the fungus is known to spread (Neill 1941). Most of the mycelium forms in leaf sheaths and seeds, with smaller amounts in stems and crowns, little in the leaves, and probably none in the roots (Siegal *et al.* 1985). The fungus grows intercellularly without penetrating the host cells, and can be detected by staining the mycelium in seeds and the upper epidermis of the leaf sheath with lactophenol cotton blue. Endophyte mycelium appears as septate, sparsely branched hyphae, generally running longitudinally between cells (Latch & Christensen 1982). The presence of endophyte can also be detected using enzyme-linked immunosorbent assays (ELISA) (Musgrave 1984).

The close association between endophyte and host plant is considered to be a mutualistic relationship. The fungus benefits by protection within the plant and dissemination through the seed, and as it apparently lacks any other form of

propagation, *A. lolii* requires an infected host grass for continued survival, and thus may be considered as an ecologically obligate symbiont (Siegal *et al.* 1985). The benefits the endophyte confers to perennial ryegrass are complex and involve increased stress-tolerance. Studies by Funk *et al.* (1985) show better performance under drought, heat and other stresses, better regrowth, and greater resistance to weed invasion by endophyte-infected plants. Latch *et al.* (1985b) demonstrated that clones of *L. perenne* 'Grasslands Nui' infected with *A. lolii* showed increased yields, and significant increases in total leaf area, tiller numbers, and growth of leaves, pseudostems, and roots. Although Neill (1941, 1952) compared herbage yields from single plants infected with endophyte and found no significant differences, Latch *et al.* suggest that this was because the variation in plant genotypes was not taken into account. Their results indicated that *A. lolii* had a large effect on plant growth.

The stress-tolerance conferred on endophyte-infected ryegrass plants is not only characterised by enhanced plant growth, but also resistance to overgrazing by herbivores and resistance to insect attack. Several insect pests have been shown to be affected by the plant-fungus interaction, and the principle aim of this study was to investigate the relationship between *A. lolii* infected perennial ryegrass and the larvae of *G. mutans*. One chemical isolated from endophyte infected ryegrass, peramine, acted as a feeding deterrent for Argentine stem weevil adults (Rowan & Gaynor 1985), and during the course of the study, an opportunity arose to compare the effects of peramine on the larvae of *G. mutans*. The biology of *G. mutans* was also investigated, as information collected during the endophyte research emphasised the intricacies of *Graphania* speciation. Although the study was laboratory based, the final aim was to examine possible interactions between *G. mutans*, endophyte, and perennial ryegrass within the pasture ecosystem.

CHAPTER TWO

BIOLOGY OF GRAPHANIA MUTANS

Introduction:

In the course of studying the effects of endophyte on *G. mutans*, it was noted that there were differences in the rate of larval development for different cohorts reared on either perennial ryegrass or a general purpose artificial diet (Appendix I). The four cohorts monitored were produced by four different *G. mutans* females obtained by light-trapping, and the larvae of each cohort were taken from a single egg batch. Figure 1 compares the pupation dates of the four cohorts and illustrates the differences in the rate of development. It can be seen that two of the cohorts pupated much earlier on both ryegrass and artificial diet. These were called "fast-track" larvae because of their rapid development, and the other two cohorts "slow-track" larvae. There was relatively little variation between siblings; all the larvae within a cohort were either fast-track or slow-track.

Another developmental difference between the two types of *G. mutans* was that slow-track larvae required an additional one or two ecdyses before reaching pupation, compared to fast-track larvae reared on the same food. This can be seen in the table below, which describes the number of instars of the fast-track and slow-track larvae reared on artificial diet and endophyte-free ryegrass.

	NUMBER OF INSTARS	
	Artificial Diet	Endophyte-free Ryegrass
FAST-TRACK LARVAE	mostly 6 some 7	all 7
SLOW-TRACK LARVAE	mostly 7 some 8	8, 9, or 10

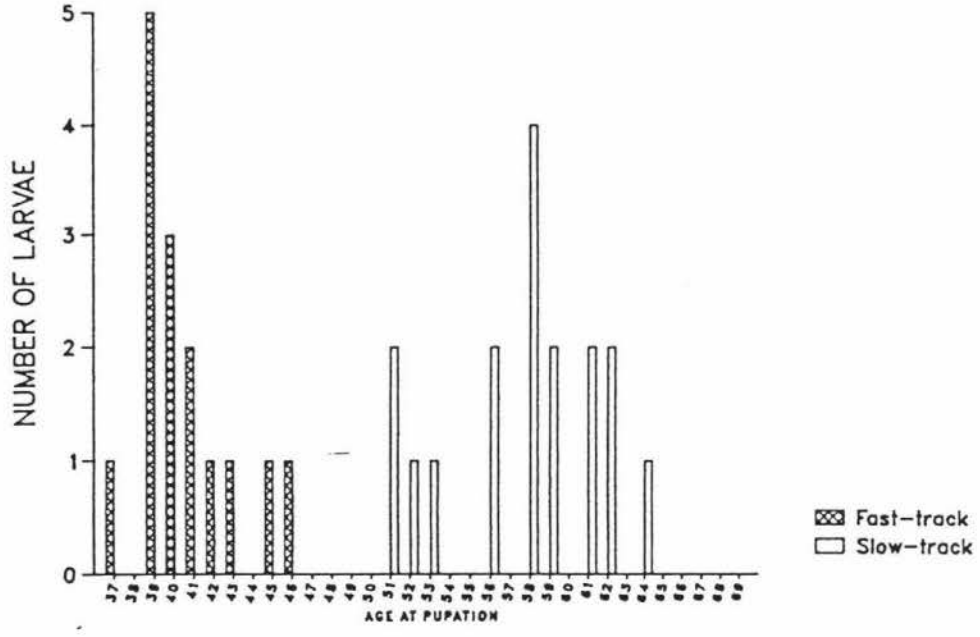

FIGURE 1a: Artificial Diet

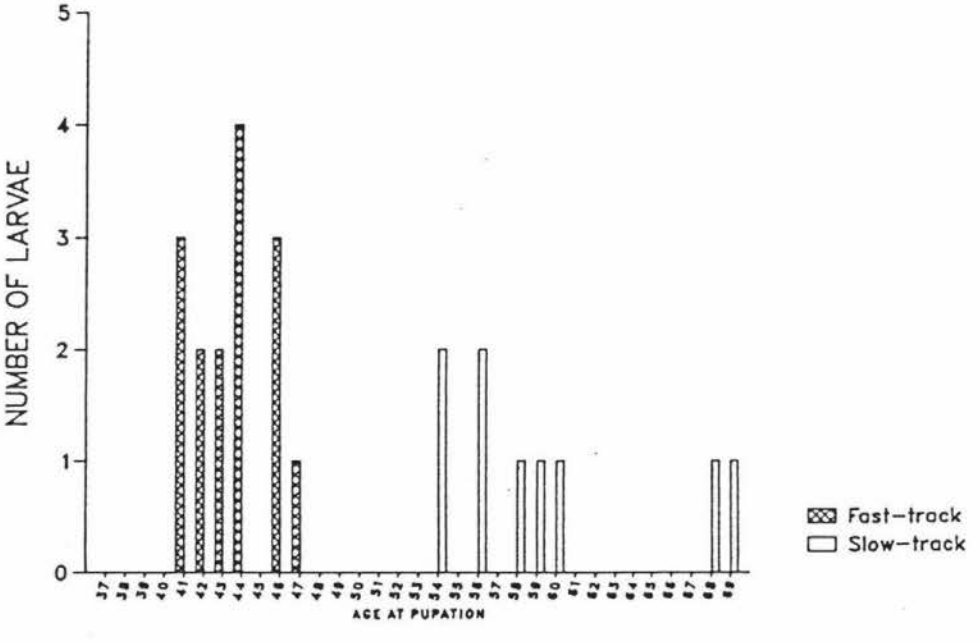

FIGURE 1b: Endophyte-free Ryegrass

FIGURE 1: Age at pupation (in days) of fast-track and slow-track *G. mutans* reared on artificial diet or endophyte-free perennial ryegrass leaves.

From this table it can be seen that larvae of *G. mutans* exhibited a developmental polymorphism (Schmidt & Lauer 1977), by which a few larvae of both fast-track and slow-track groups developed through supernumerary instars. It was the total number of moults for all larvae, including those with supernumerary instars, which differed when reared on the same diet. The additional moults of the slow-track larvae occurred about halfway through larval development; the fast-track larvae developed more rapidly through instars three, four and five.

The first section of this chapter describes the life history of *G. mutans*. Here the fast-track and slow-track larvae are assumed to be identical; there were no apparent morphological differences between them. The second section provides a more detailed comparison of some parameters of fast-track and slow-track *G. mutans* larval development on artificial diet.

SECTION 1: GRAPHANIA MUTANS LIFE HISTORY

Introduction:

Like many of the *Graphania* species, little is known about the life history of *G. mutans*, despite its abundance. Although adults are easily obtained by light-trapping, finding larvae in the field is much more difficult. Hudson (1892) stated that the larva was "best obtained by overturning logs and stones, when it may be discovered among the grass and other plants growing round their edge". Nearly one hundred years later, much 'overturning of logs and stones' revealed a distinct paucity of larvae.

Small larvae were sometimes found in pasture by lifting the leaves of plantain, or searching among the roots of clover plants showing signs of caterpillar feeding damage. When laboratory-reared larvae were placed on turfs in a controlled temperature room, it was observed that the larger larvae (fifth and sixth instar) burrowed into the soil, generally re-appearing at night to feed. The small larvae (first to third instar) tended to stay on the foliage, particularly the stems of ryegrass and petioles of clover.

To overcome the difficulties associated with finding *G. mutans* in the field, laboratory-reared insects were used in this study. This section summarises the life history of *G. mutans* reared in controlled temperature rooms on artificial diet or perennial ryegrass.

(1) Eggs:

G. mutans eggs have been found between a blade and stem of withered grass (Quail 1901), on a lucerne leaflet (MacFarlane 1970), and hundreds of batches were found on the underside of leaves of white magnolia and red flowering gum (Watt 1913). Watt also found eggs on windows and walls of houses and other "seemingly most unsuitable places", an observation confirmed on March 29, 1987 when a female *G. mutans* was seen laying eggs on the weatherboards of the Entomology Division building, DSIR, Palmerston North. In captivity the adults will oviposit at night on a variety of materials (paper, plastic, metal), the eggs generally being deposited on the lower surfaces of the object.

The eggs have been described by Quail (1901) and Watt (1913), and are illustrated in Plate 1. Usually 0.60mm - 0.65mm in diameter and 0.40mm high with a flattened base, the eggs are laid in batches in neat regular rows. In general there is only one layer of eggs, but occasionally rows of eggs are stacked on top of each other. The egg batches may be as small as 6 eggs, or well in excess of 200. The number of egg batches also varies; most moths lay one batch, others lay three or more. As the eggs develop, their colour changes from a creamy yellow or pale green, to a light brown with blackish speckles just prior to hatching. Infertile eggs collapse without colour change.

In addition to stating that the eggs of *G. mutans* were very subject to the attacks of parasites, Watt (1913) reported finding two varieties of eggs which differed "quite enough to make one believe at first sight that they belong to different species". Watt identified minor differences in sculpture and micropyle, and clear colour differences between his 'true' *G. mutans* eggs and those of "Variety A" and "Variety B". In this study, variation in egg colour was observed, but further research is needed before differences in *G. mutans* eggs could be linked to fast-track and slow-track larval development.

(2) Larvae:

Eggs laid on paper by captive females hatched after 6-7 days in a controlled temperature room (21°C ± 2°C), much earlier than the eggs laid on the wall of the Entomology Division building, which hatched after 12 days. Emerging through the top of the egg, the larvae eat the empty eggshell and then descend to the ground on long lengths of 'silk'. Neonate larvae feed on the lower epidermis and mesophyll tissues of plants, while larger larvae can devour all parts of the leaf blade. The morphology of ryegrass-reared larvae is described below.

The first instar larva is initially a light brown colour, with large setae prominent all over the body, and as noted by Quail (1901), the larva progresses in a "semi-looper" manner. Plate 2 illustrates first instar larvae and the remains of their eggshells. Having fed, the body of the larva is the same colour as the ryegrass leaves, mainly because the body wall is almost transparent in the early instars. The primary colour is pale green, with flecks of yellow, brown, pink, and black appearing as they develop.

As the size of the body increases, so the sparsely distributed black setae become less prominent. The spiracles are present as shiny black circles with coffee-coloured valves. With the second instar comes the white lateral line characteristic of *G. mutans* larvae, running just beneath the spiracles. Under the microscope this 'line' appears as a concentration of the white speckles found all over the body. Three other white lines are present, all slightly fainter: one along the dorsal midline and one on each side of the dorsal surface.

Early in instar three, the dorsal surfaces of the larva are coloured the dark green of the ryegrass leaf; the ventral surface being much paler, with many white and yellow flecks. On either side of the spiracles and above the white lateral line appear the first signs of black pigment. By the end of the third instar, and throughout the fourth, these regions become distinct black lines. The dorsal surface appears mottled, with obvious white flecks and concentrations of black and dark green pigments in pits and creases of the body wall. The head capsule remains green, with black ocelli. Plate 3 shows a fourth instar larva on a ryegrass leaf.

The extra ecdyses associated with the slow-track larvae occur about halfway through larval development. At this time, the third, fourth, fifth, and sometimes sixth instars may appear virtually identical. However, it is always the final two instars prior to

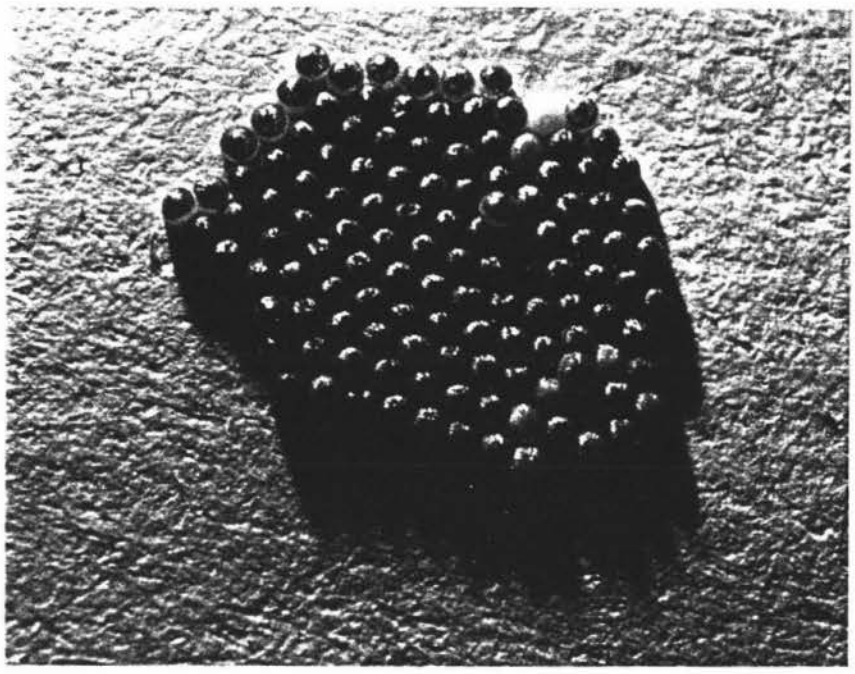

PLATE 1: *G. mutans* egg batch laid on filter paper. The pale green eggs are infertile.

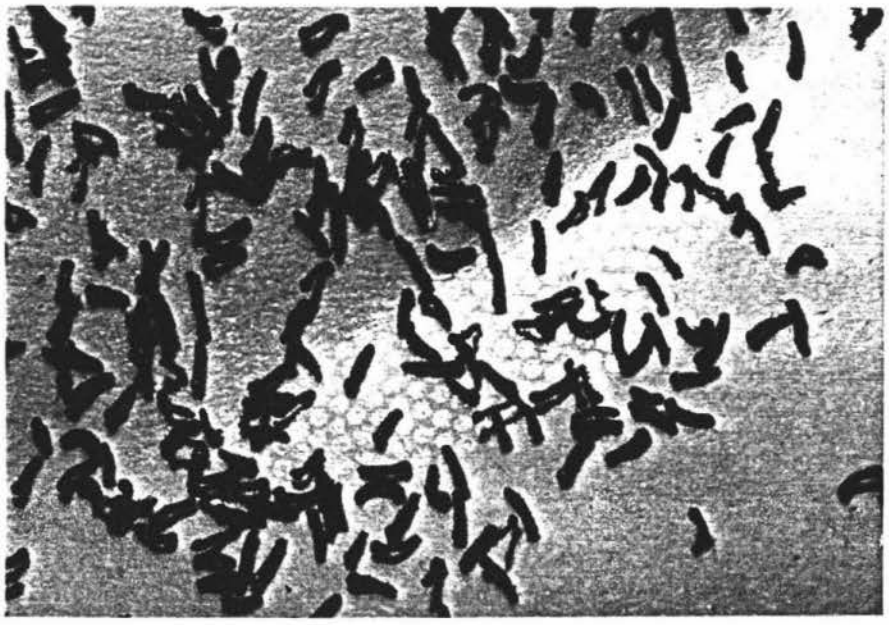

PLATE 2: First instar *G. mutans* larvae surrounding the remains of their eggshells.

pupation which display the characteristic markings associated with mature *G. mutans* larvae. These markings are gradually assumed during the instar preceding the final two stages, regardless of the number of moults before it.

The dorsal surface of the final two instars prior to pupation is no longer green. Instead, brown and black predominate. On close examination, it can be seen that all surfaces are flecked with a number of different colours: white, yellow, green, red, and brown. The lateral line is now broad and black, and a series of black triangular markings extend along the dorsal surface. The head capsule is also light brown, but the ventral surface of the body remains pale green. An example of typical seventh instar ryegrass-reared *G. mutans* larval markings is shown in Plate 4. The colouration of the larvae depends on the rearing medium. Diet-reared larvae are seldom green, the predominant colours being light brown and black, as shown in Plate 5.

Hudson (1892, 1928) and Quail (1901) have described and illustrated *G. mutans* larvae, and both authors have noted that a few larvae have remained either a uniform green or brown colour during the penultimate instar. This was also observed in a very small number of individuals reared on both ryegrass (where the larvae remained a uniform green), and on artificial diet (where they appeared a uniform pink colour).

Several days prior to pupation, the larvae stop eating. The subsequent loss in body weight leads to a decrease in size which causes the exoskeleton to become increasingly wrinkled. Just before pupating, the larva appears compressed, much like a concertina, and is curled into a crescent shape. In the field the larva may construct a cocoon amongst moss on fallen trees, but generally buries itself in earth prior to pupation (Hudson 1892, 1928). In the laboratory, the larva attempts to create a cocoon using chewed filter paper and faeces.

(3) Pupae:

Hudson (1928) wrote that *G. mutans* overwintered in the pupal state. If pupating in summer, only a short time was spent as pupae. Pupation in autumn meant that emergence did not take place until the following spring. However, this concept of hibernation has been questioned for many *Graphania* species. Fox (1970) described *G. mutans* as "cold resistant", one of the several species that were caught on Mount

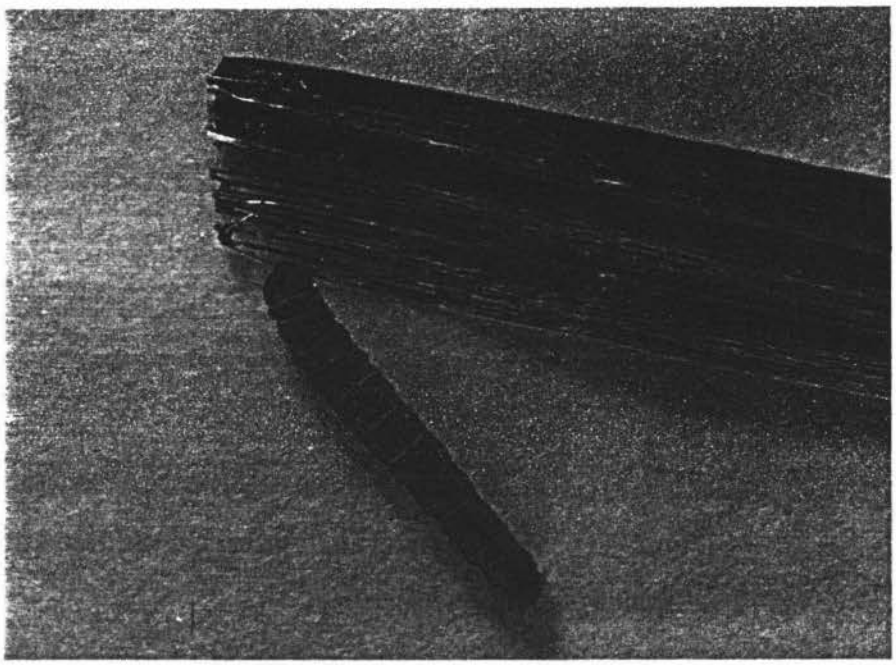

PLATE 3: Fourth instar *G. mutans* larva reared on perennial ryegrass leaves. Feeding damage is also shown.

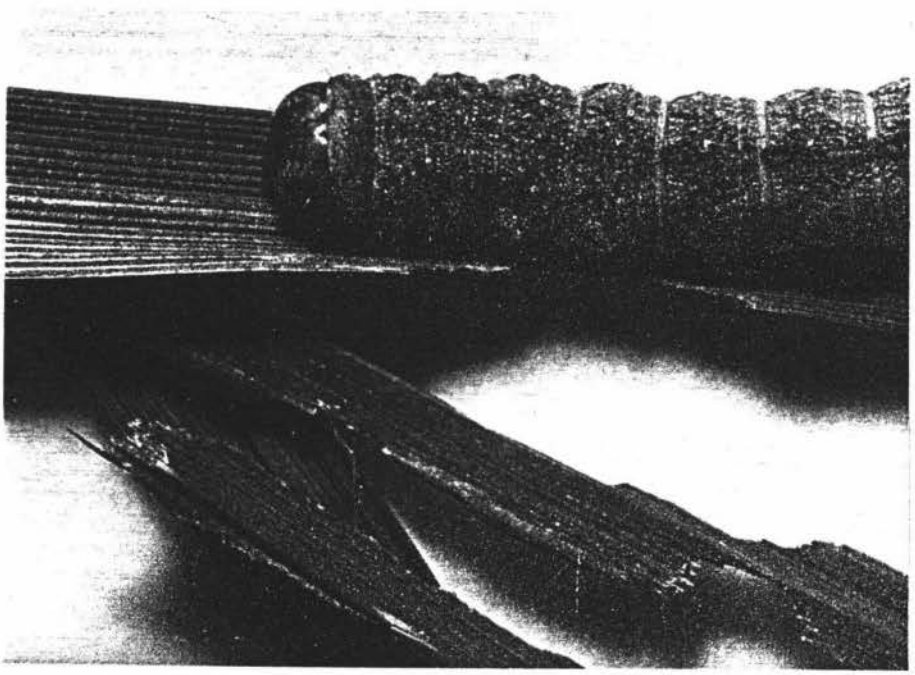

PLATE 4: Seventh instar fast-track *G. mutans* larva reared on perennial ryegrass leaves. Feeding damage is also shown.

Egmont even in mid-winter. Gaskin (1966a) stated that "hibernation has been assumed by many of the older entomologists because they came from England and their thinking was conditioned by a deciduous flora". It is unlikely that *G. mutans* hibernates in the accepted sense of the term, as in lowland New Zealand, extreme winter temperatures are very unusual. The availability of food may be more important in determining generation time (Gaskin 1966a).

The reddish-brown pupae of *G. mutans* have been described and illustrated by Hudson (1892, 1928) and Quail (1901), and are pictured in Plate 6.

(4) Adults:

The taxonomy of several *Graphania* species is "singularly complicated" (J.S. Dugdale pers. comm.). In the literature there is much confusion surrounding the identity of *G. mutans* and: *G. averilla* (Hudson 1928), *G. furtiva* (Macfarlane 1970), *G. astristriga* (Cumber 1951, MacFarlane 1970), *G. scutata* (Gaskin 1966a), *G. xanthurgramma* and *G. beata* (J.S. Dugdale pers. comm.). *G. mutans* moths have been described and illustrated by Hudson (1892, 1928, 1939), Gaskin (1966a), and Forster & Forster (1970). Male and female *G. mutans* adults are pictured in Plates 7 and 8.

Gaskin (1966a) states that "the best points of distinction are that this species does not have the mark of the reniform margined with black on its outer edge, and that the outer line of the forewings has only one tooth-shaped break in it". There is no green on the forewings and their hindwings are greyish-brown. There is considerable variation in the adults of this species, especially in the coloration of their forewings. Males are distinguished by the warm brown colouring of their forewings, head, thorax and abdomen; while the forewings of the female are generally clouded with dark grey, their head and body being a light grey colour. The antennae of the male are slightly more bipectinate than in the female (Hudson 1928).

The moths used in this study were confirmed as *G. mutans* by P.J. Watts and J.S. Dugdale. Morphologically, fast-track and slow-track adults did not exhibit consistent differences in structural characters usually considered to be specific (J.S. Dugdale pers. comm.).

PLATE 5: Recently moulted sixth instar fast-track *G. mutans* larva reared on artificial diet.

PLATE 6: *G. mutans* pupa. The gap between abdomen and thorax on the ventral surface eventually closed over.

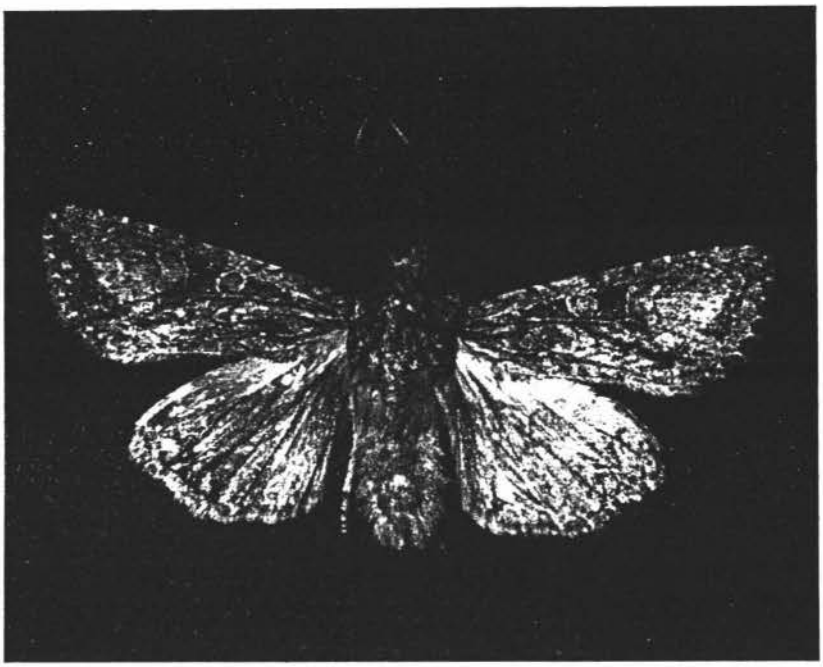

PLATE 7: Male *G. mutans* adult.

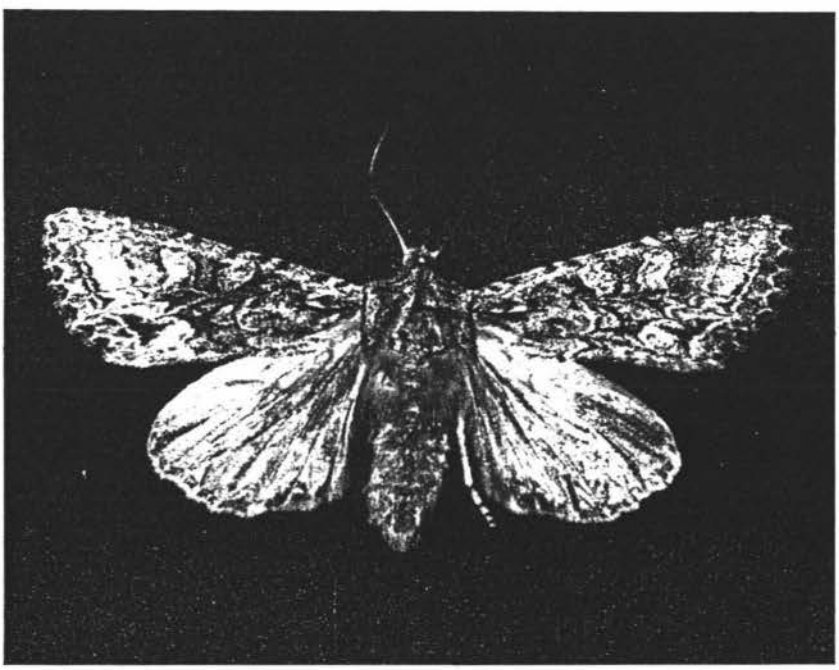

PLATE 8: Female *G. mutans* adult

SECTION 2: FAST-TRACK AND SLOW-TRACK GRAPHANIA MUTANS

Introduction:

One objective of these investigations was to develop convenient and rapid methods of identifying each of the larval instars of *G. mutans* to facilitate experiments on the effects of endophyte. In the course of evaluating one such method, it was noted that the larvae under investigation were developing much slower than usual on artificial diet (see below). This, in addition to results of an endophyte experiment being carried out at the same time (see Chapter 3, Section 3), led to the hypothesis that there were fast-track and slow-track *G. mutans*.

The experiment mentioned above was originally designed to ascertain the weights of *G. mutans* larvae reared on artificial diet. Once the larvae under investigation were recognised as slow-track, the aim of the experiment was modified to identify parameters of slow-track larval development. In this section, the results of this experiment are contrasted with the development of fast-track larvae reared on artificial diet.

Methods:

Neonate slow-track larvae from one egg batch were placed individually into 20 numbered petri dishes lined with moistened filter paper. These larvae were reared on artificial diet (Plate 2) and kept in a tupperware container (30cm x 20cm) within a controlled temperature room, (21°C ± 2°C under 18h light: 6h dark photoperiod). Every three days the diet and filter paper were changed and the larvae weighed on a Mettler balance accurate to four decimal places. Initial pupal weights were recorded with dates of pupation and eclosion. If a caterpillar had moulted, the new head capsule width (HCW) was measured using a compound microscope fitted with an eyepiece micrometer.

As there is no overlap between HCW of different stadia, measuring HCW is a simple and accurate way of determining instar, at least for larvae on a known diet and under standard conditions. The larvae must be examined each day, for they soon consume both cast off skin and head capsule. This often removes the only sign of ecdysis, as there are few morphological changes associated with the development of instars three, four and five. However, in this experiment the larvae were only examined every three days, and it was often difficult to determine if a larva had moulted, particularly at the time when the slow-track larvae were developing through supernumerary instars. This meant that some of the records of HCW for slow-track larvae reared on artificial diet were incomplete, and led to problems assessing the number of the instars of certain individuals (see Results).

The records of fast-track larval development came from an experiment where larvae were reared on a peramine-free artificial diet (Chapter 4, Section 2). Although these larvae were reared on artificial diet containing 20g of cellulose powder, their development was very similar to other fast-track larvae reared on the standard diet. These larvae were examined daily and complete records of the number of instars and HCW were obtained. Differences in the continuously distributed variables (HCW, weights, and stage durations) were analysed using ANOVA and the Student-Newman-Kuels multiple range test (Zar 1974).

Results:

(1) Number of Larval Instars:

As seen in Section 1, the majority of fast-track larvae reared on artificial diet developed through 6 instars, with only two larvae possessing the supernumerary instar. In contrast, the slow-track larvae developed through either 7 or 8 instars. Only two larvae with the supernumerary instar were definitely identified, but because there were gaps in the moulting records, it was possible that three more larvae possessed the additional instar. The larvae were grouped according to their total number of instars and were abbreviated as follows (the number of larvae in each group is written in parentheses):

F6 = fast-track larvae with 6 instars (15)
F7 = fast-track larvae with 7 instars (2)
S7 = slow-track larvae with 7 instars (16)
S8 = slow-track larvae with 8 instars (2)

One of the slow-track larvae developed through 10 instars, eventually dying after 11 weeks, well before it was ready to pupate. Its maximum weight during this time was only 20% of the average maximum weight of the slow-track larvae, and its tenth instar HCW was equivalent to that of other slow-track larvae at the sixth instar. Because this individual was so different from the others it was not considered in the results.

(2) Larval Weight:

Figure 2 illustrates the differences in mean larval weights of the slow-track and fast-track larvae. Only the results of the 6-instar fast-track and the 7-instar slow-track larvae were plotted as the small number of individuals in both F7 and S8 groups make a plot of mean weights inappropriate. (A complete description of the results is given in Appendix II). The difference in the rate of larval development is also shown in Table 1, with the slow-track larvae reaching a significantly lighter maximum larval weight some two weeks later than their fast-track counterparts. Fast and slow-track larvae with an additional instar required longer to reach their maximum larval weight, although this trend was not statistically significant.

(3) Head Capsule Width:

From Figure 3 it can be seen that the plots of HCW against instar are curvilinear and a comparison of the four multiple regressions (Zar 1974), indicates that the curves are significantly different ($F=119.36$, $p \ll 0.001$). However, Table 1 shows that there are no significant differences in HCW for the instar prior to pupation. It would appear then, that fast-track and slow-track larvae reared on artificial diet reach the same final HCW by developing through a variable number of instars. It can be seen in Figure 4 that although all the larvae have similar HCW for the first three instars, an increase in the number of ecdyses leads to a decrease in HCW for each stage, and those larvae possessing additional instars have the largest final HCW.

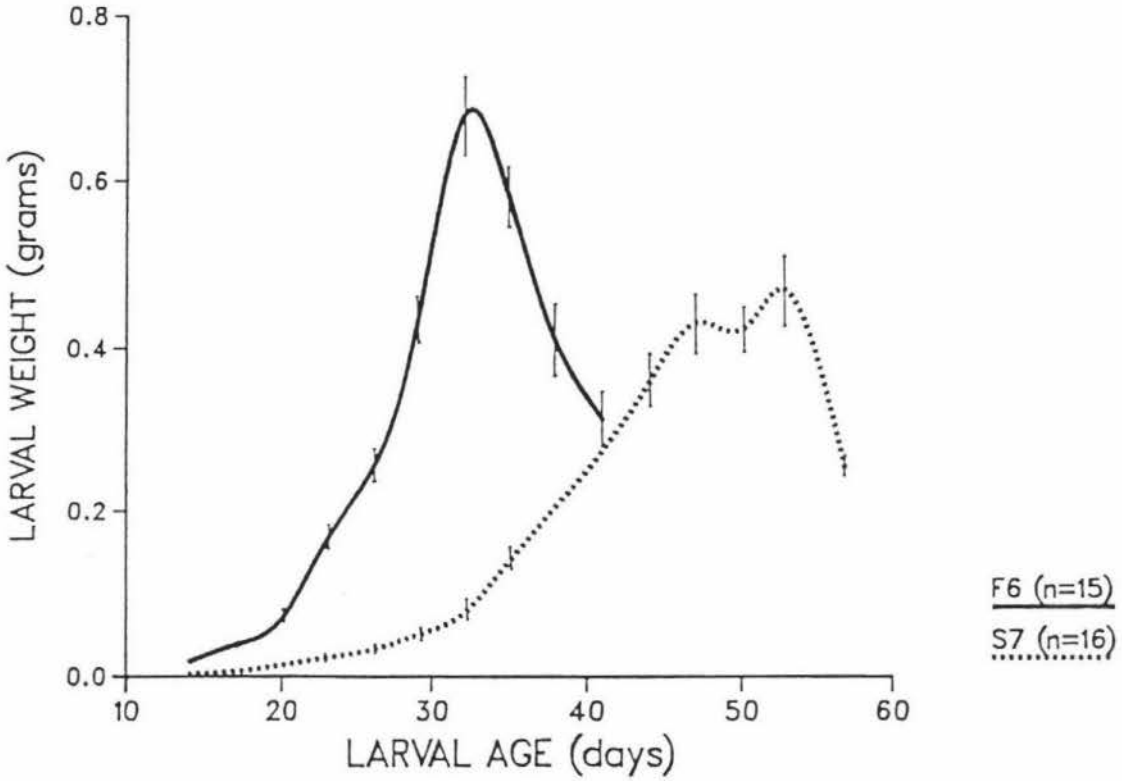

FIGURE 2: Mean larval weight ± SEM of fast-track and slow-track *G. mutans* reared on artificial diet.

F6 = six and seven instar fast-track larvae
S7 = seven and eight instar slow-track larvae

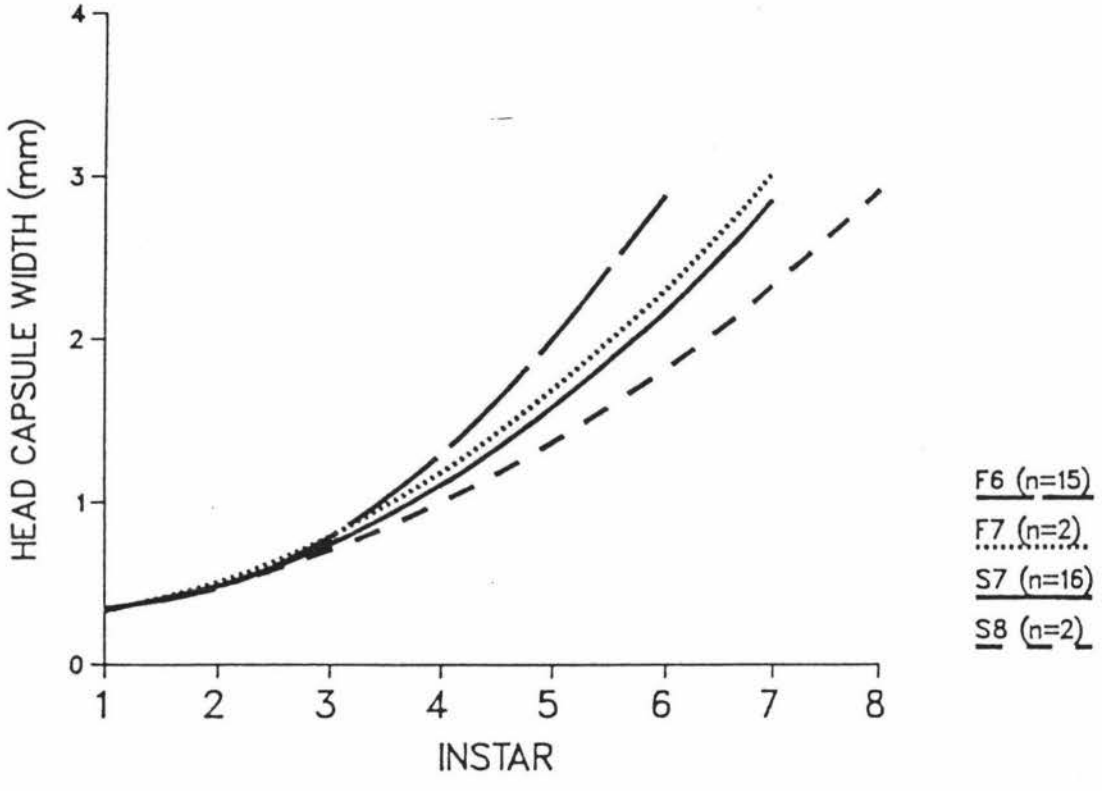

FIGURE 3: HCW regressions of fast-track and slow-track *G. mutans* reared on artificial diet.

F6 & F7 = six and seven instar fast-track larvae
S7 & S8 = seven and eight instar slow-track larvae

Regression Equations:

F6: $HCW = 0.096\ instar^2 - 0.165\ instar + 0.418$
F7: $HCW = 0.055\ instar^2 + 0.011\ instar + 0.245$
S7: $HCW = 0.055\ instar^2 - 0.020\ instar + 0.305$
S8: $HCW = 0.037\ instar^2 + 0.035\ instar + 0.264$

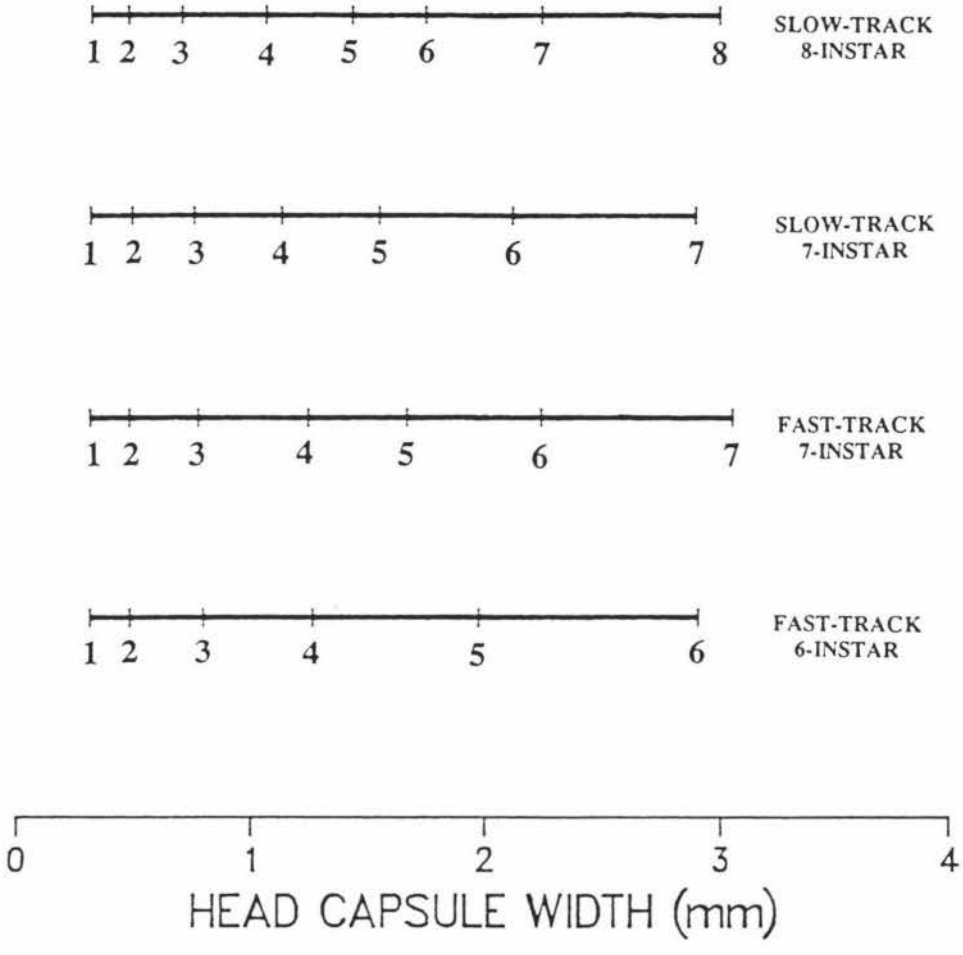

FIGURE 4: Mean HCW for each instar of fast-track and slow-track *G. mutans* reared on artificial diet.

(4) Pupation:

The trend in larval weights was repeated at pupation, with the significantly heavier fast-track pupae (Table 1) forming much earlier than the slow-track pupae (Figure 1). Although only three fast-track adults eclosed, it was apparent that they spent more time in the pupa than slow-track *G. mutans* (Table 1). There was no association between the sex of the adult and the number of larval instars.

(5) Mortality:

The survival of *G. mutans* within the laboratory was poor, with only small numbers of fast-track and slow-track adults eclosing. Most deaths occurred as pupae, and this may have been because the diet's ingredients did not fulfil the dietary requirements of developing *G. mutans*. Some aspects of the rearing method may also have been inadequate, as pupating larvae were prevented from constructing cocoons, and humidity levels varied greatly prior to eclosion. The few adults generated which were not malformed, failed to produce fertile eggs.

Reared on artificial diet, a small number of larvae died before reaching pupation. Larvae affected by this 'pestilence' at first appeared to be very sluggish and not eating the diet. Their colour changed from brown to pale yellow, and their body appeared full of fluid and was pulpy to the touch.

Discussion:

The contrasting results of these two experiments emphasise the differences between fast-track and slow-track *G. mutans* larvae. Fast-track larvae develop more rapidly, reaching maximum larval weight and pupation much earlier than slow-track larvae. They also grow much larger, with a heavier maximum larval weight and initial pupal weight. These results represent very large developmental differences between cohorts of what is recognised as one 'species', but because of the failure of laboratory reared *G. mutans* to produce offspring, it is, as yet, not possible to test if fast-track and slow-track *G. mutans* are reproductively distinct.

TABLE 1: Comparison of fast-track and slow-track *G. mutans* reared on artificial diet.

F6 & F7 = six and seven instar fast-track larvae
S7 & S8 = seven and eight instar slow-track larvae

PARAMETER	LARVAE	MEAN ± SEM (n)	ANOVA	STUDENT-NEWMAN-KUELS TEST					
				F6 vs F7	F6 vs S7	F6 vs S8	F7 vs S7	F7 vs S8	S7 vs S8
MAXIMUM LARVAL WEIGHT(g)	F6	0.751 ± 0.025 (15)	F=15.29 p<0.001	q=2.089 NS	q=9.406 p<0.001	q=4.032 p<0.025	q=2.411 NS	q=1.463 NS	q=0.460 NS
	F7	0.661 ± 0.040 (2)							
	S7	0.557 ± 0.017 (16)							
	S8	0.577 ± 0.013 (2)							
DAYS TO REACH MAX LARVAL WEIGHT	F6	33.4 ± 0.576 (15)	F=81.70 p«0.001	q=5.442 p<0.001	q=20.291 p«0.001	q=13.515 p«0.001	q=4.261 p<0.01	q=6.077 p<0.001	q=3.841 p<0.025
	F7	42.5 ± 1.500 (2)							
	S7	49.6 ± 0.816 (16)							
	S8	56.0 ± 6.000 (2)							
HCW OF FINAL INSTAR (mm)	F6	2.90 ± 0.021 (15)	F=2.97 NS						
	F7	3.06 ± 0.060 (2)							
	S7	2.89 ± 0.024 (16)							
	S8	3.00 ± 0.000 (2)							
INITIAL PUPAL WEIGHT(g)	F6	0.311 ± 0.010 (13)	F=17.29 p«0.001		q=8.249 p<0.001	q=3.239 p<0.05			q=0.827 NS
	S7	0.247 ± 0.006 (16)							
	S8	0.260 ± 0.013 (2)							
DAYS TO REACH PUPATION	F6	39.9 ± 0.431 (13)	F=106.06 p«0.001		q=18.829 p«0.001	q=13.206 p<0.001			q=4.000 p<0.01
	S7	57.5 ± 1.024 (16)							
	S8	65.0 ± 6.000 (2)							
DAYS SPENT AS PUPA	F6	21.3 ± 0.667 (3)	F=8.18 p<0.01		q=5.699 p<0.005	q=2.929 NS			q=1.216 NS
	S7	18.9 ± 0.239 (16)							
	S8	19.5 ± 0.500 (2)							

Recently, morphologically indistinguishable populations of *Planotortrix excessana* have been found which are pheromonally and therefore reproductively distinct, non-interbreeding sibling species (Foster *et al.* 1987). Sibling or cryptic species have been described as "species that morphologically resemble each other so closely that they can be recognised only after careful study of biochemical, cytological, or behavioural traits" (Diehl & Bush 1984). The identification of fast-track and slow-track larval development in this study suggests that *G. mutans* may also be composed of sibling species. This has provided an impetus for DSIR research into the pheromone systems of noctuids, particularly *G. mutans* (J.S. Dugdale pers. comm.).

Developmental differences similar to those distinguishing fast-track and slow-track *G. mutans* have been recognised in other lepidopteran species as effects of larval crowding (Leonard 1968, Hill 1986, Simmonds & Blaney 1986). Crowding was not a factor in these experiments, as all larvae were isolated within individual petri dishes. Diapause is another factor which may influence the development of lepidopteran larvae. In two species of *Agrotis*, larval diapause is characterised by a decreased rate of larval growth (Oku 1983). In these experiments diapause is unlikely to be a factor, as fast-track and slow-track *G. mutans* were obtained through the months November-March, and neither group was associated with change in environmental factors.

In these experiments, both fast-track and slow-track larvae possessing supernumerary instars showed a similar development pattern. The extra instar led to a delay in reaching maximum larval weight and pupation compared to the other larvae. Also, the HCW of the instar prior to pupation was largest for those with the extra moult. Other than these differences, there was no indication that the larvae possessing supernumerary instars behaved any differently, and all larvae were morphologically indistinguishable.

The occurrence of supernumerary instars in lepidopteran populations is well documented (Schmidt & Lauer 1977). Factors such as nutrition, temperature, humidity and photoperiod have been suspected of causing the developmental polymorphisms that produce insects with more than the "customary" number of instars. The significance of developmental polymorphisms is as yet unclear, but it is probable that the polymorphism is an adaptation allowing populations to exploit a broader ecological niche in a fluctuating environment than would be possible with a fixed pattern of development (Schmidt & Lauer 1977).

CHAPTER THREE

ENDOPHYTE AND GRAPHANIA MUTANS

Introduction:

Among the most important and widely grown pasture grasses for cattle in the United States, and sheep and cattle in New Zealand are tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*) respectively. However, tall fescue pastures infected with a fungal endophyte have been associated with poor livestock performance and symptoms of summer toxicosis (Bacon *et al.* 1977, Hoveland *et al.* 1980). The endophyte was identified as *Epichloe typhina*, but was renamed *Acremonium coenophialum* (Morgan-Jones & Gams 1982). A similar situation exists in New Zealand between *A. lolii*-infected perennial ryegrass and the incidence of ryegrass staggers (Fletcher & Harvey 1981).

Outbreaks of ryegrass staggers occur during the summer and autumn throughout New Zealand, and principally affect sheep and cattle, but also deer and horses (Cunningham & Hartley 1959). Within the flock or herd there is a wide range of susceptibility to the disease. Animals appear normal until disturbed, when the mildest clinical signs in sheep are a slight trembling of the head and neck region (Keogh 1973b). As the condition worsens there is head nodding and jerky limb movements, followed by a stiff-legged stilted gait, staggering and eventual collapse into a tetanic spasm of several minutes duration. Cattle normally collapse onto their briskets with legs splayed (Mortimer 1983).

In an outbreak of ryegrass staggers many animals may be affected, which has serious management implications as stock affected severely are unable to be disturbed in any manner or they will collapse. Stock losses however, are not usually severe, and are generally caused by accidental deaths: falling over bluffs, strangulation in fences, and drownings in water courses or troughs (Everest 1983). In addition to the clinical symptoms of ryegrass staggers, reduced liveweight gains in lambs (Fletcher 1983), and a reduction in both serum prolactin levels and liveweight gains in hoggets have been recorded (Fletcher & Barrel 1984, Fletcher 1986).

Plants are very rich in chemicals which are apparently not directly connected with the normal metabolic processes of photosynthesis, respiration and growth. These compounds were called 'secondary plant chemicals' (Fraenkel 1959,1969) because their metabolic roles within the plant were obscure . In relation to chemical interactions between organisms, the term 'allelochemics' has been proposed to replace secondary plant chemicals (Whittaker & Feeny 1971); an allelochemic being defined as a non-nutritional chemical synthesised by one species that affects the growth, health, behaviour or population biology of another species. A vast range of attractant, repellent, deterrent, stimulant, inhibitory and toxicant allelochemics exist. The chemicals implicated in ryegrass staggers have been identified as tremorgenic neurotoxins called lolitrems (Gallagher *et al.* 1981). Lolitrems elicit staggers symptoms in mice (Gallagher & Hawkes 1985), and their presence has been linked to *A. lolii* infection (Gallagher *et al.* 1982, Mortimer 1983, Gallagher *et al.* 1985). The structures of lolitrem B and C have been determined (Gallagher *et al.* 1984), and they are structurally related to known mycotoxins which suggests, but does not prove, that they are produced by the endophyte (Gaynor & Rowan 1986).

It is now possible to establish pastures with little or no endophyte in them, and animal production from these pastures is greatly enhanced (Hoveland *et al.* 1980). However, unless ryegrass staggers is a severe problem, New Zealand farmers tend to accept endophyte-infected pastures because of their increased stress-tolerance. In addition to enhanced plant growth (Chapter 1), the presence of endophyte in ryegrass can confer resistance to a variety of insects. The objective of this series of experiments was to determine how *A. lolii*-infected perennial ryegrass affects the larvae of *Graphania mutans*.

SECTION 1: ENDOPHYTE CHOICE TESTS

Introduction:

Endophyte has been linked to perennial ryegrass resistance for several major insect pests, where plant resistance to insect attack can be defined as the collective heritable characteristics by which the plant reduces the possibility of successful utilisation by

the insect (Beck 1965). Most studies of plant resistance place a low priority on obtaining a complete knowledge of the causes of the resistance, the emphasis being placed on breeding for resistance for insect control. An empirical approach proposed by Painter (1951), proved a workable compromise between mere categorisation of phenomena and the basic study of causative factors or processes (Horber 1980). The first mechanism of resistance, 'nonpreference', is the insects' response to plants that lack the characteristics to serve as hosts, resulting from negative reactions or avoidance during the search for food, oviposition sites, or shelter. Kogan and Ortman (1978) proposed to substitute 'antixenosis' for the term nonpreference, as it is parallel to 'antibiosis' and conveys the idea that the plant is avoided as a 'bad-host'.

In the United States the presence of endophyte has been shown to be linked with a reduction in feeding damage by sod webworm (*Crambus* spp.) (Funk *et al.* 1983), and the bluegrass billbug (*Sphenophorus parvulus*) (Ahmad & Funk 1983, Ahmad *et al.* 1986). Hardy *et al.* (1985) showed that fall armyworm (*Spodoptera frugiperda*) neonate larvae chose uninfected over endophyte-infected perennial ryegrass and consumed more of the fungus-free leaves. In New Zealand a series of studies with Argentine stem weevil (*Listronotus bonariensis*) have linked reduced adult feeding and oviposition with the presence of endophyte (Prestidge *et al.* 1982, Gaynor & Hunt 1983, Barker *et al.* 1983, Barker *et al.* 1984a, Prestidge *et al.* 1985). However, the presence of endophyte does not deter the feeding of several aphid species (Johnson *et al.* 1985, Latch *et al.* 1985a). These findings suggest that infected ryegrasses contain antixenotic compounds, or feeding deterrents, which affect most, but not all, insect herbivores. The objective of this set of experimental choice tests was to quantify specific behavioural responses by *G. mutans* fast-track larvae to endophyte-infected ryegrass.

Methods:

The leaf material used in both these tests came from 'Grasslands Nui' high endophyte perennial ryegrass plants grown from seed in 42cm x 30cm horticultural planter trays filled with a mixture of potting mix and sand and 'Osmocote' fertiliser. All plants were kept in one glasshouse bay and watered regularly. The plants were examined for the presence of endophyte by staining the mycelia in the leaf sheath epidermis with lactophenol cotton blue.

The leaves were removed by cutting just above the ligule with scissors and care was taken that the larvae in the experiments received plant tissue of approximately the same age. This was determined by the position of the leaf on the tiller: only those leaves beneath the terminal expanding blade, representing new growth, were used, corresponding to leaf ages 2 and 3 of Hardy *et al.* (1986).

The larval feeding preference tests were conducted with both neonate and newly moulted sixth instar *G. mutans* fast-track larvae.

(1) First Instar Larvae:

One hundred neonate larvae from one egg batch were divided into groups of 5 and placed in the centre of 20 petri dishes (9cm diameter) lined with moistened filter paper. Positioned side by side in the petri dish were similar aged, 6cm long, single leaf blades of endophyte-infected and endophyte-free perennial ryegrass. Because feeding preference tests are affected by which portion of the ryegrass leaf is used (see Appendix III), all leaf material was taken from the bottom of the excised leaf laminae.

Petri dishes were placed on trays within transparent plastic bags and kept in a controlled temperature room at 21°C ± 2°C under a photoperiod of 18:6 (L:D). The distribution of larvae on the leaves within each dish was recorded after 18 and 40 hours. At the end of the experiment, larval feeding damage on each grass blade was assessed using a subjective feeding scale. Visual estimates of feeding damage were scored using the following criteria: 0 = no feeding; 1 = slight feeding; 2 = moderate feeding; 3 = considerable feeding.

Results of both larval distribution and feeding damage were analysed using Wilcoxon's matched pairs signed rank test (Siegel 1956). At the conclusion of the experiment, a sample of the larvae were reared for two weeks on ryegrass to confirm that they developed as 'fast-track' larvae.

(2) Sixth Instar Larvae:

The larvae used in the two trials of this experiment had developed from the eggs of two different females. They were reared individually in petri dishes lined with

moistened filter paper and fed artificial diet. The petri dishes were sealed in airtight tupperware containers and kept in the controlled temperature room. All the larvae had moulted for the last time within two days of each other, and had been starved for the 24 hours preceding each trial.

In the first trial, each of 28 larvae were positioned in the centre of a 9cm diameter petri dish lined with moistened filter paper (Plate 9). Lying side by side in the petri dish were similar aged single portions of endophyte-infected and endophyte-free leaves, the latter being cut 1cm longer to facilitate recognition should the blades be moved during feeding. Larval consumption was measured by comparison of the wet weight of the individual leaf blades before and after feeding, and results were analysed using Wilcoxon's matched pairs signed rank test. Both trials ran for 60 minutes, so no correction factor for natural water loss was necessary. In the second trial the same method was used with 25 larvae.

Results:

(1) First Instar Larvae:

After 18 hours there was no significant difference in the numbers of larvae found on the endophyte-infected and endophyte-free leaves (Table 4a). Many of the larvae were found walking around the petri dishes and did not appear to have been feeding as they were not green in colour and there was little faeces on the filter paper. After 40 hours, a significant preference had appeared, with almost three times the number of larvae found on endophyte-free leaves in preference to endophyte-infected (Table 2a). A significant preference for endophyte-free plant material was also apparent from the amount of feeding damage on each leaf.

(2) Sixth Instar Larvae:

From Table 2b it is apparent that endophyte-free leaves were strongly preferred. On average, half of each endophyte-free leaf was consumed during the experiment compared to less than one fifth of the endophyte-infected leaf.

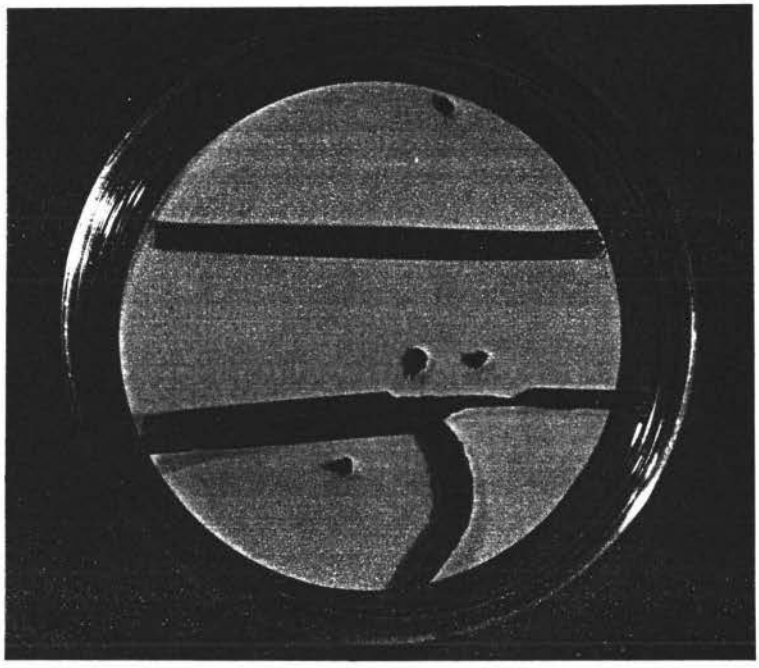

PLATE 9: Endophyte feeding preference test with sixth instar fast-track *G. mutans* larva.

TABLE 2: Results of fast-track larval feeding preference tests with endophyte-free and endophyte-infected perennial ryegrass leaves. (Results analysed using Wilcoxon's matched pairs signed rank test)

TABLE 2a: Neonate Larvae

		TREATMENT		ANALYSIS
		ENDOPHYTE-FREE RYEGRASS	ENDOPHYTE-INFECTED RYEGRASS	
18 HOURS	Total Number of Larvae on Leaves	46	37	z =-1.0342 p = 0.3011
40 HOURS	Total Number of Larvae on Leaves	61	24	z =-2.9474 p = 0.0032
	Mean Feeding Ranking	2.55 ± 0.17	1.75 ± 0.16	z =-2.1557 p = 0.0311

TABLE 2b: Sixth Instar Larvae

	PERCENTAGE OF LEAF LEFT UNEATEN		ANALYSIS
	ENDOPHYTE-FREE RYEGRASS (mean ± SEM)	ENDOPHYTE-INFECTED RYEGRASS (mean ± SEM)	
TRIAL ONE (n = 28)	52.9 ± 6.26	84.5 ± 3.93	z =-2.9311 p = 0.0034
TRIAL TWO (n = 25)	53.8 ± 6.35	84.7 ± 3.87	z =-3.0286 p = 0.0025

Discussion:

Although the results of these experiments were conclusive, there was variation between individuals in the amount of endophyte-infected leaf material consumed. This may have been due to inadequacies of the experimental method. For instance, although the infection status of each plant was confirmed, the amount of endophyte present was not quantified. Therefore, different larvae may have received leaves with varying concentrations of endophyte, thus affecting the strength of larval feeding deterrence. Even if the concentration of larval endophyte was measured, perhaps using ELISA, other fungus, or fungus/plant derived products can affect insect behaviour. Loline alkaloids for example, are associated with feeding deterrence and toxicity to some aphid species (Johnson *et al.* 1985). Until a system is devised which quantifies the biological activity of endophyte in each leaf, variation in the results obtained by feeding preference tests is likely to continue.

Another contributing variable may be within the population of *G. mutans*. A small proportion of larvae may possess a tolerance for the antixenotic compound(s) and feed undeterred on endophyte-infected leaves. Feeding preference tests are not useful for distinguishing such 'resistant' individuals. Dymock & Hunt (1987) looked for resistance to endophyte in adult Argentine stem weevil populations by testing feeding preferences twice. The majority of weevils that had fed initially on endophyte-infected leaves, fed on endophyte-free leaf material in the subsequent trial. To identify *G. mutans* resistance to endophyte, further experiments are necessary, screening much larger numbers of larvae in a succession of feeding preference trials.

The method of testing feeding preferences of polyphagous insects by choice tests is often used by agricultural entomologists and, as it is frequently impractical to use whole plants, excised leaves or leaf discs are utilised. The results of such tests are then often extrapolated to infer insect feeding preferences on whole plants in nature. Recently it has been shown that many plants that are physically damaged undergo significant changes in plant chemistry, and that these changes can decrease or increase plant palatability and the fitness of insects consuming the plants (Rhoades 1983). The chemical changes associated with this 'induced resistance' often occur quickly, and can influence the results of feeding preference tests using leaf discs or excised leaves (Risch 1985). Therefore further experiments are necessary, testing *G. mutans* feeding preference of whole plants, before any firm conclusions can be made about the effects of endophyte on *G. mutans* populations in pasture ecosystems.

Subjective feeding indices have often been used to measure feeding preferences of insects. For example, bioassays isolating feeding deterrents from endophyte-infected ryegrass have used visual estimates of Argentine stem weevil feeding damage on excised leaves and plant extracts incorporated in agar-based diets (Gaynor *et al.* 1983, Prestidge *et al.* 1985). In this study subjective feeding scales were considered unreliable because of difficulties in accurately quantifying feeding damage. Measuring the weight change of leaves before and after feeding provided much more detailed information about feeding preference. Visual estimates of feeding damage were only used in experiments with neonate larvae, where leaf consumption was too small to be accurately weighed. The results collected using this method provide only gross indications of feeding trends, but are corroborated by the results of the larval distribution on the ryegrass leaves.

From the results of these two experiments it can be seen that neonate and sixth instar fast-track *G. mutans* larvae show a distinct feeding preference for endophyte-free perennial ryegrass. This suggests that in the laboratory, excised leaves of endophyte-infected ryegrass contain at least one feeding deterrent for *G. mutans*. Having now established the presence of antixenotic compounds, the next series of experiments examines the long-term effects of feeding endophyte-infected perennial ryegrass on fast-track and slow-track *G. mutans* larvae.

SECTION 2: EFFECTS OF ENDOPHYTE ON FAST-TRACK LARVAE

Introduction:

The second mechanism of resistance proposed by Painter was 'antibiosis', which includes all the adverse affects exerted by the plant on the insects' biology, for example: survival, development and reproduction. Both categories of resistance are arbitrary and vaguely delineated. Antixenosis may be mistaken for antibiosis and vice versa, and the two interact and complement each other, intensifying resistance expressions. Although both resistance categories have been implicated in studies of the effects of endophyte-infected ryegrass on insect development, little is yet understood about the exact mechanisms of resistance.

Gaynor & Rowan (1986) suggest that reduced insect growth and increased mortality of Argentine stem weevil (*Listronotus bonariensis*) may be due to reduced consumption of endophyte-infected ryegrass. This would result from the presence of a feeding deterrent, but the research of Barker *et al.* (1984b), and Prestidge & Gallagher (1985) indicate that antibiosis is also likely to be involved. Clay *et al.*. (1985a) demonstrated developmental differences in the fall armyworm (*Spodoptera frugiperda*) although their experiments were not sufficient to determine if the observed effects were due to feeding deterrence or antibiosis. Ahmad *et al.*. (1985) reported evidence for antibiosis for the house cricket (*Acheta domesticus*) in which death occurred due to severe dysfunction of the gut. Using larvae of the Southern armyworm (*Spodoptera eridania*) fed endophyte-infected ryegrass, Ahmad *et al.* (1987) found no overt signs of neurotoxicity. Death was speedy, and due to interference with vital physiological processes causing extreme weight loss.

It is clear that the allelochemicals associated with endophytes have various effects on different taxonomic orders. The experiment described in this section was designed to evaluate the long-term effects of feeding endophyte-infected ryegrass to *G. mutans* fast-track larvae.

Methods:

Immediately upon hatching, 40 larvae from one egg batch were placed individually in numbered petri dishes (9cm diameter) lined with moistened filter paper. Leaves from endophyte-infected ryegrass plants were fed to half the larvae, the other half receiving leaves from uninfected plants. Leaves were added as required to ensure an excess of food, and old leaves and faeces were regularly removed. All larvae were fed the bottom 8cm of leaves of the same relative age,(as outlined in Section 1). The petri dishes were kept on trays within transparent plastic bags to maintain high humidity in a constant temperature room (21°C ± 2°C under a photoperiod of 18:6 (L:D) and relative humidity of 60%), and positions were regularly randomised to eliminate possible within room effects.

All the ryegrass plants used in this experiment were genotypically identical. The plants were old and root bound, so they were trimmed and subdivided into groups of about 10 tillers. These were replanted in 7cm plastic pots using a mixture of potting mix and

sand and 'Osmocote' fertiliser. The pots were kept on metal trays within one glasshouse bay and were watered every day. Some months earlier they had been checked for the presence of endophyte using the lactophenol cotton blue stain for mycelia in the leaf sheath, and it was assumed that their infection status had not changed. However, at the completion of the experiment, when the plants were rechecked, not all the plants thought to contain endophyte were infected.

The development of the larvae was monitored daily. At each moult the date was recorded and the new HCW of the larva measured using an eyepiece micrometer. The larvae were weighed every three days on a Mettler balance accurate to four decimal places. Initial pupal weights, the dates of pupation and eclosion, and larval and pupal survival were recorded.

The G-test with William's correction was used to analyse differences between the number of instars as well as larval and pupal survival on endophyte-infected and endophyte-free plants. Differences in the continuously distributed variables (HCW, weights, and stage durations) were analysed using ANOVA and the Student-Newman-Kuels multiple range test (Zar 1974).

Results:

(1) Number of Larval Instars:

Fast-track larvae fed on endophyte-free leaves developed through 7 instars, one more than the majority of larvae reared on artificial diet. Of the larvae fed endophyte-infected leaves, 60% developed through 7 instars, and the other 40% had 8 instars before pupation. This association between endophyte and the number of instars is highly significant ($G=10.827$, $p=0.001$), and it is important when looking at the results below, that the effects of endophyte are not confused with the characteristics of the extra instar. The larvae were grouped according to their total number of instars and were abbreviated as follows (the number of larvae in each group is written in parentheses):

E-7 = larvae reared on endophyte-free ryegrass with 7 instars (16)
E+7 = larvae reared on endophyte-infected ryegrass with 7 instars (10)
E+8 = larvae reared on endophyte-infected ryegrass with 8 instars (7)

(2) Larval Weight:

Figure 5 illustrates the long-term differences in mean larval weights of the fast-track larvae fed either endophyte-free, or endophyte-infected ryegrass (a complete description of the results is given in Appendix IV). The bell-shaped curves of the E-7 and E+7 larvae are similar, although the mean weights of the larvae fed endophyte-free ryegrass were consistently higher. The E+8 larvae had the lowest mean weight until the time of their last moult, after which they began to gain weight steadily.

From Table 3, the maximum weights of the E-7 larvae were significantly greater than both groups of endophyte-reared larvae which were of similar weight. The two 7-instar groups reached maximum weight at approximately the same time, both significantly earlier than the E+8 larvae.

(3) Head Capsule Width:

From Figure 6 it can be seen that the regressions of HCW against instar are curvilinear and a comparison of the three multiple regressions (Zar 1974), indicates that the curves are significantly different ($F=178.80$, $p\ll0.001$). The HCW of the first three instars for both treatments were very similar, and were slightly smaller on average than those of diet reared larvae at an equivalent stage. However, by the time of their pupation, the head capsules of all ryegrass-reared larvae were much larger than those reared on artificial diet.

During the later instars, larvae reared on endophyte-free ryegrass had the largest HCW, with the E+7 larvae being consistently smaller. The differences in capsule width between these two and the E+8 larvae became more pronounced as they developed, and it was only the extra moult that allowed the 8 instar larvae to 'catch up' in terms of HCW. From Table 3 it can be seen that at the final instar, the HCW of the E-7 and the E+8 larvae were much the same, and both were larger than the E+7.

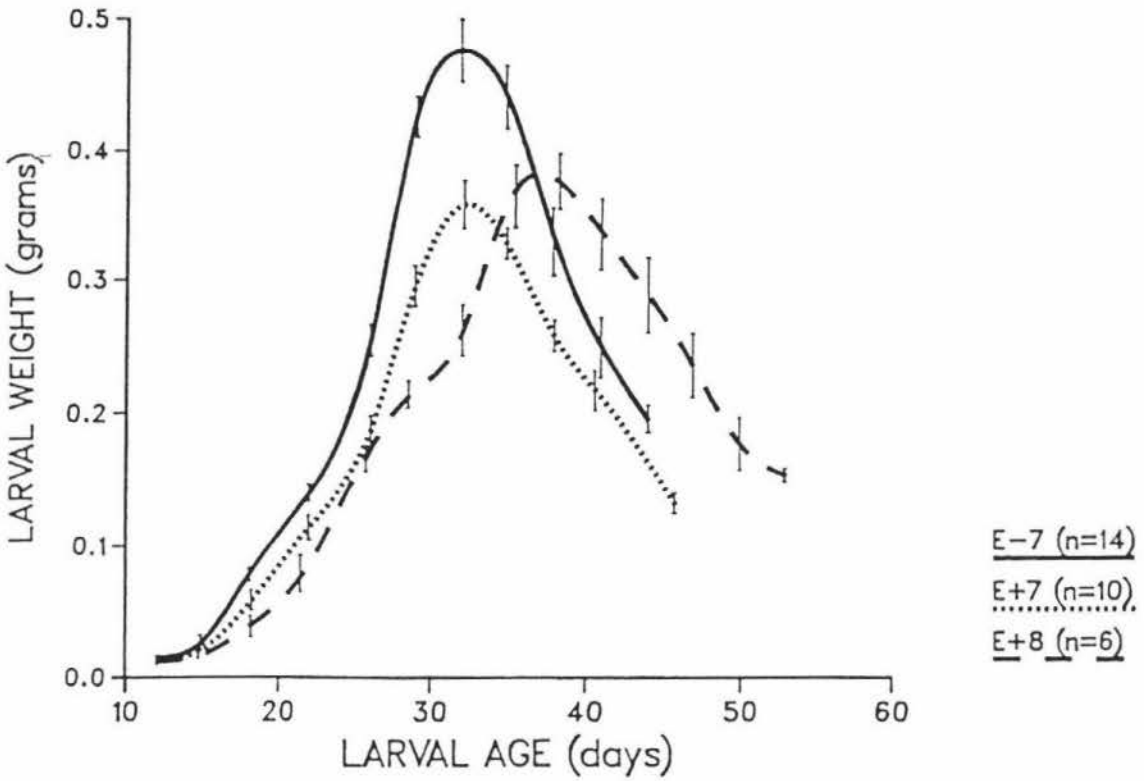

FIGURE 5: Mean larval weight ± SEM of fast-track larvae reared on endophyte-free and endophyte-infected perennial ryegrass leaves.

E-7 = seven instar larvae reared on endophyte-free ryegrass
E+7 & E+8 = seven and eight instar larvae reared on endophyte-infected ryegrass

There was very little difference between the moulting dates for the first three ecdyses. At the fourth and fifth moults, larvae fed endophyte-free ryegrass ecdysed earlier than both types of larvae fed infected leaves, but there were no significant differences at moult 6, the final moult of the 7-instar larvae (Table 3). The last moult for the E+8 larvae occurred about a week later.

(4) Pupation:

From Table 3 it can be seen that the endophyte-free pupae were formed first and weighed more than both groups of the endophyte-reared pupae. Of those fed endophyte, there was little difference in pupal weights, but their mean dates of pupation were separated by almost a week.

(5) Mortality:

The 'pestilence' that affected *G. mutans* larvae reared on artificial diet was present in this experiment. The symptoms were very similar, with sluggishness and weight loss associated with a change in colour from the characteristic green of the ryegrass leaf to pale yellow. Only a small number of larvae developed this flaccid, fluid-filled condition, and their was no association between endophyte and the number of deaths caused by this 'pestilence' (Table 4).

Most of the mortality occurred at pupation. All but one of the endophyte-free reared larvae pupated successfully, which was in marked contrast to the larvae fed endophyte. Only 60% of these larvae pupated, and the majority of these were malformed. Often the pupae were black and shrivelled, leaking a black fluid onto the filter paper.

Significant differences occurred in both the numbers of larvae that pupated and the numbers which eclosed (Table 4). Of the 9 adults that appeared in the endophyte-free treatment: three failed to completely emerge from the pupa, two had malformed wings, and four appeared to be perfectly normal. Only one adult from the endophyte treatments appeared to be normal, the other two had badly malformed wings. The moths were transferred into a large container to determine if they would mate, but no fertile eggs were laid.

TABLE 3: Comparison of the development of fast-track larvae reared on endophyte-free and endophyte-infected perennial ryegrass leaves.
E-7 = seven instar larvae reared on endophyte-free ryegrass
E+7 & E+8 = seven and eight instar larvae reared on endophyte-infected ryegrass

PARAMETER	LARVAE	MEAN ± SEM	(n)	ANOVA	STUDENT-NEWMAN-KUELS TEST		
					E-7 vs E+7	E-7 vs E+8	E+7 vs E+8
MAXIMUM LARVAL WEIGHT(g)	E-7	0.490 ± 0.021	(14)	F=11.58	q=6.555	q=4.019	q=1.031
	E+7	0.361 ± 0.016	(10)	p<0.001	p<0.001	p=0.025	NS
	E+8	0.397 ± 0.024	(6)				
DAYS TO REACH MAX LARVAL WEIGHT	E-7	34.8 ± 0.494	(14)	F=17.89	q=1.201	q=8.346	q=6.524
	E+7	35.6 ± 0.400	(10)	p«0.001	NS	p«0.001	p<0.001
	E+8	39.5 ± 0.671	(6)				
HCW OF FINAL INSTAR (mm)	E-7	3.30 ± 0.022	(15)	F=10.59	q=5.642	q=1.143	q=5.530
	E+7	3.17 ± 0.024	(10)	p<0.001	p<0.001	NS	p<0.005
	E+8	3.34 ± 0.024	(6)				
DAYS TO REACH INSTAR SEVEN	E-7	26.2 ± 0.200	(15)	F=3.77			
	E+7	26.7 ± 0.260	(10)	NS			
	E+8	25.6 ± 0.369	(7)				
INITIAL PUPAL WEIGHT(g)	E-7	0.194 ± 0.009	(14)	F=14.14	q=7.363	q=3.273	q=1.922
	E+7	0.127 ± 0.005	(7)	p<0.001	p<0.001	p<0.05	NS
	E+8	0.153 ± 0.003	(3)				
DAYS TO REACH PUPATION	E-7	43.8 ± 0.515	(14)	F=14.57	q=2.916	q=7.553	q=5.007
	E+7	46.0 ± 1.020	(7)	p<0.001	p=0.05	p<0.001	p<0.005
	E+8	51.7 ± 1.860	(3)				

TABLE 4: Comparison of the mortality of fast-track *G. mutans* reared on endophyte-free and endophyte-infected perennial ryegrass leaves.
(Results analysed using the G-test with William's correction, sample sizes are in parentheses)

PARAMETER	ENDOPHYTE-FREE RYEGRASS	ENDOPHYTE-INFECTED RYEGRASS	ANALYSIS
PESTILENCE MORTALITY	25% (5/20)	11% (2/19)	G = 1.379 NS
PUPATION SUCCESS	93% (14/15)	59% (10/17)	G = 5.493 p < 0.025
ECLOSION SUCCESS	57% (8/14)	30% (3/10)	G = 3.673 p < 0.06

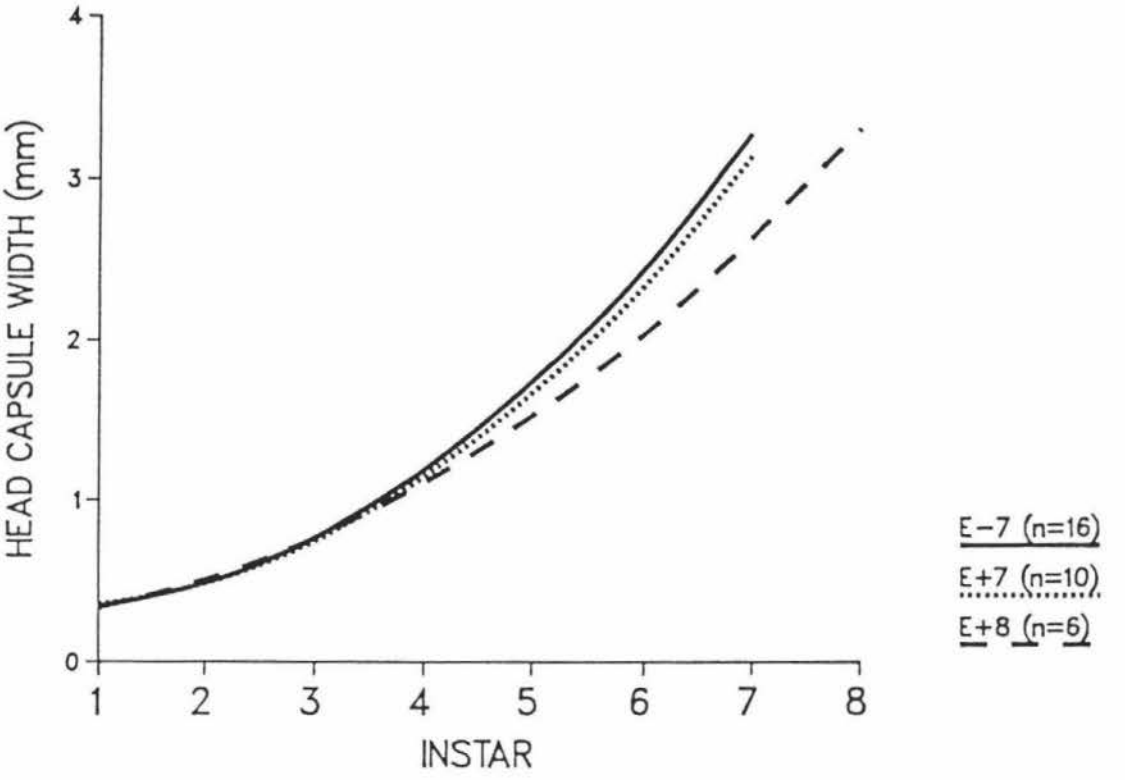

FIGURE 6: HCW regressions of fast-track larvae reared on endophyte-free and endophyte-infected perennial ryegrass leaves.

E-7 = seven instar larvae reared on endophyte-free ryegrass
E+7 & E+8 = seven and eight instar larvae reared on endophyte-infected ryegrass

Regression Equations:

E-7: $HCW = 0.069\ instar^2 + 0.065\ instar + 0.344$
E+7: $HCW = 0.067\ instar^2 + 0.073\ instar + 0.366$
E+8: $HCW = 0.042\ instar^2 + 0.046\ instar + 0.252$

Discussion:

Fast-track *G. mutans* larvae reared on artificial diet generally developed through 6 instars, but on endophyte-free ryegrass the number of instars is increased to 7. Also the maximum larval weight of fast-track *G. mutans* reared on endophyte-free ryegrass leaves is only two thirds that of the diet-reared larvae, but the extent of pupal mortality is the same for both. This may be because *G. mutans* is generally associated with dicotyledonous plants like plantain and dock, and a diet of perennial ryegrass may not be completely suited to larval development (J.S. Dugdale pers. comm.).

The nutrition of lepidopterans can influence not only the number of moults, but also the proportion of instar groups (Leonard 1970a, Wigglesworth 1972). None of the fast-track *G. mutans* reared on endophyte-free ryegrass possessed the supernumerary instar found in 10% of diet-reared fast-track larvae. However, the presence of endophyte shifted the proportion of fast-track larvae with the extra instar up to 40%. Schmidt & Lauer (1977) suggest that a change in ratio of one instar group to another could reflect a change in quality of the host on which the insects feed. Certainly the reductions in body weight, HCW, and initial pupal weights, plus the increased mortality displayed by larvae reared on endophyte-infected ryegrass, support the hypothesis that the presence of endophyte is associated with a decrease in the suitability of ryegrass as a host for *G. mutans*.

The ryegrass plants used in this experiment had been screened for the presence of endophyte some months before the study began. However, when they were rechecked once the larvae had pupated, not all the plants thought to contain endophyte were infected. Those which still contained the fungus had relatively low levels of endophyte compared to the plants germinated from seed and used in Section 1. All the larvae received the same amount of endophyte on average, as leaves were taken at random from all plants, but the concentration of that endophyte was relatively low. This experiment should be repeated to determine if the effects of endophyte on fast-track *G. mutans* larval development are more marked when larvae are reared on uniformly higher concentrations of *A. lolii*.

In addition to the variation in endophyte infection, the concentration of endophyte found in the leaves of individual plants may have varied as the experiment progressed. New leaves from established tillers of tall fescue are known to outgrow the fungal endophyte initially and remain free of its effects for a short time (Hardy *et al.* 1986).

At the beginning of this experiment, the tillers were relatively uncrowded and rapid growth occurred forming long, wide leaves. As the experiment continued, crowding of tillers in the pots increased, the rate of growth decreased, and the leaves produced were shorter and narrower. It is possible that larvae received an increasing amount of endophyte as the experiment progressed, as the endophyte has longer to enter the leaves of crowded tillers with reduced rates of growth.

This experiment has shown that endophyte reduces the body weights, HCW, and initial pupal weights, besides delaying pupation and decreasing pupal survival of 7-instar fast-track *G. mutans* larvae. The mechanisms that produced these effects are unclear. The developmental differences could be due to effects of antixenosis, antibiosis, or a combination of both. In the following section, this experiment is repeated with the aim of examining in more detail the mechanisms of ryegrass resistance to *G. mutans* larave.

SECTION 3: EFFECTS OF ENDOPHYTE ON SLOW-TRACK LARVAE

Introduction:

To further investigate the effects of endophyte on fast-track larvae the experiment described in Section 2 was repeated. After less than three weeks it became clear that the development of these larvae on ryegrass was not as rapid as those of the first experiment: these were slow-track larvae. Therefore, in this section, the long-term effects of feeding endophyte-infected ryegrass to *G. mutans* slow-track larvae are examined.

Methods:

In this experiment, emphasis was placed on determining whether endophyte induced ryegrass resistance was due to antixenosis or antibiosis. Using the same method as described in Section 2, 40 slow-track larvae from one egg batch were reared on endophyte-infected or endophyte-free ryegrass. If developmental differences were due

to the presence of feeding deterrents, larvae fed endophyte-infected leaves would be expected to eat less than larvae reared on endophyte-free ryegrass. Therefore, after two weeks, the amount of leaf material given to each larva each day was weighed, and the uneaten remains removed and reweighed the next day. The values for the amount of leaf biomass consumed by the larvae of the two treatments were analysed using the two sample T-test.

In this study, all plants were screened for the presence of *A. lolii* before the experiment began, and again the concentration of endophyte was relatively low. The old root bound plants were subdivided into groups of only 5-6 tillers to try and avoid the severe overcrowding that occurred towards the end of the first experiment. All the pots were kept on metal trays within one glasshouse bay and were watered daily. The position of trays containing endophyte-infected and endophyte-free plants within the bay was regularly randomised to minimise possible within room effects.

Results:

(1) Number of Larval Instars:

In this experiment there was no association between endophyte and the number of instars of the slow-track larvae (G=0.485). Larvae fed on endophyte-free leaves developed through 8, 9, or 10 instars; one or two more than those reared on artificial diet. Larvae fed endophyte-infected leaves developed through 8, 9, 10, or 11 instars. Again larvae were grouped according to their total number of instars and were abbreviated as follows (the number of larvae in each instar group is written in parentheses):

E-8 = larvae reared on endophyte-free ryegrass with 8 instars (5)
E-9 = larvae reared on endophyte-free ryegrass with 9 instars (8)
E-10 = larvae reared on endophyte-free ryegrass with 10 instars (1)
E+8 = larvae reared on endophyte-infected ryegrass with 8 instars (4)
E+9 = larvae reared on endophyte-infected ryegrass with 9 instars (6)
E+10 = larvae reared on endophyte-infected ryegrass with 10 instars (2)
E+11 = larvae reared on endophyte-infected ryegrass with 11 instars (1)

In the experiment with fast-track larvae (Section 2), it was apparent that the possession of an additional instar had significant effects on most of the recorded parameters. With slow-track larvae showing so many additional instars and so few larvae in each group, few differences between the endophyte-infected and endophyte-free treatments were significant.

(2) Larval Weight:

Figure 7 shows the long-term differences in mean larval weight for slow-track larvae fed on endophyte-free and endophyte-infected leaves (a complete description of results is given in Appendix V). It can be seen that the weight gain for the first four instars of all larvae was similar. After 30 days, the larvae reared on endophyte-free leaves rapidly gained weight and development followed the characteristic bell-shaped curve. The presence of endophyte in the ryegrass produced an almost linear plot of weight gain, with all but one of the larvae failing to successfully pupate.

Comparison of the endophyte-infected and endophyte-free treatments revealed few statistically significant differences but some trends were seen (Table 5). The endophyte-free reared larvae tended to be slightly heavier and developed slightly faster than those fed endophyte-infected leaves. This trend applied to all but one larva: the single endophyte reared 9-instar larva that successfully pupated. This insect was 0.1g heavier than any of the other endophyte-reared larvae and followed a growth curve very similar to that of the E-9 group (Figure 7b).

(3) Head Capsule Width:

The HCW of the first three instars of all the larvae were very similar and it was not until the third moult that it became apparent that some larvae were developing more rapidly than others. Larvae of both treatments with the biggest increases in HCW at each moult developed through the fewest instars and finished with the smallest HCW at pupation. Table 5 shows that there were significant differences in final HCW associated with the extra instar, for example: E+10 » E+9 » E+8. The larger the number of moults, the greater the final HCW appeared to be, regardless of the presence or absence of endophyte.

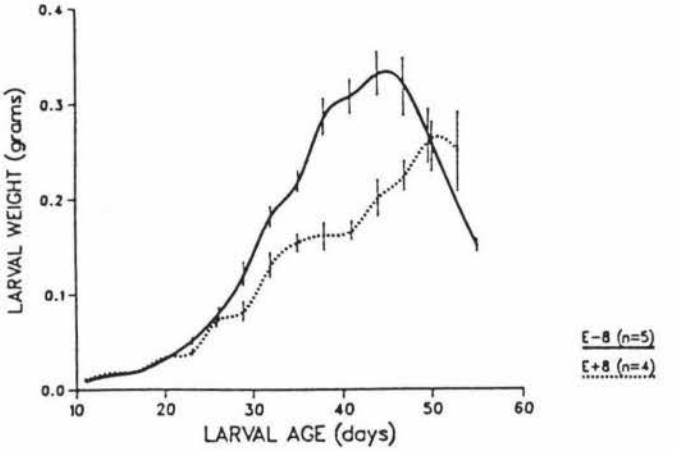

FIGURE 7a: Eight Instar Larvae

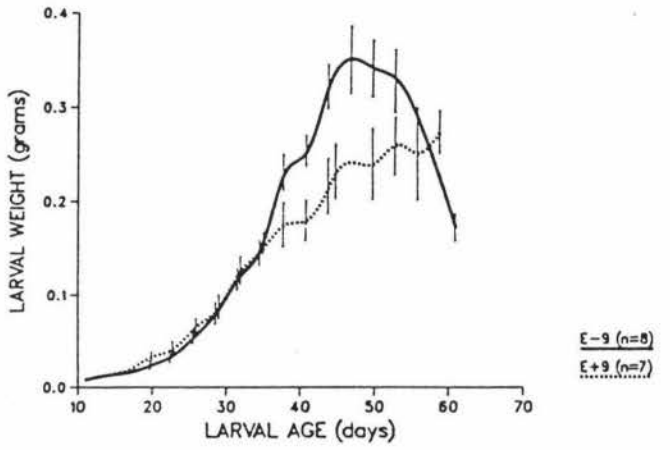

FIGURE 7b: Nine Instar Larvae

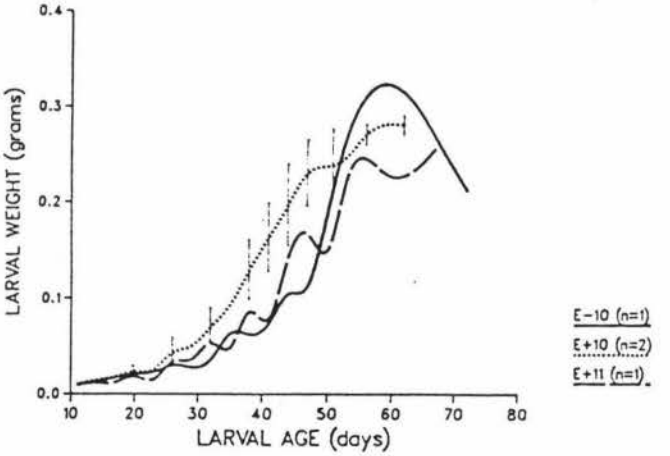

FIGURE 7c: Ten and Eleven Instar Larvae

FIGURE 7: Mean larval weight ± SEM of slow-track larvae reared on endophyte-free and endophyte-infected perennial ryegrass leaves.

E-8, E-9 & E-10 = eight, nine and ten instar larvae reared on endophyte-free ryegrass
E+8, E+9, E+10 & E+11 = eight, nine, and ten instar larvae reared on endophyte-infected ryegrass

When the E+11 larva was three weeks old it appeared to have all the symptoms of the 'pestilence' infection which eventually killed most of the larvae reared on endophyte (see Mortality). After not eating for several days it appeared to 'recover' and developed through 11 instars, but it never achieved the weight of the other endophyte-reared larvae (see Figure 7). It is probable that it never achieved its full HCW either, so this individual was not considered in the results.

There was very little difference in the moulting dates of the larvae of either treatment for the first 6 ecdyses. At moult 7, the last for the 8-instar larvae, it appeared that endophyte increased the length of time between moults (Table 5). This trend continued at moult 8, although again, the sample size was too small to draw any firm conclusions.

(4) Pupation:

Endophyte had a major effect at pupation as only one larva reared on endophyte-infected ryegrass successfully pupated compared to 8 reared on endophyte-free leaves. Because of this no comparisons could be made of endophyte-free and endophyte-infected weights and dates of pupation. Similarly, the sample size of the pupae reared on endophyte-free ryegrass was so small that no significant results were obtained (Table 6), although it did appear that those pupae from 9 instar larvae pupated later and were slightly heavier than those from 8 instar larvae.

(5) Mortality:

Nearly three quarters of the larvae in this experiment died from the 'pestilence' described in earlier chapters. It affected half of the larvae fed endophyte-free leaves, and significantly, over 90% of the larvae reared on endophyte (Table 7). Having reached pupation, the chances of survival were much greater (Table 7). The emerging adults however, were very small, but all looked in good condition; only three had slightly crumpled wingtips.

TABLE 5: Comparison of the development of slow-track larvae reared on endophyte-free and endophyte-infected perennial ryegrass.

E-8, E-9 & E-10 = eight, nine and ten instar larvae reared on endophyte-free ryegrass
E+8, E+9, E+10 & E+11 = eight, nine, ten and eleven instar larvae reared on endophyte-infected ryegrass

PARAMETER	LARVAE	MEAN ± SEM	(n)	ANOVA	STUDENT-NEWMAN-KUELS TEST									
					E-8 vs E-9	E-8 vs E+8	E-8 vs E+9	E-8 vs E+10	E-9 vs E+8	E-9 vs E+9	E-9 vs E+10	E+8 vs E+9	E+8 vs E+10	E+9 vs E+10
MAXIMUM LARVAL WEIGHT(g)	E-8	0.341 ± 0.024	(5)	F=2.93 NS										
	E-9	0.381 ± 0.028	(7)											
	E-10	0.267 ± 0.000	(1)											
	E+8	0.252 ± 0.024	(3)											
	E+9	0.313 ± 0.033	(4)											
	E+10	0.273 ± 0.007	(2)											
	E+11	0.245 ± 0.000	(1)											
DAYS TO REACH MAX LARVAL WEIGHT	E-8	43.4 ± 1.470	(5)	F=3.32 p<0.05	q=2.440 NS	q=2.261 NS	q=3.999 NS	q=4.440 p<0.05	q=0.285 NS	q=2.679 NS	q=2.819 NS	q=1.351 NS	q=2.261 NS	q=1.192 NS
	E-9	46.6 ± 0.702	(7)											
	E-10	67.0 ± 0.000	(1)											
	E+8	47.0 ± 1.732	(3)											
	E+9	49.3 ± 1.436	(4)											
	E+10	51.5 ± 4.500	(2)											
	E+11	56.0 ± 0.000	(1)											
HCW OF FINAL INSTAR (mm)	E-8	3.10 ± 0.052	(5)	F=16.30 p<0.001	q=3.765 p<0.025	q=2.740 NS	q=3.586 p=0.05	q=8.777 p<0.001	q=6.503 p<0.001	q=0.242 NS	q=6.587 p<0.001	q=6.117 p<0.005	q=10.599 p<0.001	q=6.066 p<0.001
	E-9	3.26 ± 0.038	(8)											
	E-10	3.32 ± 0.000	(1)											
	E+8	2.96 ± 0.043	(4)											
	E+9	3.26 ± 0.050	(5)											
	E+10	3.64 ± 0.040	(2)											
	E+11	3.40 ± 0.000	(1)											
DAYS TO REACH INSTAR EIGHT	E-8	35.0 ± 0.245	(5)	F=5.89 p<0.025	q=1.125 NS	q=4.890 p<0.025	q=1.121 NS	q=1.962 NS	q=6.567 p<0.005	q=2.696 NS	q=3.010 NS	q=4.190 p<0.025	q=1.895 NS	q=1.228 NS
	E-9	33.9 ± 0.170	(8)											
	E-10	39.0 ± 0.000	(1)											
	E+8	40.0 ± 0.842	(4)											
	E+9	36.0 ± 0.330	(7)											
	E+10	37.5 ± 1.768	(2)											
	E+11	41.0 ± 0.000	(1)											
DAYS TO REACH INSTAR NINE	E-9	41.9 ± 0.789	(8)	F=3.26 NS										
	E-10	47.0 ± 0.000	(1)											
	E+9	45.7 ± 1.500	(6)											
	E+10	46.0 ± 3.000	(2)											
	E+11	50.0 ± 0.000	(1)											

TABLE 6: Comparison of the pupae of slow-track *G.mutans* reared on endophyte-free and endophyte-infected perennial ryegrass leaves.

E-8 & E-9 = pupae from eight and nine instar larvae reared on endophyte-free ryegrass

E+9 = pupa from nine instar larva reared on endophyte-infected ryegrass

PARAMETER	LARVAE	MEAN ± SEM	(n)	ANOVA
INITIAL	E-8	0.149 ± 0.006	(3)	F=1.63
PUPAL	E-9	0.172 ± 0.013	(5)	NS
WEIGHT(g)	E+9	0.155 ± 0.000	(1)	
DAYS TO	E-8	55.3 ± 0.667	(3)	F=2.83
REACH	E-9	61.3 ± 2.648	(5)	NS
PUPATION:	E+9	57.0 ± 0.000	(1)	

TABLE 7: Comparison of the mortality of slow-track *G. mutans* reared on endophyte-free and endophyte-infected perennial ryegrass leaves.

(Results analysed using the G-test with William's correction, sample sizes are in parentheses)

PARAMETER	ENDOPHYTE-FREE RYEGRASS	ENDOPHYTE-INFECTED RYEGRASS	ANALYSIS
PESTILENCE MORTALITY	50% (9/18)	90% (17/19)	G = 7.079 p < 0.01
PUPATION SUCCESS	89% (8/9)	50% (1/2)	INSUFFICIENT DATA
ECLOSION SUCCESS	75% (6/8)	100% (1/1)	INSUFFICIENT DATA

(6) Leaf Biomass Consumed:

The total amount of leaf material consumed for the duration of the experiment was 23.339g of endophyte-free ryegrass, almost twice as much as the 13.715g of endophyte-infected leaves. These figures represent the total biomass eaten by all larvae, and were only slightly affected by the significant differences in mortality of larvae reared on both types of ryegrass. From Figure 8 it can be seen that the period of increased consumption of endophyte-free leaf material was between days 35-50, corresponding to the eighth and ninth instars of all larvae. For the first 50 days of development, larval mortality in both treatments was the same, and it was only when the majority of larvae had started pupating, and therefore more or less stopped eating, that the differences in survival appeared.

The average amount of endophyte-free leaf material consumed each day was 0.476g ± 0.059, compared to the 0.264g ± 0.023 of endophyte-infected ryegrass leaves. Analysis using the two-sample T-test showed that this was a significant difference ($t=-3.35$, $p<0.01$). From Figure 8 it can be seen that there was some variation in the daily consumption of ryegrass, and this can be linked to the number of larvae ecdysing, because for the two or three days preceding each moult, larvae stop eating. Since the moulting dates for the two treatments were not synchronous, there were occasions when there were more larvae eating endophyte-infected leaves than endophyte-free.

Discussion:

Most of the trends found in the first experiment (Section 2) were repeated in this experiment, although the situation was complicated because of the small number of larvae in each instar group. Larval weight, HCW, pupation and mortality were all influenced to some extent by the presence of endophyte. The experiment should be repeated using plants with a much higher concentration of endophyte, and with a larger number of larvae to obtain significant differences between instar groups.

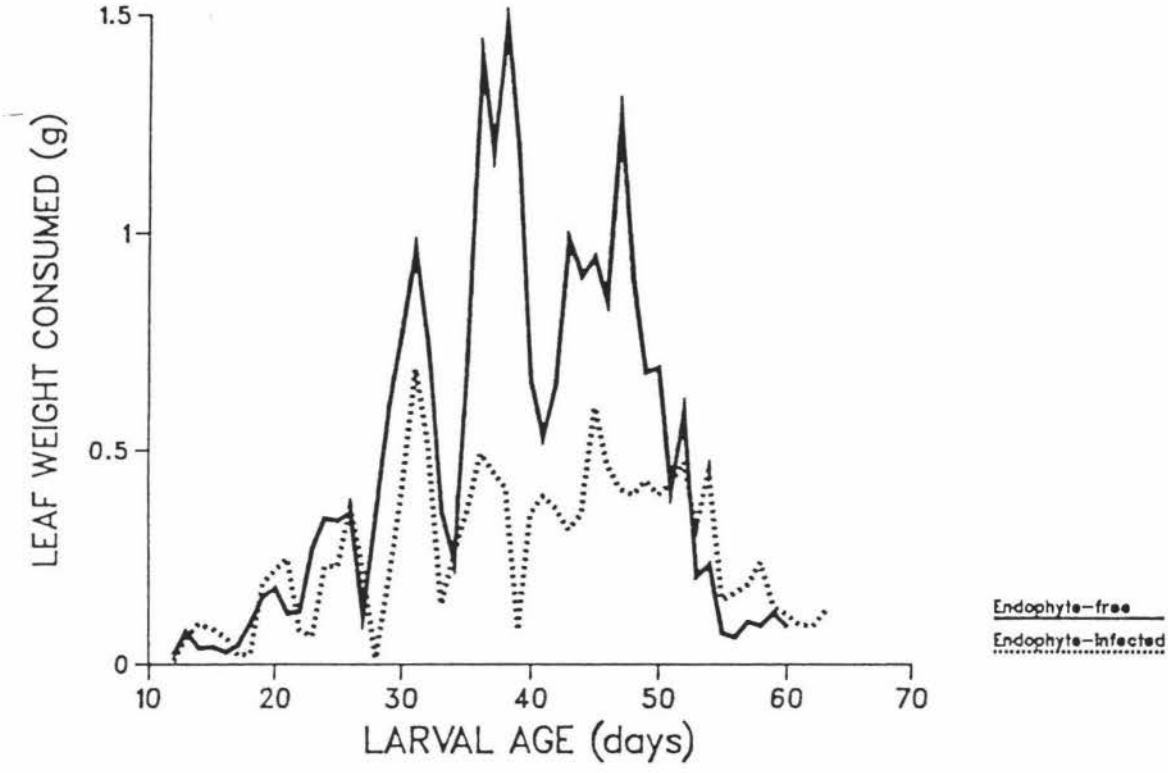

FIGURE 8: Total amount of leaf material consumed daily by slow-track larvae reared on endophyte-free and endophyte-infected perennial ryegrass leaves.

As discussed in Section 2, the number of instars of lepidopterans can be influenced by their diet. In this experiment, the increase in the number of instars from mostly 7 when reared on artificial diet, to 8, 9, or 10 when reared on ryegrass, implies that perennial ryegrass leaves may not be suited to slow-track *G. mutans* larval development. That the presence of endophyte was not linked to a change in the proportion of larvae in the different instar groups, suggests that there were was no difference in the quality of endophyte-infected and endophyte-free ryegrass. However, the reduction in larval weights, the delay in reaching those weights, and the increased mortality associated with endophyte infection indicates that the presence of *A. lolii* in perennial ryegrass does confer some resistance against slow-track *G. mutans*.

The presence of feeding deterrent(s) first shown in feeding preference tests (Section 1) was confirmed in this experiment by the reduced larval consumption of endophyte-infected ryegrass. It is likely that this reduced feeding is due to antifeedant products associated with endophyte infection, but less actual consumption may also be caused by the smaller size of the larvae. Antixenosis may then be responsible for some of the differences in the parameters recorded. For example, larvae which consume less leaf material would be expected to weigh less and have smaller HCW. However, the development of the single E+9 larva that pupated, indicates that antibiosis may also be involved in ryegrass resistance. This individual developed much faster and was much heavier than any others reared on endophyte, and was the only larva with the characteristic bell-shaped plot of weight gain. This suggests that the individual possessed a 'tolerance' to the presence of endophyte, not shared by the other larvae of the endophyte treatment.

If the resistance of the endophyte-infected ryegrass was due entirely to the antixenotic effects of feeding deterrent(s), it would be expected that the increased weight of this individual was due to increased consumption of leaves compared to the other larvae reared on endophyte. This was not the case. Some larvae reared on endophyte-infected leaves consumed more ryegrass than the 'tolerant' individual, but weighed considerably less. Therefore, the single E+9 larva that pupated appeared to be resistant not to the feeding deterrent(s), but to the effects of endophyte that decrease larval weight gain. By repeating this experiment with larger numbers of larvae, tolerance to the antibiosis effects of endophyte-infected perennial ryegrass within the *G. mutans* population could be further investigated.

SECTION 4: CONCLUSION

From the results of Sections 2 and 3, it can be seen that the developmental differences between fast-track and slow-track *G. mutans* larvae reared on perennial ryegrass were similar to those of the larvae reared on artificial diet (Chapter 2, Section 2). Reared on ryegrass under standard conditions, fast-track larvae pupated earlier (Figure 1), had fewer instars (Table 1), and gained weight much more quickly than slow-track larvae (Figure 9). Perennial ryegrass may not have been completely suited to *G. mutans* larval development, as both types had an increased number of ecdyses prior to pupation, and maximum larval weights and initial pupal weights were much less than those of the larvae reared on artificial diet. Further experiments are necessary to examine the development of fast-track and slow-track larvae when reared on a recognised host plant, such as plantain or dock.

Further work is also required on the 'pestilence' that affected both fast-track and slow-track larvae. Infected *G. mutans* larvae displayed symptoms similar to those caused by a nuclear polyhedrosis virus which infects another noctuid, the silver Y moth (*Chrysodeixis erisoma*), leaving cadavers limp and flaccid (Roberts 1979). It is unclear why the presence of endophyte influenced the 'pestilence' infection of slow-track larvae and not fast-track. Reared on artificial diet, there was no difference between the infection rates of both types of *G. mutans*.

In this chapter, it has been demonstrated that endophyte has antixenotic effects on *G. mutans*. The presence of feeding deterrent(s) in endophyte-infected leaves was shown in the feeding preference tests of Section 1, and again in Section 3, when slow-track *G. mutans* consumed more endophyte-free ryegrass leaves. The effects of endophyte on both fast-track and slow-track larval development were to decrease larval weight, HCW, and number of successful pupations. However these differences cannot be attributed solely to the antixenotic effects of endophyte infection. Metabolic inhibitors may also have affected the development of *G. mutans* slow-track larvae, as discussed in Section 3. Within the laboratory then, the presence of endophyte does confer resistance upon perennial ryegrass against *G. mutans* larvae, with strong antixenosis and possible antibiosis effects.

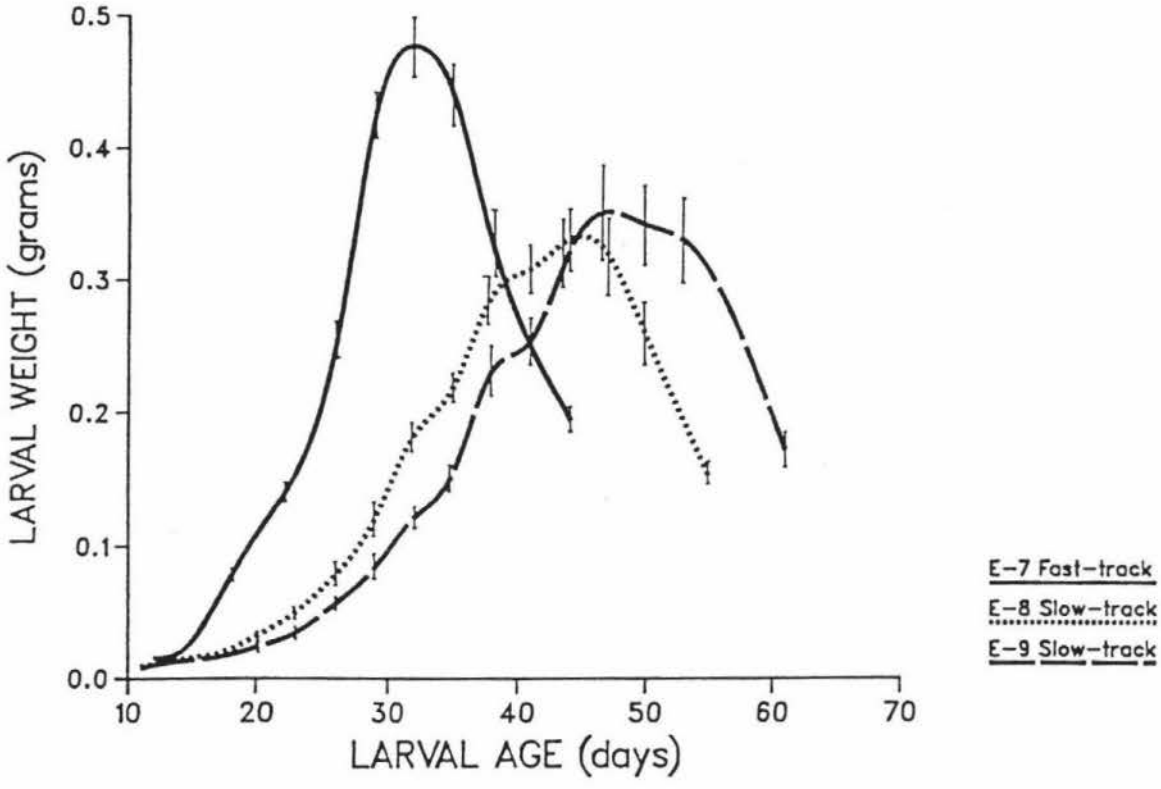

FIGURE 9: Mean larval weight ± SEM of fast-track and slow-track *G. mutans* larvae reared on endophyte-free and endophyte-infected perennial ryegrass leaves.

E-7 = seven instar larvae reared on endophyte-free ryegrass
E-7 & E-8 = seven and eight instar larvae reared on endophyte-free ryegrass

CHAPTER FOUR

PERAMINE AND GRAPHANIA MUTANS

Introduction:

Work carried out with Argentine stem weevil has shown that *A. lolii* infected perennial ryegrass contains several different allelochemic compounds. Two potent lolitrem neurotoxins from staggers-producing pasture have been isolated (Gallagher *et al.* 1981), and their structures determined (Gallagher *et al.* 1984). Lolitrem B is not a feeding deterrent to adult Argentine stem weevil (Prestidge & Gallagher 1985), but stem weevil larvae reared on diet containing lolitrem B exhibit slower development rates and higher mortality.

Using a choice bioassay based on plant extracts incorporated into artificial diets, a compound which deters adult Argentine stem weevil feeding has been isolated (Gaynor *et al.* 1983, Rowan & Gaynor 1985). This compound, peramine, differs chemically from the lolitrem neurotoxins, and has not been associated with ryegrass staggers. Peramine may be a fungal metabolite, and appears very important in reducing the number of adult weevils, and hence the number of eggs and larvae present on infected plants (Gaynor & Rowan 1986).

Prestidge *et al.* (1985) have also reported the presence of a stem weevil feeding deterrent from cultures of *A. lolii*, and the properties of the active substance were compatible with peramine, although no pure material was isolated. They concluded that the deterrent compounds produced in culture were not phytoalexins (defined by McLaren (1986) as fungitoxic substances which are synthesised by the plant in response to pathogen attack), but did not rule out the possibility that the plant-fungus interaction may produce other compounds, some of which may be phytoalexins, which have a deterrent action against insect herbivores. The objective of this series of experiments was to discover what effect peramine has on the larvae of *G. mutans*.

SECTION 1: PERAMINE CHOICE TESTS

Introduction:

In Chapter 3 it was shown that fast-track *G. mutans* larvae, like Argentine stem weevil, preferred eating perennial ryegrass leaves free from endophyte infection. Using the same methods, the objective of this set of experiments was to determine if the chemical which affects Argentine stem weevil, peramine, is also an effective feeding deterrent for *G. mutans* fast-track larvae.

Methods:

The methods were identical to those used in the tests involving endophyte-infected leaves (Chapter 3, Section 1). Two batches of artificial diet were prepared. One contained peramine at 10ppm, the peramine having been incorporated into the diet with cellulose powder. The second diet contained only the cellulose blank as the control.

The first experiment involved first instar larvae. Small plugs from each diet were placed side by side in a 9cm petri dish lined with moistened filter paper. One hundred neonate larvae from one egg batch were divided into groups of five and placed in the centre of 20 such dishes, which were kept in the controlled temperature room on trays within transparent plastic bags. The distribution of larvae on the diet was counted after 16 and 40 hours, and the results analysed using Wilcoxon's matched pairs signed rank test. Because of the rough texture of the diet it was not possible to score the feeding of the neonate larvae, so no estimate of consumption was obtained.

The sixth instar larvae used in the second experiment were reared individually on artificial diet within petri dishes in the controlled temperature room. All the larvae had moulted within two days of commencing the experiment, and had been starved for the preceding 24 hours. Two plugs of diet, one containing peramine, were placed side by side in a 9cm petri dish lined with moistened filter paper. One larva was placed in the centre of each dish and kept for 18 hours in the controlled temperature room on trays

within transparent plastic bags. The difference in weight of the plugs before and after feeding measured larval feeding preference. Control plugs of diet kept in a similar fashion showed less than a 1% change in weight so a correction factor for natural water loss was not needed. The experiment was repeated twice: firstly with 15, and then a month later with 30 larvae.

Results:

The results for the two trials are shown in Table 8. In both cases peramine had no influence on the feeding preference of the *G. mutans* fast-track larvae.

Discussion:

In Chapter 3, Section 1, neonate and sixth instar fast-track *G. mutans* larvae demonstrated a significant feeding preference for excised perennial ryegrass leaves free from endophyte infection. Using the same methods, peramine at 10ppm had no effect on fast-track larval feeding preference. As discussed in Chapter 3, Section 1, the inability to quantify the biological activity of endophyte in leaves was one of the main problems associated with feeding preference tests. Using peramine incorporated in artificial diet, this is no longer a source of variation. The presence or absence of a known concentration of peramine is the only difference between the two batches of diet, as both contain exactly the same ingredients.

It would appear from these results that the *G. mutans* feeding deterrent present in endophyte-infected ryegrass is not peramine. Argentine stem weevil adults respond to the presence of peramine at concentrations as low as 0.1ppm (J.J. Dymock pers. comm.), yet at 10ppm, peramine had no effect on the feeding preference of fast-track larvae. It is possible, however, that peramine may be present at higher concentrations within the endophyte-infected plant. Research has shown that 'Grassland Nui' perennial ryegrass plants contain concentrations of peramine ranging between 4ppm - 40ppm (D.D. Rowan pers. comm.). Further experiments are necessary to determine if higher concentrations of peramine act as a feeding deterrent for fast-track *G. mutans* larvae.

TABLE 8: Results of fast-track larval feeding preference tests with peramine-free and peramine-treated artificial diet.
(Results analysed using Wilcoxon's matched pairs signed rank test)

TABLE 8a: Neonate Larvae

		TREATMENT		ANALYSIS
		PERAMINE-FREE DIET	PERAMINE-TREATED DIET	
16 HOURS	Total Number of Larvae on Diet	23	33	z =-0.9825 p = 0.3259
40 HOURS	Total Number of Larvae on Diet	43	42	z =-0.0218 p = 0.9826

TABLE 8b: Sixth Instar Larvae

	PERCENTAGE OF DIET LEFT UNEATEN		ANALYSIS
	PERAMINE-FREE DIET (mean ± SEM)	PERAMINE-TREATED DIET (mean ± SEM)	
TRIAL ONE (n = 15)	74.1 ± 0.05	74.8 ± 0.06	z =-0.1704 p = 0.8647
TRIAL TWO (n = 30)	61.0 ± 0.05	72.2 ± 0.04	z =-1.2650 p = 0.2059

SECTION 2: EFFECTS OF PERAMINE ON FAST-TRACK LARVAE

Introduction:

Although recent studies have shown peramine to be a feeding deterrent for Argentine stem weevil, little is known about the long-term effects of feeding peramine to insects. Preliminary experimental results have shown increased larval mortality and decreased rates of development for Argentine stem weevil larvae reared on peramine included in an artificial diet (J.J. Dymock pers. comm.). The objective of this experiment was to determine if peramine had any effects on the development of *G. mutans* fast-track larvae and to compare the results with the long-term effects of endophyte outlined in Chapter 3.

Methods:

Using the methods outlined in Chapter 3, Section 2, 40 larvae were divided into two groups and reared on small plugs of diet either with peramine, or without peramine. The concentration of the peramine was 10ppm and came from the same batch used in the peramine choice tests (Section 1). Extra diet was added as required to ensure an excess of food, and every three days the diet and filter paper were changed, and the larvae weighed. The results were collected and analysed as for the experiment on the long-term effects of endophyte on fast-track larvae.

Results:

(1) Number of Larval Instars:

Of the larvae reared on peramine-free diet, 90% developed through 6 instars, with only two larvae possessing the supernumerary instar. The effect of peramine was to significantly shift the proportion of larvae with the extra instar up to 50% ($G=5.827$, $p<0.025$). The larvae of the experiment could be divided into five groups (the number of larvae in each group is written in parentheses):

P-6 = larvae reared on P- diet with 6 instars (15)
P-7 = larvae reared on P- diet with 7 instars (2)
P+6PUP = larvae reared on P+ diet with 6 instars that pupated (3)
P+6NOP = larvae reared on P+ diet with 6 instars that fail to pupate (5)
P+7 = larvae reared on P+ diet with 7 instars (8)

One larva reared on peramine-free diet developed through 8 instars in 6 weeks before it died of the 'pestilence' infection, well before it was ready to pupate. Its maximum weight was 0.025g, only 4% of the average maximum weight of the other peramine-free reared larvae. Because this individual was so different from the others it was not considered in the results.

(2) Larval Weight:

Figure 10 shows the larval weights for the different groups (a complete description of the results is given in Appendix VI). The characteristic bell-shaped curves of the P-6 and P+6PUP larvae were very similar, reaching maximum larval weight over a week earlier than the other groups (Table 9). The P-7 and P+7 larvae were also similar, both curves being skewed to the right. In between the two was the P+6NOP curve. These larvae were lighter than both the P-6 and P+7 groups, the only significant difference in maximum larval weight (Table 9).

(3) Head Capsule Width:

From Figure 11, it can be seen that peramine had little affect on HCW. The significant differences between the regressions of HCW against instar were associated with the additional moult ($F=124.18$, $p<<0.001$). The two 7-instar groups were much smaller until their final moult, when their HCW was significantly larger than that of all the 6-instar larvae (Table 9).

The first three moults of each group occurred at approximately the same time. After that, the pattern was much the same as for the larval weight results: both the P-6 and P+6PUP moulted earlier than the P+6NOP, P-7, and the P+7 larvae (Table 9).

TABLE 9: Comparison of the development of fast-track larvae reared on peramine-free and peramine-treated artificial diet.

P-6 & P-7 = six and seven instar larvae reared on peramine-free diet
P+6PUP, P+6NOP & P+7 = pupating and non-pupating six and seven instar larvae reared on peramine-treated diet

PARAMETER	LARVAE	MEAN ± SEM	(n)	ANOVA	STUDENT-NEWMAN-KUELS TEST									
					P-6 vs P-7	P-6 vs P+6PUP	P-6 vs P+6NOP	P-6 vs P+7	P-7 vs P+6PUP	P-7 vs P+6NOP	P-7 vs P+7	P+6PUP vs P+6NOP	P+6PUP vs P+7	P+6NOP vs P+7
MAXIMUM LARVAL WEIGHT(g)	P-6	0.751 ± 0.025	(15)	F=4.38 p<0.01	q=1.812 NS	q=2.396 NS	q=4.989 p<0.01	q=0.692 NS	q=0.166 NS	q=1.449 NS	q=2.108 NS	q=1.455 NS	q=2.686 NS	q=5.050 p<0.01
	P-7	0.661 ± 0.040	(2)											
	P+6PUP	0.651 ± 0.058	(3)											
	P+6NOP	0.581 ± 0.036	(5)											
	P+7	0.771 ± 0.033	(8)											
DAYS TO REACH MAX LARVAL WEIGHT	P-6	33.4 ± 0.576	(15)	F=21.69 p<0.001	q=6.011 p<0.001	q=0.314 NS	q=6.162 p<0.001	q=12.235 p<0.001	q=5.174 p=0.05	q=1.605 NS	q=0.786 NS	q=4.630 p<0.01	q=7.895 p<0.001	q=3.445 p=0.05
	P-7	42.5 ± 1.500	(2)											
	P+6PUP	33.0 ± 1.000	(3)											
	P+6NOP	39.8 ± 0.735	(5)											
	P+7	43.8 ± 1.556	(8)											
HCW OF FINAL INSTAR (mm)	P-6	2.90 ± 0.021	(15)	F=4.87 p<0.005	q=3.759 NS	q=0.151 NS	q=0.460 NS	q=4.925 p<0.01	q=2.995 NS	q=3.667 NS	q=0.964 NS	q=0.456 NS	q=3.255 p<0.05	q=4.149 p<0.05
	P-7	3.06 ± 0.060	(2)											
	P+6PUP	2.91 ± 0.048	(3)											
	P+6NOP	2.89 ± 0.015	(5)											
	P+7	3.02 ± 0.031	(9)											
DAYS TO REACH INSTAR SIX	P-6	27.8 ± 0.509	(15)	F=4.18 p<0.01	q=2.178 NS	q=0.467 NS	q=4.941 p<0.01	q=3.422 p<0.025	q=2.107 NS	q=1.089 NS	q=0.314 NS	q=3.882 NS	q=2.550 NS	q=2.107 NS
	P-7	30.5 ± 0.500	(2)											
	P+6PUP	27.3 ± 0.333	(3)											
	P+6NOP	32.0 ± 1.140	(5)											
	P+7	30.1 ± 0.948	(9)											
DAYS TO REACH INSTAR FIVE	P-6	21.4 ± 0.456	(15)	F=4.50 p<0.01	q=3.620 NS	q=0.120 NS	q=3.518 p<0.025	q=4.668 p<0.01	q=3.068 NS	q=1.086 NS	q=0.968 NS	q=2.592 NS	q=3.066 NS	q=0.271 NS
	P-7	25.0 ± 0.000	(2)											
	P+6PUP	21.3 ± 0.333	(3)											
	P+6NOP	23.8 ± 0.800	(5)											
	P+7	24.0 ± 0.782	(9)											

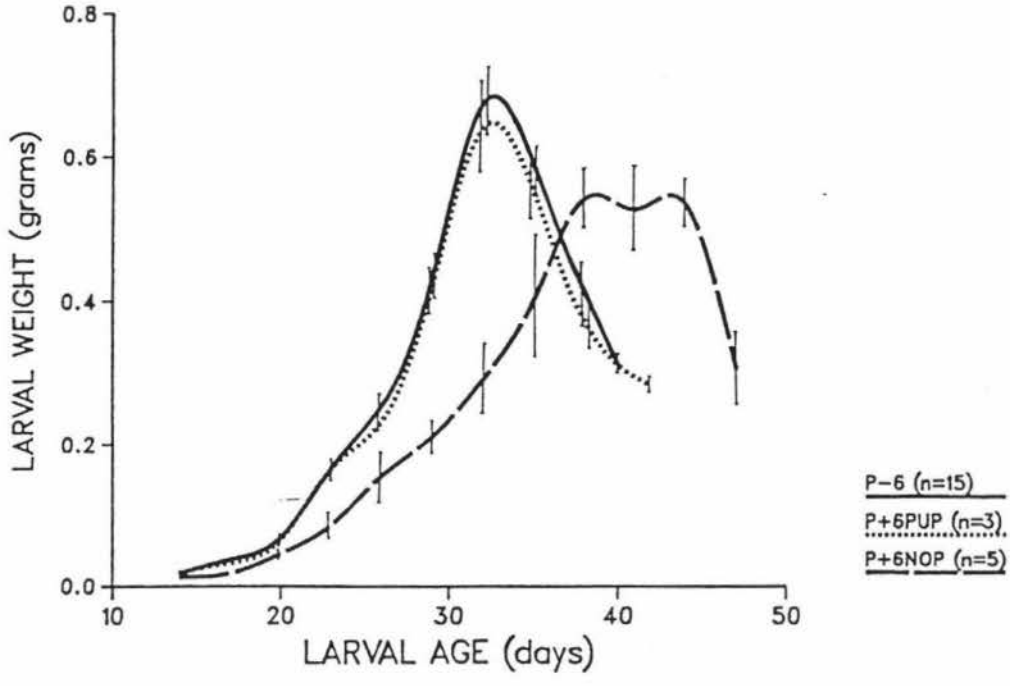

FIGURE 10a: Six Instar Larvae

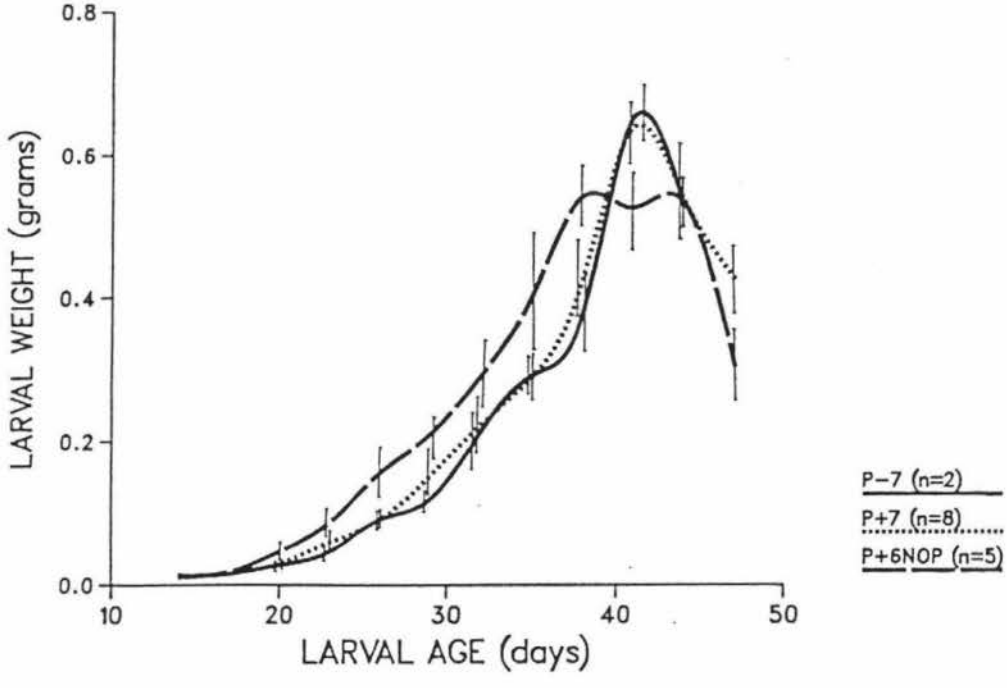

FIGURE 10b: P+6NOP and Seven Instar Larvae

FIGURE 10: Mean larval weight ± SEM of fast-track larvae reared on peramine-free and peramine-treated artificial diet.

P-6, P-7 = six and seven instar larvae reared on peramine-free diet
P+6PUP, P+6NOP & P+7 = pupating and non-pupating six and seven instar larvae reared on peramine-treated diet

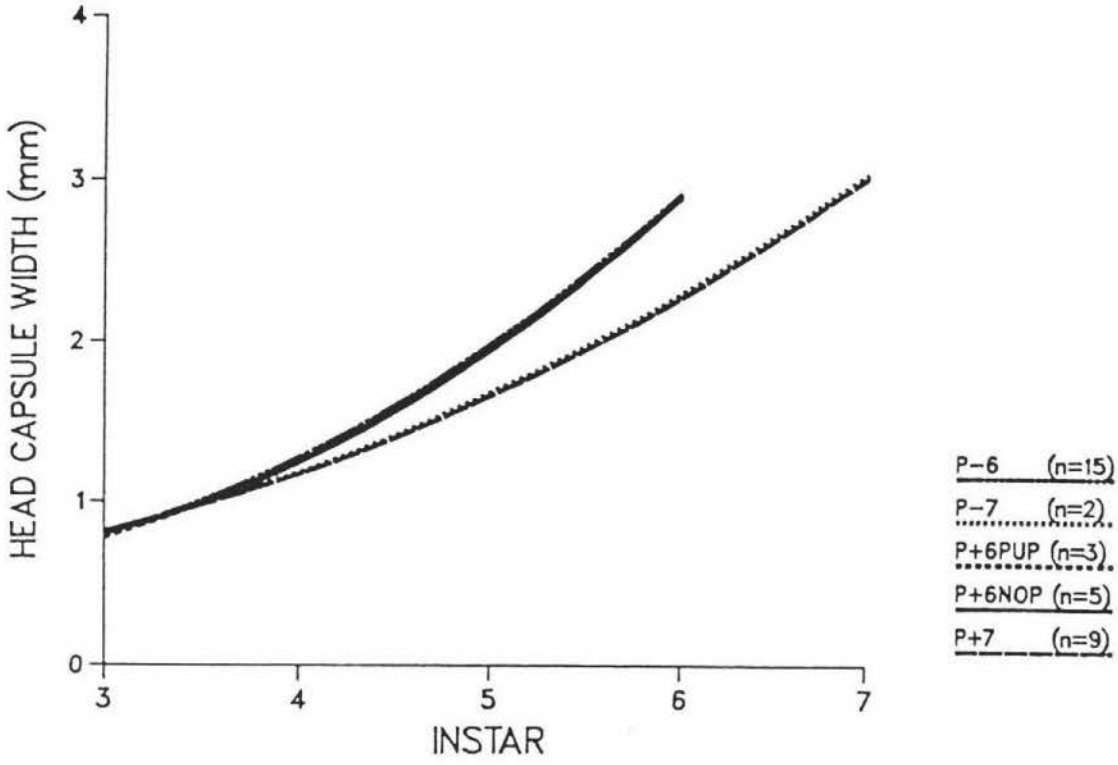

FIGURE 11: HCW regressions of fast-track larvae reared on peramine-free and peramine-treated artificial diet.

P-6, P-7 = six and seven instar larvae reared on peramine-free diet
P+6PUP, P+6NOP & P+7 = pupating and non-pupating six and seven instar larvae reared on peramine-treated diet

Regression Equations:

P-6: $HCW = 0.117\ instar^2 - 0.348\ instar + 0.791$
P-7: $HCW = 0.063\ instar^2 - 0.073\ instar + 0.462$
P+6PUP: $HCW = 0.113\ instar^2 - 0.313\ instar + 0.700$
P+6NOP: $HCW = 0.130\ instar^2 - 0.473\ instar + 1.050$
P+7: $HCW = 0.065\ instar^2 - 0.098\ instar + 0.523$

(4) Pupation:

From Table 10 it can be seen that the number of peramine-free reared larvae that successfully pupated (see below) was over four times that of those reared on peramine. Those of the P-6 and P+6PUP groups that successfully pupated showed no significant differences in the date of pupation and initial pupal weights. However, if all the larvae that reached pupation (successful or otherwise) are considered, peramine can be seen to have delayed the date of pupation of 6-instar larvae.

(5) Mortality:

Deaths due to 'pestilence' infection occurred mostly before pupation and were not significantly influenced by the presence of peramine (Table 11). There was, however, a significant difference between the number of peramine-free and peramine-infected deaths at pupation (Table 11), with most of the larvae fed peramine dying at this time. Many cadavers were blackened half larval - half pupal forms, with the brown pupal case present, but the head capsule and exoskeleton of the larval form not properly cast off. The rest were shrivelled black and malformed, leaking black fluid onto the filter paper.

Many of the newly formed peramine-free pupae had a hole in the ventral surface of the brown pupal case. This gap between abdomen and thorax eventually closed over (Plate 6), but not before many pupae leaked black fluid. Of all the larvae that pupated, only three of the peramine-free treatment eclosed. The adults looked in very good condition but failed to produce fertile eggs.

Discussion:

Many of the effects of peramine on the recorded parameters of fast-track *G. mutans* development were very similar to those displayed by fast-track larvae reared on endophyte-infected ryegrass leaves (Chapter 3, Section 2). Peramine increased the number of larvae with the supernumerary instar, reduced larval weight, delayed

TABLE 10: Comparison of the pupae of fast-track *G. mutans* reared on peramine-free and peramine-treated artificial diet.

P-6 & P-7 = pupae from six and seven instar larvae reared on peramine-free diet

P+6, P+6PUP & P+7 = pupae from six and seven instar larvae reared on peramine-treated diet

PARAMETER	LARVAE	MEAN ± SEM (n)	ANOVA	STUDENT-NEWMAN-KUELS TEST		
				P-6 vs P+6	P-6 vs P+7	P+6 vs P+7
DAYS TO REACH PUPATION: ALL LARVAE	P-6	40.7 ± 0.630 (15)	F=23.86	q=4.530	q=9.583	q=4.907
	P-7	55.0 ± 0.000 (1)	p«0.001	p<0.005	p<0.001	p<0.005
	P+6	45.0 ± 1.558 (7)				
	P+7	51.0 ± 1.095 (5)				
DAYS TO REACH PUPATION: SUCCESSFUL LARVAE	P-6	39.9 ± 0.431 (13)	F=2.00			
	P+6PUP	41.7 ± 0.333 (3)	NS			
INITIAL PUPAL WEIGHT(g)	P-6	0.311 ± 0.010 (13)	F=3.51			
	P+6PUP	0.281 ± 0.009 (3)	NS			

TABLE 11: Comparison of the mortality of fast-track *G. mutans* reared on peramine-free and peramine-treated artificial diet.

(Results analysed using the G-test with William's correction, sample sizes are in parentheses)

PARAMETER	PERAMINE-FREE DIET	PERAMINE-TREATED DIET	ANALYSIS
PESTILENCE MORTALITY	6% (1/17)	25% (4/12)	G = 2.421 NS
PUPATION SUCCESS	81% (13/16)	25% (3/12)	G = 5.493 p « 0.001
ECLOSION SUCCESS	23% (3/13)	0% (0/3)	INSUFFICIENT DATA

pupation, and increased mortality of fast-track larvae. Although peramine, unlike endophyte, had no effect on HCW, these results suggest that the presence of peramine is associated with a decrease in the suitability of the artificial diet for *G. mutans* larval development.

The presence of both endophyte and peramine significantly lowered the number of successful pupations, creating instead, malformed black and bleeding pupae, or, blackened half larval - half pupal forms. These types of pupation failures were found but very rarely in larvae reared on endophyte-free or peramine-free diet. Any antixenotic effects of endophyte or peramine that may have contributed to this mortality are likely to have been amplified by the inadequacies of the rearing method. That perennial ryegrass may not be suitable for *G. mutans* development has already been discussed (Chapter 3), yet even those reared on peramine-free artificial diet showed very poor pupal survival. The development of a satisfactory rearing method is a priority for future research, as the effects of peramine on larval development would be more apparent if greater numbers of larvae on artificial diet successfully eclosed.

The main difference between the two experiments was the presence of three individuals that appeared unaffected by the presence of peramine. These larvae, the P+6PUP group, possessed a 'tolerance' not found in the fast-track larvae reared on endophyte, and in only one slow-track larva. Unlike the other 6-instar larvae reared on peramine, the 'tolerant' individuals successfully pupated, and their development followed the same pattern as the P-6 group. Such 'tolerance' to peramine also occurs in Argentine stem weevil populations. Most stem weevil larvae reared on peramine showed reduced development rates and increased mortality, but a small number of individuals pupated as normal (J.J. Dymock pers. comm.).

SECTION 3: CONCLUSION

In this chapter it has been shown that peramine,the Argentine stem weevil feeding deterrent isolated from endophyte-infected perennial ryegrass, has no antixenotic effects on *G. mutans* fast-track larvae at 10ppm concentration. This result indicates

that the feeding preference for endophyte-free ryegrass leaves shown in Chapter 3, is likely to be caused by some other factor.

Peramine however, does influence fast-track *G. mutans* larval development. As peramine is not a feeding deterrent, the reduced larval weight, delayed pupation, increased mortality, and increased number of larvae possessing the supernumerary instar, must be the consequence of antibiosis. The presence of peramine therefore, may explain in part, many of the effects of endophyte on fast-track larval development recorded in Chapter 3, Section 2.

Peramine is known to be only one of a range of chemicals produced by the interaction between endophyte and perennial ryegrass, any of which may influence *G. mutans*. For example, the lolitrem neurotoxins associated with ryegrass staggers, are known to influence Argentine stem weevil larval development. Further experiments testing the lolitrem chemicals and other alkaloids, are necessary before the nature of perennial ryegrass resistance to *G. mutans* can be fully determined.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSIONS

The taxonomy of the genus *Graphania* is complicated partly because of the similarity in appearance of several species, and also because of variation in morphological characters within populations. The need for a revision of *Graphania* taxonomy has been recognised (MacFarlane 1970), yet in this study there was evidence to suggest that simply redefining the structural characteristics considered to be specific for *G. mutans* may not be sufficient to separate biological species. The presence of sibling species within populations of *P. excessana* has shown (Foster *et al.* 1987) that it is no longer possible to be dogmatic about the taxonomy of many lepidopterans.

By examining the development of different larval cohorts reared on both perennial ryegrass and artificial diet, it was possible to separate *G. mutans* into two groups. The fast-track group developed much more rapidly, and were always much larger as larvae and pupae than those of the slow-track group. Despite these distinct differences in larval development, adults of the fast-track and slow-track cohorts possessed the morphological characteristics of what is recognised as the *G. mutans* species.

Further research is necessary into the possibility of sibling species of *G. mutans*. A rearing method should be developed which is capable of producing several generations of *G. mutans* within the laboratory as it is important to determine if fast-track and slow-track adults will interbreed, and whether the difference in larval development is inherited. The mating systems of *G. mutans* should also be investigated in a manner similar to that used with *P. excessana* (Foster *et al.* 1987). Such studies of biochemical, cytological and behavioural traits would be of value not only for the taxonomy of *G. mutans*, but for many other species that morphologically resemble each other.

The presence of the fungal endophyte *A. lolii* within perennial ryegrass has been shown to affect both types of *G. mutans*. Feeding preference tests demonstrated that the fungus-plant interaction produced at least one feeding deterrent which influenced both neonate and sixth instar fast-track larvae. Slow-track larvae reared on endophyte-infected ryegrass leaves were shown to consume less leaf material than those reared on

endophyte-free ryegrass. Reduced feeding could either be a function of the antifeedant properties associated with infection, or less actual consumption due to the smaller size of the larvae, or a combination of both.

The long-term effect of rearing fast-track and slow-track larvae on endophyte-infected ryegrass was to decrease larval weight, HCW, rate of development, and the number of successful pupations in comparison to larvae fed leaves from endophyte-free ryegrass. These developmental differences were probably caused by a combination of antixenosis and antibiosis. Larvae were shown to eat less endophyte-infected leaf material, yet the 'tolerance' of one slow-track larva to the presence of endophyte, without increased leaf consumption, suggested that antibiosis effects were also involved.

Similar experiments have been conducted investigating the effects of *A. lolii* on other noctuid larvae. Hardy *et al.* (1985) showed that fall armyworm (*Spodoptera frugiperda*) neonate larvae strongly preferred endophyte-free leaf blades. Reduced larval weight gains and extended developmental times were clearly evident. Larvae maintained on endophyte-infected leaves required nearly 1.5 additional days to reach a satisfactory weight to ensure pupation. The high mortality of *G. mutans* about the time of pupation, may have been because few of the larvae reared on endophyte achieved that 'satisfactory weight to ensure pupation'. This is particularly applicable to the slow-track larvae, where the only successfully pupating individual had a maximum weight 0.1g heavier than any of the other larvae reared on endophyte-infected ryegrass. Alternatively, death may have been caused by subtle antibiosis effects not manifested until pupation.

Clay et al. (1985a) showed that the presence of endophyte not only retarded growth and development of *S. frugiperda* but also increased larval mortality. The toxic effects of endophyte were more marked on the closely related southern armyworm (*S. eridania*), as larvae feeding on basal parts of infected ryegrass plants died within 48 hours. The antibiosis effects of endophyte were less marked than this for *G. mutans*, but the toxic effects were greater for *G. mutans* than *S. frugiperda*. Clearly, even within the same lepidopteran family, there are different responses to the allelochemics produced by the fungus-plant interaction. More than one class of compound may be associated with endophytic grasses (Bacon *et al.* 1986), and each may affect insects differently according to the biochemical and physiological attributes of the insect. This is clearly demonstrated by the different responses to the feeding deterrent peramine.

Extracted from endophyte-infected ryegrass, the antifeedant compound peramine has been shown to deter Argentine stem weevil feeding at very low concentrations. However, peramine incorporated in artificial diet had no effect on the feeding preference of *G. mutans* fast-track larvae. This suggests that peramine is a relatively specific plant defence, and possibly only a deterrent for a small range of specialist herbivores like Argentine stem weevil. Endophyte-infected perennial ryegrass plants must contain other allelochemics, some of which do influence the feeding preference of polyphagous Lepidoptera like *G. mutans*.

Although not possessing the same antixenotic properties, many of the long-term effects of peramine on the development of *G. mutans* fast-track larvae were similar to those caused by endophyte in perennial ryegrass. Therefore, some of the antibiosis effects of endophyte may be attributable to the toxicity of peramine to *G. mutans* larvae. It is likely that other allelochemics are also involved, as peramine, unlike endophyte, had no effect on HCW. Several individual larvae showed a 'tolerance' to peramine in artificial diet, but 'tolerance' to endophyte was very rare. The single E+9 larva that successfully pupated must have possessed biochemical and physiological mechanisms to detoxify peramine, and the other allelochemics conferring resistance to endophyte-infected ryegrass.

Before discussing the possible adaptive significance of endophyte-induced resistance, it is important to recognise that none of the experiments were carried out under natural conditions. Inferences about *G. mutans* in the pasture ecosystem extrapolated from laboratory studies should not be accepted uncritically. For example, all endophyte experiments used excised leaf blades, and it is known that induced chemical changes in cut foliage can influence feeding preferences, especially of generalist phytophagous insects (Risch 1985). Also, peramine may have different properties within the plant compared to diet. Biologically active compounds in the presence of other synergistic chemicals may have increased toxicity (Scriber 1984). The concentration of peramine at the site of *G. mutans* feeding is also unknown.

Hardy *et al.* (1985) stated that grasses of high nutritional quality may compensate for the negative effects associated with fungal infections. Perennial ryegrass may not be highly suited to *G. mutans* development, which may mean that the resistance properties of endophyte-infected plants are better expressed. Environmental conditions

can also play a role in determining endophyte toxicity. Farm animals grazing endophyte-infected pastures usually exhibit toxicity symptoms most strikingly when under heat stress (Funk *et al.* 1983). In current experiments, the poor diet, coupled with inadequate rearing methods, may have placed the *G. mutans* larvae under additional stress and this increased the effects of endophyte.

The nature of endophyte infection itself is subject to variation. Many different strains of *A. lolii* have been identified, each producing varying concentrations of allelochemics such as peramine (J.J. Dymock pers. comm.). The situation can be further complicated by the presence of other fungi. Infection by the vesicular-arbuscular mycorrhizal fungi *Glomus fasciculatum* has recently been shown to reduce the level of resistance to Argentine stem weevil conferred on ryegrass by *A. lolii* (Barker 1987).

The effects of endophyte upon the reproductive biology and population dynamics of *G. mutans* needs to be determined. It has been demonstrated that endophyte affects larval development, but the essential function of metabolism is to catalyse the transmission of genetic information (Calow 1977). Quantification of the effects of endophyte on reproductive success is necessary, not only for *G. mutans*, but for many other insects as well. Without information on the fecundity of endophyte-reared adults and their progeny, it is impossible to gauge the influence of *A. lolii* on herbivores within the pasture ecosystem.

Endophyte infection in perennial ryegrass populations will decrease or increase depending on the differential fitness of uninfected and infected plants. This is because *A. lolii* is only disseminated through the vegetative growth of hyphae into the developing seeds of its host. The high levels of infection found in pastures strongly suggests that an advantage is held by infected plants, or at least indicates no disadvantage (Clay *et al.* 1985a).

Resistance to insect herbivores is one possible advantage for infected ryegrass, as defoliation reduces the photosynthetic capacity of the plant. Endophyte-infected plants would be at a selective advantage in a mixed population if uninfected plants were exclusively eaten. The results of this study indicate that *G. mutans* larvae would tend to avoid endophyte-infected ryegrass plants. However it is possible that *G. mutans*

larvae could prefer dicotyledonous hosts like dock, plantain or clover to grasses, though the responses of *G. mutans*, a generalist herbivore, suggest that other generalist phytophagous insects would also be negatively affected by endophyte-infected ryegrass.

The relationship between *G. mutans* and endophyte-infected ryegrass is complicated, and clearly much experimental work remains to be done. The development of an adequate rearing method would provide opportunities to further explore the differences between fast-track and slow-track *G. mutans*. It would also enable the insect's reproductive biology to be examined, with emphasis on the effects of endophyte on reproductive success. However, laboratory studies can only provide a limited amount of information. Until research is conducted on natural populations of this abundant species, the relationship between endophyte, perennial ryegrass and *G. mutans* will remain unclear.

APPENDIX I

Composition of *G. mutans* artificial diet.

The diet used was Pritam Singh's 'Diet 1115':

Ingredients:

lima beans	175g
bran	75g
pasturised brewers yeast	20g
sucrose	30g
methyl-p-hydroxy benzoate	1.5g
sorbic acid	1.0g
agar	30g
water	1000ml

The ingredients are mixed in a large beaker and cooked in the microwave for 7 minutes. The hot ingredients are then blended. When cooled to approximately 70°C blend in:

vitamin C	3g
50/50 linseed oil/maise oil	1ml
10% formaldehyde	4ml

The molten diet is poured into a beaker and then adjusted to pH4.5 using 1N HCl or 3M KOH. The diet is then poured into a suitable mould.

APPENDIX II

Mean larval weight ± SEM of fast-track and slow-track *G. mutans* larvae reared on artificial diet (Graph 2).

F6 = six instar fast-track larvae
S7 = seven instar slow-track larvae
Mean age at pupation is written in parentheses
¶ = Number of larvae that successfully pupated

Larval Age (days)	Mean Larval Weight (g) ± SEM	
	F6	S7
	(n = 15)	(n = 16)
14	0.017 ± 0.001	0.003 ± 0.000
17	0.038 ± 0.004	0.006 ± 0.001
20	0.069 ± 0.005	0.013 ± 0.001
23	0.166 ± 0.013	0.022 ± 0.003
26	0.254 ± 0.019	0.032 ± 0.003
29	0.430 ± 0.027	0.049 ± 0.007
32	0.677 ± 0.047	0.074 ± 0.009
35	0.581 ± 0.034	0.136 ± 0.017
38	0.410 ± 0.043	
41	0.311 ± 0.035	0.274 ± 0.026
44	(40.7 ± 0.63)	0.360 ± 0.032
47	¶ = 13	0.430 ± 0.036
50		0.422 ± 0.028
53		0.469 ± 0.042
57		0.247 ± 0.006
		(57.5 ± 1.02)
		¶ = 16

APPENDIX III

Preliminary choice test experiments.

A preliminary trial was conducted to determine if *G. mutans* larvae showed a preference for any region of the ryegrass leaf. Twenty leaves from endophyte-infected and endophyte-free ryegrass plants were removed by cutting just above the ligule with scissors. Each leaf was cut into two equal lengths. The top half ('Tip'), and the bottom half ('Base') were placed side by side in a petri dish lined with moistened filter paper. One fourth-instar fast-track *G. mutans* larva was placed into each dish, and all dishes were kept on trays in transparent plastic bags within a controlled temperature room for 18 hours. The difference in weight of each blade before and after feeding was used to measure feeding preference, and the results were analysed using Wilcoxon's matched pairs signed rank test. Leaves treated in a similar fashion showed less than a 1% loss in weight due to natural water loss so no correction factor was necessary.

In the Table below, it can be seen that the larvae preferred feeding on the base of endophyte-free ryegrass leaves, yet there was no preference shown for either portion of the endophyte-infected leaves.

	PERCENTAGE OF LEAF LEFT UNEATEN		ANALYSIS
	BASE (mean ± SEM)	TIP (mean ± SEM)	
Endophyte-free	82.7 ± 0.03	93.8 ± 0.02	z =-2.5386 p = 0.0111
Endophyte-infected	86.8 ± 0.02	87.7 ± 0.36	z =-0.5973 p = 0.5503

This may be because in *L. perenne* 'Grassland Nui', endophyte is more concentrated at the base of the lamina than at the tip (S. Pilkington pers. comm.). The deterrent effects of the endophyte may be sufficient to nullify whatever feeding stimulus is present in the base of the endophyte-free ryegrass leaf, thus explaining the lack of feeding preference. Because feeding preference tests are affected by which portion of the leaf blade is used, the leaf material in all experiments involving ryegrass and endophyte was taken from the bottom of each excised leaf lamina.

APPENDIX IV

Mean larval weight ± SEM of fast-track larvae reared on endophyte-free and endophyte-infected perennial ryegrass leaves (Graph 5).

E-7 = seven instar larvae reared on endophyte-free ryegrass
E+7 & E+8 = seven and eight instar larvae reared on endophyte-infected ryegrass
Mean age at pupation ± SEM is written in parentheses
¶ = Number of larvae that successfully pupated

Larval Age (days)	Mean Larval Weight (g) ± SEM		
	E-7 (n = 14)	E+7 (n = 10)	E+8 (n = 6)
12	0.015 ± 0.001	0.011 ± 0.001	0.012 ± 0.002
15	0.027 ± 0.002	0.021 ± 0.001	0.015 ± 0.001
18	0.077 ± 0.004	0.055 ± 0.004	0.038 ± 0.003
22	0.139 ± 0.005	0.113 ± 0.008	0.085 ± 0.009
26	0.255 ± 0.011	0.187 ± 0.007	0.173 ± 0.016
29	0.426 ± 0.015	0.297 ± 0.015	0.217 ± 0.008
32	0.476 ± 0.023	0.357 ± 0.019	0.259 ± 0.019
35	0.439 ± 0.024	0.325 ± 0.014	0.359 ± 0.029
38	0.329 ± 0.025	0.257 ± 0.012	0.377 ± 0.021
41	0.249 ± 0.023	0.211 ± 0.014	0.335 ± 0.027
44	0.194 ± 0.009	0.127 ± 0.005	0.288 ± 0.028
47	(43.8 ± 0.52)	(46.0 ± 1.02)	0.234 ± 0.025
50	¶ = 14	¶ = 7	0.176 ± 0.020
53			0.153 ± 0.003
			(51.7 ± 1.86)
			¶ = 3

APPENDIX V

Mean larval weight ± SEM of slow-track larvae reared on endophyte-free and endophyte-infected perennial ryegrass leaves (Graph 7).

E-8, E-9 & E-10 = eight, nine and ten instar larvae reared on endophyte-free ryegrass
E+8, E+9, E+10 & E+11 = eight, nine, and ten instar larvae reared on endophyte-infected ryegrass
Mean age at pupation ± SEM is written in parentheses
¶ = Number of larvae that successfully pupated

LARVAL AGE (days)	MEAN LARVAL WEIGHT(g) ± SEM						
	E-8 (n = 5)	E-9 (n = 8)	E-10 (n = 1)	E+8 (n = 4)	E+9 (n = 7)	E+10 (n = 2)	E+11 (n = 1)
11	0.009 ± 0.000	0.008 ± 0.000	0.009 ± 0.000	0.011 ± 0.000	0.008 ± 0.000	0.010 ± 0.001	0.010 ± 0.000
14	0.015 ± 0.001	0.013 ± 0.001	0.013 ± 0.000	0.018 ± 0.001	0.013 ± 0.001	0.015 ± 0.002	0.011 ± 0.000
17	0.019 ± 0.001	0.016 ± 0.001	0.017 ± 0.000	0.020 ± 0.001	0.020 ± 0.002	0.019 ± 0.001	0.012 ± 0.000
20	0.032 ± 0.002	0.024 ± 0.002	0.021 ± 0.000	0.034 ± 0.002	0.033 ± 0.004	0.024 ± 0.006	0.018 ± 0.000
23	0.051 ± 0.004	0.035 ± 0.004	0.023 ± 0.000	0.039 ± 0.003	0.042 ± 0.007	0.026 ± 0.003	0.015 ± 0.000
26	0.079 ± 0.008	0.057 ± 0.003	0.030 ± 0.000	0.072 ± 0.005	0.066 ± 0.008	0.043 ± 0.016	0.034 ± 0.000
29	0.121 ± 0.013	0.084 ± 0.009	0.028 ± 0.000	0.082 ± 0.010	0.086 ± 0.013	0.050 ± 0.001	0.036 ± 0.000
32	0.182 ± 0.010	0.120 ± 0.007	0.036 ± 0.000	0.130 ± 0.013	0.125 ± 0.016	0.070 ± 0.019	0.053 ± 0.000
35	0.218 ± 0.010	0.154 ± 0.007	0.063 ± 0.000	0.154 ± 0.009	0.149 ± 0.015	0.093 ± 0.004	0.048 ± 0.000
38	0.287 ± 0.019	0.230 ± 0.019	0.062 ± 0.000	0.161 ± 0.014	0.175 ± 0.023	0.128 ± 0.031	0.085 ± 0.000
41	0.318 ± 0.018	0.254 ± 0.017	0.073 ± 0.000	0.166 ± 0.010	0.180 ± 0.021	0.162 ± 0.036	0.078 ± 0.000
44	0.331 ± 0.023	0.323 ± 0.023	0.104 ± 0.000	0.201 ± 0.019	0.217 ± 0.029	0.197 ± 0.043	0.142 ± 0.000
47	0.318 ± 0.030	0.351 ± 0.036	0.112 ± 0.000	0.224 ± 0.016	0.232 ± 0.019	0.230 ± 0.035	0.166 ± 0.000
50	0.258 ± 0.024	0.341 ± 0.030	0.188 ± 0.000	0.263 ± 0.032	0.240 ± 0.038	0.237 ± 0.067	0.149 ± 0.000
53	0.149 ± 0.006	0.329 ± 0.033	0.266 ± 0.000	0.250 ± 0.042	0.260 ± 0.031	0.246 ± 0.027	0.222 ± 0.000
56	(55.3 ± 0.67)	0.172 ± 0.013	(died day 72)	¶ = 0	0.210 ± 0.022	0.270 ± 0.010	0.245 ± 0.000
59	¶ = 3	(61.3 ± 2.65)			(57.0 ± 0.00)	(died=day 65)	0.229 ± 0.000
		¶ = 5			¶ = 1		(died day 70)

APPENDIX VI

Mean larval weight ± SEM of fast-track larvae reared on peramine-free and peramine-treated artificial diet (Graph 10).

P-6 & P-7 = six and seven instar larvae reared on peramine-free diet
P+6PUP, P+6NOP & P+7 = pupating and non-pupating six and seven instar larvae reared on peramine-treated diet.
Mean age at pupation ± SEM is written in parentheses
¶ = Number of larvae that successfully pupated

LARVAL AGE (days)	MEAN LARVAL WEIGHT(g) ± SEM				
	P-6 (n = 15)	P-7 (n = 2)	P+6PUP (n = 3)	P+6NOP (n = 5)	P+7 (n = 8)
14	0.017 ± 0.001	0.014 ± 0.001	0.019 ± 0.001	0.013 ± 0.002	0.010 ± 0.001
17	0.038 ± 0.004	0.015 ± 0.001	0.032 ± 0.001	0.018 ± 0.003	0.015 ± 0.002
20	0.069 ± 0.005	0.027 ± 0.001	0.065 ± 0.001	0.046 ± 0.007	0.029 ± 0.003
23	0.166 ± 0.013	0.045 ± 0.003	0.165 ± 0.010	0.086 ± 0.018	0.058 ± 0.008
26	0.254 ± 0.019	0.090 ± 0.005	0.232 ± 0.003	0.155 ± 0.034	0.090 ± 0.009
29	0.430 ± 0.027	0.119 ± 0.001	0.417 ± 0.032	0.210 ± 0.022	0.152 ± 0.023
32	0.677 ± 0.047	0.214 ± 0.050	0.642 ± 0.066	0.292 ± 0.048	0.223 ± 0.025
35	0.581 ± 0.034	0.293 ± 0.003	0.543 ± 0.038	0.405 ± 0.085	0.291 ± 0.035
38	0.410 ± 0.043	0.376 ± 0.055	0.370 ± 0.034	0.541 ± 0.041	0.423 ± 0.054
41	0.318 ± 0.018	0.650 ± 0.026	0.281 ± 0.009	0.166 ± 0.010	0.180 ± 0.021
44	(39.9 ± 0.01)	0.527 ± 0.091	(41.7 ± 0.33)	0.533 ± 0.034	0.538 ± 0.051
47	¶ = 13	¶ = 0	¶ = 3	0.305 ± 0.051	0.426 ± 0.047
				¶ = 0	¶ = 0

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