The bacterial flora of lepidopterous larvae, with special reference to those of *Zygaena* (Fab.).

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by

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ABSTRACT

Analysis of the bacterial flora of Zygaena larvae from several colonies was made. Marked differences in midgut flora were consistently found in larvae from different colonies, even though the bacteria associated with the food plants were essentially the same. In all larvae certain bacteria on the food plant were eliminated in the midgut to produce the flora characteristic of that colony. The mechanism by which this selective inhibition occurred was investigated, and a system is described which produces in vitro the inhibition found in vivo. The significance of the bacterial flora of the larvae is discussed.

A system for the aseptic rearing of Zygaena larvae is described, which may be adapted for other phytophagous insects, also a method for the fluorescent labelled antibody tracing of cellular antigens in insect tissues.

During a population study of a colony of Zygaena filipendulae (L.) and Z. lonicerae (von Schev.) (Lepidoptera, Zygaenidae) it was found that microbial diseases caused only a low percentage of mortality among the larvae. Results indicated that in years of high larval densities, food shortage would be a factor tending to limit population size. In 1967, both populations suffered heavy mortality as a result of parasitism by Telenomus (Hymenoptera, Scelionidae).

First report is made of a polyhedrosis virus infection of the family Zygaenidae.

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INTRODUCTION

Steinhaus (1949), after extensive reviews of the literature, concluded that, at that time, "no large accurate qualitative analysis of the bacterial flora of the alimentary tracts of insects has been made". He drew attention to the considerable confusion as to the true identity of a large number of the bacteria described, and to the confused and often misleading nomenclature. In a later review, Bucher (1963) expressed the opinion that much of the early work in insect bacteriology was of little value, as most bacteria were inadequately described, and most workers assumed that their isolates were new species, assigning them either erroneously to the genus Bacillus, or to the genera Bacterium and Coccobacillus, which are no longer recognised as valid. It is only during the last twenty years that insect microbiologists have used adequate methods in bacterial systematics and identification. The greater part of this more recent work has been concerned with the microflora of dying insects, and relatively little is known of the normal flora. This imbalance of knowledge was realised by Brooks (1963), who gave as her primary reason for considering the microbiota of healthy insects "the valid assumption that a sound understanding of the abnormal is based on a knowledge of the normal". Similar sentiments were expressed by Stevenson (1966). Almost all the studies of the bacterial flora of healthy insects have been made on insects reared under laboratory conditions, and, with some exceptions, the few studies on field collections have been made with numerically small, single samples of insects. The present study attempted to identify the bacteria associated

with certain field populations of lepidopterous larvae, and to determine, if possible, the effect of the bacterial flora on the survival of the host.

POPULATION STUDY OF ZYGAENA FILIPENDULAE AND Z. LONICERAE.

Selection of population.

The selection of a population of Lepidoptera with which to work was determined by several considerations. The population should be relatively dense, so that some epizootic disease would be more likely to occur, be detected, and its effects measured.

Although there are always difficulties attendant on the estimation of an insect population in the field, the species chosen should not present major difficulties in this respect. Identification should be possible without the need for lengthly examination, and the taxonomy of the group should be well established. The behaviour of the insect is also important. Migratory insects or those with a subterranean stage would present sampling problems which would make their study impractical for the present work.

During the Autumn of 1965, and the Spring of 1966 many areas were sampled, and information sought from several University Departments in the South-East of England. Regional Officers of the National Agricultural Advisory Service were also consulted. In the late Spring of 1966 attention was drawn to an outbreak of the brown tail moth Euproctis chrysorrhoea (Huebner) on Canvey Island, Essex. When visited, this population was found to be extremely dense, the third and fourth instar larvae numbering between 100 and 200 per cubic metre of food plant. This species over-winters as the larva, the stage in which most diseases of Lepidoptera occur, also the larvae are gregarious, forming communal tents in which any disease would be more likely to become epizootic. The population was

centred on an extensive area of waste land, the larvae feeding on hawthorn Crataegus monogyna Jacq., and sloe Prunus spinosa L. It seemed, therefore, that this population met all the criteria previously mentioned, and consequently work commenced on this species in the Spring of 1966. Much of the groundwork of the surface sterilisation and aseptic dissection techniques later perfected for Zygaena species was done with E. chrysorrhoea.

During both field and laboratory work with E. chrysorrhoea, there developed an acute form of urticaria, which obliged the author to seek medical treatment. Although a course of antihistamine injections was received, the allergy grew increasingly more distressing, and the project was abandoned on medical advice in June, 1966. Results from this work are detailed in the Appendix.

On an earlier visit to Dungeness it had been noted that there was a dense population of burnet moth larvae Zygaena filipendulae (L.) and Z. lonicerae (von Schev.) on the strip of grassland alongside the sallow scrub on which small numbers of E.chrysorrhoea larvae were feeding. Also feeding on the sallow was a small population of larvae of the lackey moth Malacosoma neustria (L.). It was decided to make the Dungeness area the main study area, and to work primarily on the burnet larvae, because these were so numerous. The E. chrysorrhoea and M. neustria populations would be kept under observation for incidence of disease.

Description of Study Area and Experimental Plots.

The study area was on Dunge Beach, Kent, at latitude 50° 55' 30" N., and 0° 58' 00" East, and forms part of the Dungeness Nature Reserve of the Royal Society for the Protection of Birds. The author is grateful to the Warden for permission to work on the Reserve. The area is composed largely (98%) of shingle in "fulls" or ridges, the shingle originating from the cliffs and superficial deposits to the West of Hastings. A detailed account of the geology of the Dungeness area will be found in Drew (1864) and Osborne White (1928). Excavation of the shingle had made several lakes, and one of these served as the Western boundary of the grassland which formed the study area. The Eastern and Southern boundary was formed by dense scrub sallow, Salix atrocinerea Brot. The Northern boundary was less well defined, the grassland gradually dissappeared and here shingle predominated with only occasional scrub of broom, Sarothamnus scoparius (L.) Wimm. The area of grassland enclosed by the boundaries described was 420 metres long (North to South) and 30 to 50 metres wide (East to West). A discontinuous ridge of bare shingle, 2 to 5 metres wide, divided the study area lengthways into two approximately equal areas of grassland. To facilitate location and reference in any future distribution studies, a base-line running East to West was established at the extreme Northern end of the study area, and marked with small cairns built of the shingle. Further cairns were built at 50 metre intervals Southwards from the base-line along the Eastern and Western boundaries of the area.

South (1948), lists a variety of vetches and clovers as the food plants of the larvae of Zygaena loniceraе and Z. filipendulae. These plants differed in their distribution over the study area, which therefore could not be treated as a uniform habitat. Experimental plots were accordingly marked out in areas of different communities, differences in the proportions and numbers of Papilionaceous species being the deciding factors. Tansley's (1946) method of ranking plants according to their abundance was used. Each plot was 5 metres long (North to South) by 2 metres wide, and rectangular in shape. The four corners of each plot were marked by small cairns built of the shingle. The location of the plots, and occurrence of Papilionaceous plants within them are shown in Table 1.

Table 1.

Plot location and occurrence of Papilionaceous plants, Dungeness study area.

Plot No.	Metres S. of base-line	Papilionaceae	Frequency
1	0	Lotus corniculatus L.	Occ.
		Trifolium repens L.	Ra.
2	60	Lotus corniculatus L.	Dom.
		Trifolium repens L.	Ra.
3	120	Lotus corniculatus L.	Ab.
		Trifolium repens L.	Freq.
4	150	Lotus corniculatus L.	Ab.
		Lotus uliginosus Schk.	Ab.
		Trifolium repens L.	Freq.
5	180	Lotus corniculatus L.	Ab.
		Trifolium repens L.	Ab.
6	210	Lotus corniculatus L.	Ra.
		Trifolium repens L.	Freq.
7	270	Melilotus altissima Thuill.	Freq.
		Trifolium dubium Sibth.	Freq.
		Trifolium repens L.	Freq.
8	420	Lotus corniculatus L.	Ra.
		Trifolium repens L.	Ra.

Dom. - Dominant Occ. - Occasional Thuil. - Thuillier

Ab. - Abundant Ra. - Rare Schk. - Schkuhr

Freq. - Frequent L. - Linnaeus Sibth. - Sibthorp

Population Estimates.

Previous studies of the bacterial flora of healthy insects in the field have relied solely on laboratory identification of the bacteria in field-collected samples of insects. In no case has the population been estimated, or the changes in population size recorded during the sampling of insects for the bacteriological study. No attempt has been made to correlate changes in the bacterial flora with changes in the population, or indeed to investigate whether the flora has any affect on the host population at all. It was decided therefore to attempt to estimate the population of Zygaena at Dungeness, in addition to sampling it for study of the bacterial flora.

As mentioned earlier, the Zygaena population on the study area appeared very dense, amd a sampling routine which would give a reliable index of the population at an acceptable "cost" level was required. "Cost" level was particularly important because only a few hours per month would be spent on the study area. The sampling routines used were different for the different developmental stages of the moth, and were modified, as the result of experience, in the second season.

Estimation of Numbers of Cocoons.

When work commenced with Zygaena, most of the larvae at Dungeness had pupated. Because the larvae move to near the tops of the stems of grasses in order to pupate, the conspicuous yellowish cocoons are easily counted. The method of sampling was to count the cocoons in 56 quadrats of 1 sq. metre each, distributed over the entire study area in a regular grid

pattern. Each quadrat was separated from the next by 30 metres North to South and by 8 metres East to West. The results are shown in Table 2. These figures were low because many of the larvae had not yet pupated when the count was made. A second count of cocoons was made at the end of June, 1966, but by this time experimental plots had been established, and the counts made within the plots. A steel wire ring 40 cms. diameter was used as a standard area (= 0.125 sq. metres), and the ring cast so as to fall more or less randomly within the plot. Once the cocoons within the ring had been counted, the grass stem was bent over, thus avoiding the possibility of counting the same cocoons twice. As a further precaution against overlap, three plastic seed-markers were pushed into the shingle at equidistant points around the circumference of the ring. In this way even areas with no cocoons were identified as already counted.

No evidence of predation of cocoons was seen in either season at Dungeness, nor were any signs of pupal parasitism noted. (The situation at Thursley Common, Surrey is very different - the Zygaena cocoons there are prey to birds, possibly warblers, and host to the Tachinid parasite Phryxe.) Cocoons of Zygaena are persistent and remain as evidence of pupation, or at least maturation of the larva, even after attack by birds or emergence of parasites. Many persist for more than a year, but after this length of time they have weathered to a light grey colour, and were easily distinguished from the bright white or yellow cocoons of the current season. Cocoon counts recorded are for current cocoons only, cocoons of the previous year are not included.

The results are shown in Table 3. Cocoons were also counted in 1967, but in this season the numbers were smaller, and it was possible to count the whole of each experimental plot. The results are given in Table 4.

Table 2.

Numbers of cocoons of Zygaena spp. in 1 m^2 at Dungeness Study Area. 5.6.66.

Row interval 30 metres, column interval 8 metres

				Row Totals	Metres S. of Base-line
1	2	4	3	10	30
2	0	0	4	6	60
5	2	6	8	21	90
8	5	2	0	15	120
0	6	3	9	18	150
5	16	32	27	80	180
9	17	25	45	96	210
5	24	12	15	56	240
0	4	7	11	22	270
0	1	2	0	3	300
0	0	0	2	2	330
2	4	0	11	17	360
4	2	10	16	32	390
12	14	3	0	29	420
Column Totals	53	97	106	151	407	

Table 3.

Numbers of Zygaena cocoons in 0.125 m^2, Dungeness Study Area. 30.6.66.

Quadrat No.	Plot 4	Plot 6	Plot 7	Plot 8	
1	2	4	4	4	
2	2	0	1	0	
3	4	3	2	0	
4	1	9	1	2	
5	2	4	4	2	
6	1	3	3	4	
7	0	0	0	1	
8	1	5	2	2	
9	2	6	2	4	
10	3	0	0	0	
11	5	6	0	4	
12	0	5	4	4	
13	1	2	4	0	
14	0	5	1	2	
15	0	6	0	1	
16	0	0	6	1	
17	0	18	0	7	
18	0	19	1	0	
19	0	2	3	2	
Total Cocoons	24	97	38	40	= 199

Percentage of cocoons emerged at time of count*

Plot 4	Plot 6	Plot 7	Plot 8
54	64	92	87

Percentage of total cocoons emerged = 72.

* See note at foot of Table 4.

Table 4.

Cocoons of Zygaena in experimental plots of 10 square metres each on Dungeness Study Area. 4.7.67.

Plot No.	Metres S. of baseline	Total Cocoons	Cocoons emerged*	% emerged
1	0	44	24	55
2	60	58	39	67
3	120	39	23	59
4	150	33	16	49
5	180	22	12	55
6	210	25	14	56
8	420	28	17	61
		249	145	58

* Cocoons emerged at time of count. These were noted separately so that adjustment could be made to counts of adults.

In laboratory conditions 97% of cocoons emerged successfully.

Estimation of Numbers of Imagines.

It was noted that the moths would not fly when the wind speed exceeded 15 knots (0.5 m/sec). On calm days, or on those when there was only a gentle breeze, the moths were too active to allow accurate counting. On days with a wind of over 15 knots the moths remained settled on the vegetation and would only move if touched, and even then there would be only a short flight of a few centimetres. Counts were therefore made on days with a wind speed of 16 - 18 knots. Wind speed is measured hourly at

the Dungeness lighthouse, 1.5 kms. South of the study area, and monitored continuously at Lydd Airport, 3 kms. to the North-east. Actual and expected wind speed were therefore easily checked by telephone before departing for the study area to do adult counts. Each plot counted was first divided into 10, 1 square metre quadrats with taut string, and the numbers of Z. lonicerae and Z. filipendulae recorded separately. The results are shown in Table 5. Cocoons were counted at the same time and the percentage of cocoons emerged noted. The "corrected" count is the number of imagines to be expected, assuming that the adult mortality remains the same and that there is 100% emergence of cocoons.

A marked difference in distribution of the two species was noted in 1967, when Z. filipendulae occurred in large numbers at the North of the study area, Z. lonicerae in large numbers at the centre, and with both species approximately in equal numbers at the Southern end.

Table 5.

Zygaena imagines in experimental plots (10 m^2) on Dungeness Study Area. 1966

Plot	% emerged at time of count	Zygaena lonicerae			Zygaena filipendulae			Total Zygaena		
		A*	B*	C*	A*	B*	C*	A*	B*	D*
4	54	54	100	50	55	102	50	109	202	39
6	64	69	108	56	55	86	44	124	194	37
7	92	23	25	38	38	41	62	61	66	13
8	87	28	32	55	23	26	45	51	58	11
		174	265	51	171	255	49	345	520	100

A* = adults counted B* = corrected for those not yet emerged C* = corrected % D* = % counted

Table 5 continued.

1967.

Plot	% emerged at time of count	Zygaena lonicerae			Zygaena filipendulae			Total Zygaena		
		A*	B*	C*	A*	B*	C*	A*	B*	D*
1	55	19	35	28	50	91	72	69	126	24
2	67	18	27	42	25	37	58	43	64	12
3	59	16	27	44	20	34	56	36	61	11
4	49	22	45	61	14	29	39	36	74	14
5	55	15	27	55	12	22	45	27	49	9
6	56	38	68	93	3	5	7	41	73	14
8	61	22	36	43	29	48	57	51	84	16
		150	265	50	153	266	50	303	531	100

A* = Adults counted B* = Corrected for those not yet emerged C* = Corrected % D* = % counted

Laboratory Emergence of Adults and Estimation of Fecundity.

A sample of cocoons was collected at approximately the mid-point of the study area in 1966. These were taken to the laboratory and allowed to emerge. Of 164 cocoons collected, 159 (97%) emerged successfully. The numbers of the two Zygaena species were in Zygaena filipendulae 77 (48%); Z. lonicerae 82 (52%). The sex ratio for both species was 1:1. Moths of each species mated readily in the laboratory, and the females laid eggs which developed normally. Pairs of Z. lonicerae and Z. filipendulae were caged separately in the laboratory, and given fresh flowers of ragged robin, Lychnis flos-cuculi L., daily for food. Ragged robin and vipers bugloss Echium vulgare L. were the main suppliers of nectar to the adult moths at Dungeness. Fecundity was estimated in the

laboratory by counting the number of eggs laid by known numbers of females.

Zygaena lonicerae

6 pairs produced 362 eggs. Average of 6 pairs = 60 eggs per female.

Zygaena filipendulae

12 pairs produced 1299 eggs. Average of 12 pairs = 108 eggs per female.

Williams 1948, dissected a newly emerged female Z. filipendulae and found that the moth contained 280 ripe eggs and 816 egg rudiments. Dissections of females of known age showed a reduction of egg rudiments from about 800 on emergence to about 300 at the 6th. day. If the reduction in egg rudiments is due entirely to their maturation and subsequent laying, then William's data would indicate that Z. filipendulae females lay approximately 800 eggs each. At Dungeness, the moths were found not to select the larval food plant for oviposition sites, therefore it may be expected that large numbers of eggs would be produced to offset the probablehigh larval mortality which would result. However, the number of eggs laid as indicated by Williams' egg rudiment reduction data is very much higher than the number of eggs counted in the laboratory and reported above. Direct comparison cannot be made because Williams does not give any direct information on the number of eggs laid. He does, however, give a graph of the daily weight of a female moth from emergence to death. the graph shows a continuous weight loss of approximately 12 mg. per day, with two sharp decreases in weight between the second and third day, and the fifth and sixth day, which Williams states is due to egg laying. It is possible to read from Williams' graph the weight of eggs laid on each of the two occasions. After allowing for the daily loss of 12 mg., the weight of eggs laid would appear to be 34 mg. and 31 mg. Thus the

800 eggs indicated by egg rudiment reduction data would have weighed 65 mg. It must be stressed that these figures are obtained from Williams' data by inference only, and are subject to unknown errors, in particular the possibility that the moth may have fed between egg laying and the next weighing. However, the figures do indicate that Zygaena eggs, on average, would weigh approximately 0.08 mg. As a check on this figure, newly-laid Z. filipendulae eggs were collected in the laboratory and weighed as rapidly as possible on a Cahn electrobalance. Ten eggs were weighed individually, the average weight of these was 0.224 mg. If this figure is divided into the weight-loss due to egg-laying reported by Williams, then the moth could not have laid more than 290 eggs, or 152 and 138 eggs per batch on the two occassions that eggs were laid. The single batch numbers obtained by this means are in reasonable agreement with batch numbers recorded from field-emerged females at Dungeness (60 - 140 eggs per batch) and from laboratory-reared females (108 per batch). The laying of a second batch of eggs would not have been detected in the field, and was not noted in the laboratory stock.

From these results it would seem that the reduction in egg rudiments recorded by Williams is not entirely due to their maturation and subsequent laying. Although such a reduction may provide an index of age - and it was for this purpose only that Williams made his investigation, - it would seem most unlikely that a direct, absolute measure of fecundity can be obtained by this means.

Oviposition Behaviour.

Slight differences of oviposition were noted between the two species. Z. lonicerae females laid eggs usually in neat rows to form a 'raft'. Occasionally eggs were found laid in a line. However, the eggs were always in a single layer. Z. filipendulae laid eggs in an untidy heap, often the eggs were three or four deep. Both species usually laid three or four batches of 20 to 35 eggs each, although females occasionally laid all their eggs in one batch. Z. lonicerae would always oviposit on the glass or plastic of the container, rather than on the assortment of plants provided, whilst Z. filipendulae would apparently use either the sides of the container or the plant as an oviposition site.

All attempts to produce hybrids, by either cross, failed. One pair in copula was caught in the field, of which the male was Z. filipendulae and the female Z. lonicerae. The female later laid three egg batches in the characteristic pattern of Z. lonicerae, and all hatched. The resulting larvae were weak and would not feed well. All died in or before the third instar. No microbial disease was found in any of the dead hybrid larvae.

In the field, Zygaena females appeared to select oviposition sites with respect only to their physical characteristics. The plants selected all had glabrous leaves which were large enough for the moths to hang beneath, and were usually of such a height that the moths were able to keep clear of the dense vegetation near the ground. Eggs were never found on the larval food-plant, Lotus corniculatus, which is decumbent in habit and has relatively small leaves. Erect plants with large, but hairy leaves such as Urtica diocia L. were apparently also avoided. Egg masses were most frequently found on Rubus fruticosus agg., and occasionally

on a variety of other plants, for example, Potentilla palustris L., Rumex acetosa L., and Holcus lanatus L.

Egg masses were usually oval in shape, 4 mm. to 10 mm. long, and contained 20 to 60 eggs. Eggs when recently laid were bright yellow in colour.

Estimation of Numbers of Eggs.

Incidence of Egg parasitism.

Egg counts were made using the 40 cm. diameter wire loop already described. Counts were made within the experimental plots, and precautions taken as before to avoid recounting any area. On each count, every leaf within the quadrat was examined carefully on both sides for egg masses of Zygaena. By 1967 enough experience had been gained to identify the egg masses reliably to species, and these were recorded separately. In both seasons the plant on which the eggs had been laid was also noted. The results are shown in Tables 6 and 7.

In 1967 egg batches were taken to the laboratory for the aseptic rearing experiments reported on page 78. Experiments were hindered because almost all the eggs were parasitised by Telenomus sp. (Hymenoptera, Scelionidae) and so the study area was revisited and a few eggs from each of many egg batches were collected. These were taken to the laboratory, and any damaged eggs discarded. There remained 200 eggs, and these were kept in polystyrene dishes with moist filter paper until they hatched. Only 10 Zygaena larvae hatched. All the remaining eggs contained Telenomus. This high level (95%) of parasitism probably

accounted for the failure to find Zygaena larvae on the study area later, even though much experience had been gained in locating and recognising them, and an area equivalent to eight times the original experimental plot areas was sampled. No egg parasites had been seen the previous season, when many hundreds of eggs, collected in the field, had been hatched in the laboratory in connection with the microbiological work.

Table 6.

Egg Counts, 1966.

Each count was of a 0.125 m^2 circular area randomly placed within the experimental plot.

Plot 4			Laid on	Total eggs
8 counts with 0 batches,	Numbers of eggs	0	-	0
1 " "	1 " " " "	36	Rubus sp.	36
1 " "	2 " " " "	15, 40	"	55
3 " "	3 " " " "	1,12,22,22,31, 40,50,60,80	"	318

Total Zygaena eggs in Plot 4 = 409

Plot 7			Laid on	Total eggs
9 counts with 0 batches.	Numbers of eggs	0	-	0
5 " "	1 " " " "	15,21,28,31,44	Rubus sp.	139
2 " "	2 " " " "	16,37,8,1	"	62
1 " "	3 " " " "	1,10,25	"	36

Total Zygaena eggs in Plot 7 = 237

Table 6 continued.

Plot 8										Laid on	Total eggs
15 counts with 0 batches. Numbers of eggs 0										-	0
1	"	"	1	"	"	"	"	17		Rubus sp.	17
1	"	"	1	"	"	"	"	40		Rumex acetosa	40

Total Zygaena eggs in Plot 8 = 57.

Table 7.

Egg counts 1967.

Each count is of a 0.125 m^2 circular area randomly placed within the experimental plot.

Plot 1

Z. filipendulae										Laid on	Total eggs
7 counts with 0 batches. Numbers of eggs 0										-	0
3	"	"	1	"	"	"	"	26,31,98		Rubus sp.	155
											155

Z. lonic erae

10 counts with 0 batches. Numbers of eggs 0 Total eggs 0

Total Zygaena eggs in Plot 1 = 155.

Plot 2

Z. filipendulae										Laid on	Total eggs
8 counts with 0 batches. Numbers of eggs 0										-	0
1	"	"	1	"	"	"	"	30		Rubus sp.	30
1	"	"	2	"	"	"	"	24,66		"	90
											120

Table 7 continued.

Z. lonicerae

10 counts with 0 batches. Number of eggs 0 Total eggs 0

Total Zygaena eggs in Plot 2 = 120

Plot 4

	Z. filipendulae			Laid on	Total eggs
7 counts with	0	batches, Numbers of eggs	0	-	0
1 "	"	1 "	" " "	50	Rubus sp.	50
2 "	"	2 "	" " "	13,29,37,51	"	130
						180

	Z. lonicerae			Laid on	Total eggs
7 counts with	0	batches. Numbers of eggs	0	-	0
1 "	"	1 "	" " "	93	Holcus lanatus	93
2 "	"	1 "	" " "	7,15	Rubus sp.	22
						115

Total Zygaena eggs in Plot 4 = 295

Plot 6

10 counts with 0 batches. Number of eggs 0.

Total Zygaena eggs in Plot 6 = 0

Plot 8

	Z. filipendulae			Laid on	Total eggs
2 counts with	0	batches. Numbers of eggs	0	-	0
8 "	"	1 "	" " "	17,21,23,26, 26,30,33,37	Rubus sp.	213
						213

Table 7 continued.

Z. lonicerae	Laid on	Total eggs
9 counts with 0 batches. Numbers of eggs 0	-	0
1 " " 1 " " " " " 20	Rubus sp.	20
		20

Total Zygaena eggs in Plot 8 = 233

Larval food choice experiment.

As noted earlier, females of both species of Zygaena usually chose bramble, Rubus fruticosus agg., on which to lay eggs. In the field, young larvae of both species fed greedily on Lotus corniculatus and Lotus uliginosus, although on the latter plants larvae of Z. lonicerae were most often seen. The young larvae must therefore migrate from the oviposition site to the food plant, and many were seen moving down the bramble stems or descending to the foliage beneath by means of a silk thread. The small larvae, approximately 1 mm. in length, must therefore migrate a relatively enormous distance before they are able to feed. The bramble was distributed apparently randomly over the entire study area, whereas the Papilionaceous plants were not. Also, larvae were only observed to feed on two of the five Papilionaceous plants commonly found. If survival of the young larvae was dependant to some degree on the distance to be travelled before arriving at a food-plant, it was necessary to know more precisely what the food-plants were. A laboratory experiment was therefore made to determine which of the five available Papilionaceous plants was acceptable as food to the larvae. The method was as follows,

the five plants tested were those occurring at Dungeness. Two polystyrene dishes, 9 cm. in diameter, were used for each of the five plant species tested. A filter paper disc was placed in the bottom of each dish, and moistened with distilled water. Fresh sprigs of the plant were added, and then five Z. lonicerae larvae were placed in the first dish, and five Z. filipendulae larvae in the second dish. The larvae used were 2nd. or 3rd. instar. To maintain food quality, the plants in the dishes were replaced with fresh sprigs of the same species each day. All sprigs used were chosen so that the leaf area available was approximately 15 sq. mm. The experiment lasted 28 days. Result - the two species of larvae showed no difference in feeding preferences. On Lotus corniculatus and L. uliginosus, the larvae fed greedily. Trifolium repens was also completely eaten by all larvae, but less readily, and only after 48 hours starvation, during which time the larvae crawled over the T. repens but did not feed. Melilotus altissima was also ignored for 48 hours, and even when the larvae began to feed, only a small quantity, less than 10%, was eaten. None of the larvae feed on Trifolium dubium.

The laboratory experiment confirmed the field observation that Zygaena larvae fed readily only on Lotus species at Dungeness. It would be expected, therefore, that young larvae would have less chance of survival in the plots where Lotus was less dense, and that the density of Melilotus and Trifolium would have little effect on larvae survival.

It is evident that before any quantitative conclusions may be reached on larvae survival, it is necessary to have more information on the

initial numbers. All attempts to count first instar larvae in the field failed. They were never found except very close to the oviposition site, and when among ground foliage their small size and excellent camouflage made them extremely difficult to see. All larvae counted were at least second instar, when they were much larger, (2 - 3 mm., weight approximately 10 mg.), and all had presumably successfully located a Lotus plant, and had fed. To obtain some indication of the initial numbers of larvae the egg counts would have to be relied upon. Laboratory experiments had shown that normally, all undamaged eggs hatch successfully, and that the incubation period was 5 - 6 days. It was relatively easy to count the numbers of emerged and non-emerged pupae, and adjust the egg count to allow for the additional adults yet to lay eggs, but the fundamental difficulty was obtaining sufficiently accurate counts of the eggs already laid. The adults emerged over a period of 2 - 3 weeks, so that eggs laid early in the season had already hatched. Even with most patient work, the count obtained was probably a gross underestimate of real numbers, because the eggs were so difficult to find. Once the eggs had hatched, all that remained were the tiny transparent egg-membranes, which must often have been missed. Comparison between different plots in a survey of this kind must always rely on the assumption that a constant proportion of the real numbers present will be recorded per 'unit of information' gathered. It is evident from what has gone before that the proportion of real numbers present represented by the egg-counts is probably very small. Moreover, it is highly likely that this proportion is far from

constant, owing to the difficulty of detecting hatched eggs, and the very different ratios of emerged to non-emerged pupae in the different plots at the time of the egg-count. Time in the field was severely limited, and as a result, only three experimental plots could be counted. The results of the egg count are shown in Tables 6 and 7. A comparison between these counts, the amount of Lotus present, and the numbers of larvae counted 2 weeks later is shown in Table 8.

Table 8

Larval survival of Zygaena in relation to Lotus abundance.

Plot	Tot. eggs counted[1]	Eggs/Quad.[2]	Lotus abund.[3]	Larvae/Quad.[4]	Larvae/Sweep
4	409	31	2 spp. Abundant	42	33
7	237	14	Absent	0	0
8	57	3	1 sp. Rare	0	2

[1] From Table 6

[2] Circular Quadrat 0.125 m^2 in area

[3] From Table 1

[4] Larvae were counted 14 days after egg counts

On Plot 4, where there was ample Lotus, and therefore the distance to be travelled by the larvae was comparatively short, an average of 31 eggs per quadrat was counted, and larvae counts made 14 days later averaged 42 larvae per quadrat. The discopancy is more likely due to underestimation of egg numbers, as mentioned earlier, than to immigration of larvae. Plot 7, on which an average of 14 eggs per quadrat was counted, yielded no larvae 14 days later. Lotus was absent from Plot 7, but there were

ample quantities of both species of Trifolium and of Melilotus. On Plot 8, where Lotus was rare, only 3 eggs were counted, on average, in each quadrat. Normal sampling failed to reveal any larvae 14 days later, but an average of two larvae per sweep were recovered by sweeping.

Counts of larvae were made until pupation the following June; the effect of the large numbers of larvae on the Lotus in Plot 4 will be described later. Sampling on Plots 7 and 8 continued, although no larvae were recorded until two months after hatching, when a single larva was recovered from Plot 7 and from Plot 8. Thereafter no larvae were found on these plots.

It is evident that no quantitative conclusions may be drawn regarding first instar mortality from the data obtained, since the egg counts on Plot 4 could only account for 74% of the second instar larvae known to be present 14 days later, and the counts on the other plots are unlikely to be more accurate. However, the data support the more general statement that first instar mortality was very high, probably approaching 100%, on Plots 7 and 8, and that the mortality was very much lower on Plot 4. This mortality could be correlated with availability of food plant, the hypothesis being that first instar mortality was a direct or indirect result of starvation.

Estimation of Numbers of Larvae.

All counts of larvae were made within the experimental plots described earlier. Each plot was divided into ten equal areas of one metre square by means of a temporary grid of taut string. Random numbers were taken

in pairs from Fisher and Yates' (1953) tables, and used as co-ordinates in dm. for the location of the sampling ring within each square. The sampling rings were made of 7/029" electric wire covered with black P.V.C. insulation. The joins were made using tight-fitting black neoprene sleeves. Each ring was 13 cm. diameter, enclosing an area of approximately 131 cm.^{2} The ten one metre squares of each experimental plot now each contained one randomly-located sampling ring. A further sampling ring, of the same size and construction as before was placed immediately adjacent to, and touching, each of the rings already on location, and on either the North, East, South or West side, in rotation. Each experimental plot thus finally contained 20 sampling rings. A plan of each plot was made on graph paper, showing the location of the rings, then the rings were hidden to avoid removal by vandals and the temporary grid of taut string removed. To avoid killing any larvae by trampling, the rings were located and larval counts made from a wooden platform, 1 m. long by 0.5 m. wide, supported on slender legs to give working height of 15 cm. The number of Zygaena larvae in each ring was recorded directly and with as little disturbance as possible, but when there was risk of counting errors, because of large numbers or very active larvae, the larvae were collected in a aspirator or 'pooter' during counting. After the count, the larvae were returned to their habitat in as near as possible the same distribution as when they were found. Each sampling ring was very carefully searched, and those in which the vegetation was relatively high were searched in strata, i.e. above 5 cm., 5 cm. to 2.5 cm. and 2.5 cm. to ground level.

Because the habitats were different (see Table 1) a fixed time scheme was not adopted owing to the inevitable differences in counting efficiency. Each sampling ring was searched until there was confidence that all the larvae present had been counted. Larger non-active larvae were not collected in the aspirator, which was only used to count active, or very large numbers of larvae when there was a risk of recount. On average, each ring required 10 minutes to count.

It was noted earlier that there was poor larval survival on plots 7 and 8, possibly due to absence of food-plant. Counts on these areas were abandoned in early October. From 16th. October 1966, the quantity of Lotus and of Trifolium was recorded for each sampling ring counted. The Lotus was estimated visually in "units", one unit being a 3" (7.5 cm.) shoot with all leaves present. One Trifolium "unit" was one leaf, complete with all three leaflets.

During the second week of November, 1966, a youth's camp was held very close to the study area. On return to the study area on 18th. November, it was found that a large bonfire had been burnt at one end of Plot 2, also the surrounding area had been severely damaged by trampling. Obviously, further larval counts were pointless on Plot 2, and were accordingly abandoned.

Exceptionally heavy rain-fall over South-east Kent during the second two weeks of November caused the River Rother to overflow its banks, flooding much of Walland and Romney Marshes. The study area was flooded to a depth of 10 - 15 cm. between the 28th. and 30th. of November, and

the water did not subside until about 22nd. of January, 1967. Temperatures during December 1966 and January 1967 fell as low as -6°C so that a layer of ice 20 - 25 mm. thick formed on the flood waters during late December and early January. Minimum temperatures were well below freezing point for the first eight days of January. On 9th. February, when work was resumed on the study area, a count of larvae on Plot 4 showed that during the flooding, the numbers had fallen from 337 to 83, a reduction of 74%. In an effort to increase the sample size, a new experimental plot was established 30 metres North of Plot 4, now designated Plot 3.

Plot 3 was 60 metres square, and twelve sampling rings identical with those already in use, were located within the plot, using random number co-ordinates as before. A second ring was placed immediately adjacent to each of those already located, and either North, East, South or West of it, in rotation. Thus 24 rings were located.

The larval counts made on 9th. February were of larvae still in their hibernation sites, - low down on grass stems and leaves, or on the shingle beneath the vegetation. The larvae of both Zygaena species overwinter as immobile third instar and are straw coloured, matching their background very closely. The next count was made on 15th. March, when most of the larvae had moulted, and were now pale green with rows of black spots. Also the larvae were actively moving among the top of the vegetation. By this time the Lotus had almost doubled in quantity, and the larvae were feeding continually. By the end of March, the Lotus had about doubled in quantity again, and was now at a maximum, and dominant on the experimental

Plots 3 and 4. The numbers of larvae remained the same as for the middle of March.

In the middle of April, larvae were more numerous on the plots, presumably as a result of immigration from surrounding areas with little Lotus. The Lotus on the Plots now showed signs of grazing by the Zygaena larvae, and as a result, the quantity of available food had fallen. Through May and June, the amount of Lotus on the plots continued to fall due to the grazing by the larvae, and by the middle of May large areas of the plots had been almost completely defoliated, only the stalks of Lotus remaining. Larval numbers fell gradually during this period, largely as a result of emigration, larvae were seen wandering over the shingle presumably in search of food. On Plot 4 a total of only 3 shoots of Lotus remained uneaten in the 20 sampling rings. In contrast to Lotus, the Trifolium continued to grow unchecked, so that by the 13th. June, it was more than twenty times the quantity of Lotus, yet the hungry larvae did not feed on it. These results are shown graphically in Figure 1. The results for Plot 4, for which most complete data are available, is shown in Table 9.

Figure 1. Numbers of Zygaena larvae and quantity of Lotus and Trifolium in 20, 13cms. diameter sampling rings on Plot 4, Dungeness.

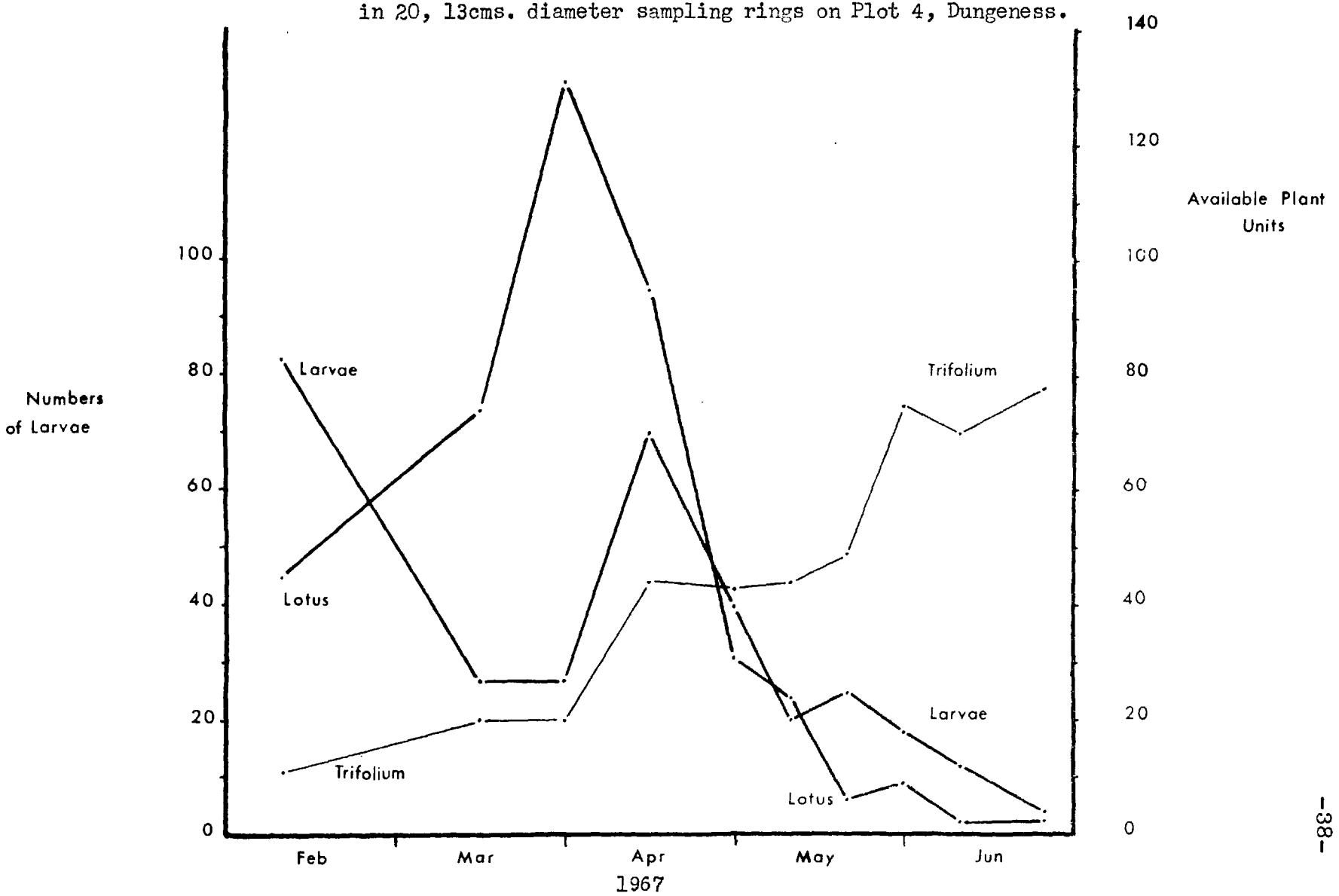


Table 9.

Summary of Larval counts, Plot 4, Dungeness study area 1966-67.

With the exception of column 1, all figures represent totals from 20 sampling rings, each 13 cm. diameter (131 cm^2).

Column	1	2	3	4	5
Date	Larvae counted	Larvae counted, corrected for ring size	Available Lotus units	Available Trifolium units	Larvae with Beauveria
6.10.66	239	?	?	?	9
16.10	195	331	15	24	9
1.11	338	338	27	15	1
18.11	314	338	10	1	0
29.11					
25.1.67	STUDY AREA FLOODED 50 - 52 DAYS				
9.2	83	83	45	11	0
		Larvae	Hibernating		
		Larvae	Active		
15.3	27	27	74	20	0
30.3	27	27	131	20	0
18.4	70	70	95	44	0
2.5	40	40	31	43	0
9.5	20	20	24	44	0
17.5	25	25	6	49	0
24.5	18	18	9	75	0
6.6	12	12	2	70	0
13.6	4	4	3	78	0

It was to be expected that Zygaena larvae would be aggregated in the field, as the eggs are laid in batches of several dozen each, and the larvae would tend to move to, and remain on Lotus plants. Calculations based on larval counts were made using the Topfit programme in the College computer. The fit of the data to the following theoretical dispersions was made:- Binomial, poisson, normal, double poisson, Neyman type A, negative binomial, truncated poisson, truncated Neyman type A, truncated negative binomial, and logarithmic.

During September, and through the hibernation period from October to February, the data showed a satisfactory fit to the negative binomial distribution, as tested by χ^2 and by the method of Lefkovitch (1966). When the larvae resumed their activities during the following Spring, the degree of aggregation, as indicated, for example, by Morisitas' Index (1962), began to fall off, and the distribution of larvae approached random.

Mortality of Zygaena at Dungeness Study Area.

The flooding of the study area during November, December, and January of 1966-67 caused a loss of 74% of the hibernating larvae. Even so there still remained enough larvae to cause selective elimination of the Lotus from the experimental plots by the following June. Many larvae must have died during the migration to find food, for many were found wandering well away from any source of Lotus. Lack of food probably also caused the death of many first-instar larvae, because the female moths select oviposition sites apparently by their physical characteristics,

and any close proximity to larval food-plant is incidental.

Hymenopterous parasites - were a major factor in causing Zygaena mortality. During March 1967, on two successive counts of larvae in the field, 2 out of 27 (approximately 7.5%) were parasitised by Apanteles zygaenarum Marshall. During the same month, of 40 larvae collected for microbial study, 4 (10%) were parasitised by Apanteles, and one contained the hyperparasite Mesochorus sp., probably M. temporalis (Thomson). During the Summer of 1967 most (95%) of Zygaena eggs at Dungeness were parasitised by Telenomus sp., and a population "crash" resulted, no larvae being found two weeks later, even after intensive sampling. A population "crash" was recorded by Lane (1961) in colonies of Z. lonicerae, but the cause was not discovered. As the mortality occured among half-grown larvae, Lane suggested that Apanteles or a virus disease might be possible causes.

Predation. Little predation of Zygaena was noted at Dungeness. The study area is on a main migration route for many birds, and large numbers pass through during each Spring and Autumn. In November 1966, a flock of 350 starlings was present on the study area for 10 - 12 days. They were observed to feed, apparently, on the experimental plots where the Zygaena larvae were hibernating, and an attempt was made to find out if the larvae were being eaten. The starlings could not be shot, because the study area was on a bird reserve, and so analysis of faecal samples for the dead capsules of Zygaena larvae was made. Fifty square metres, including

Plots 3 and 4, contained an average of 9 pellets of starling faeces per square metre. 100 faecal pellets were collected separately and taken to the laboratory, where they were shaken with water and examined microscopically for evidence of Zygaena remains. Much insect material was found, almost all of it coleopterous. Not only were elytra found, but also antennae, head capsules, and, occasionally, complete beetles. No bird predation of cocoons was found or suspected at Dungeness. No attempt was made to identify invertebrate predators, but several imagines were seen in the webs of orb spiders Aranea sp.

Fungi.

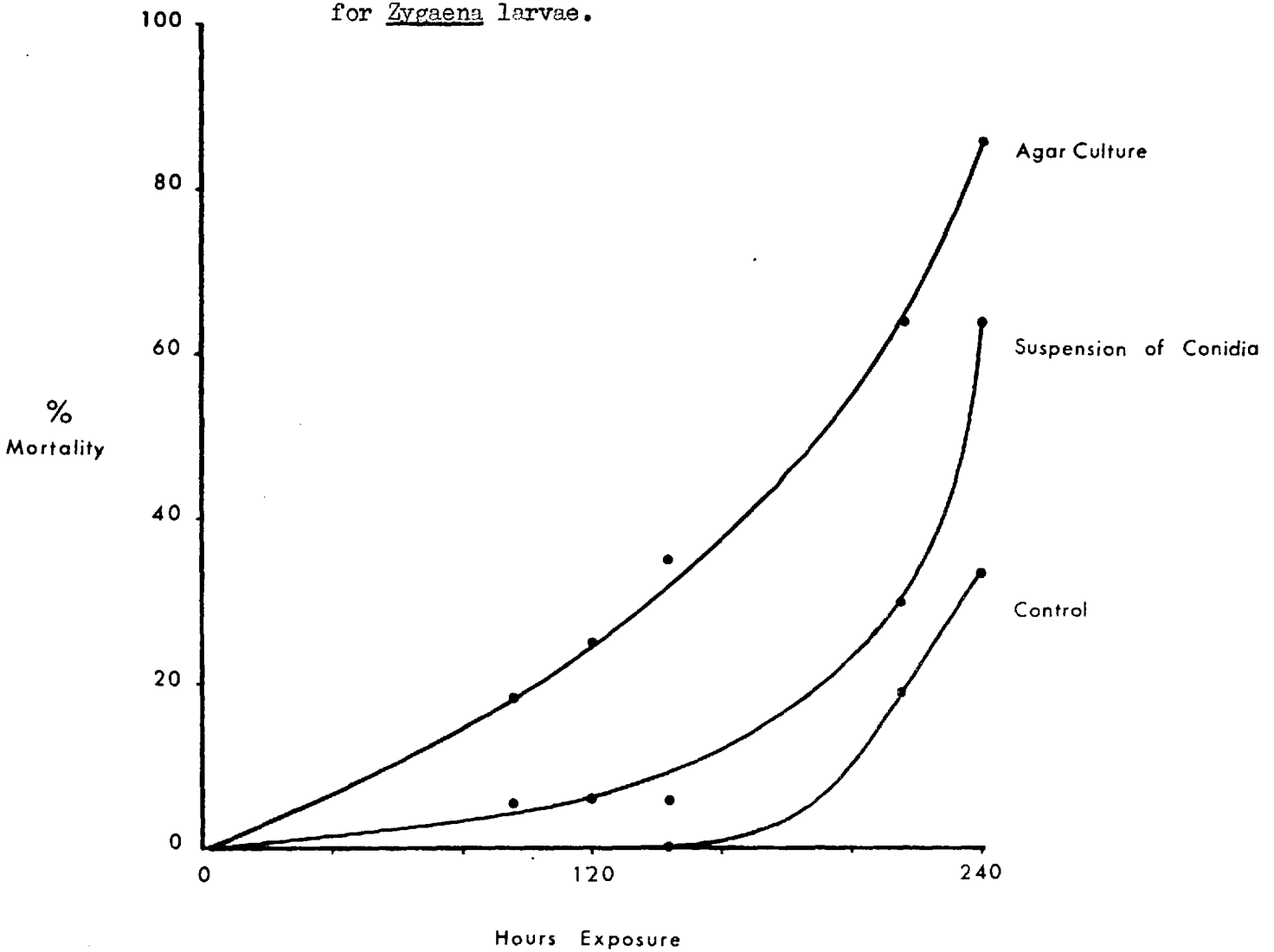
Beauveria bassiana (Balsamo) Vuillemin killed, apparently, 9 out of 239 larvae (4%) and 9 out of 195 larvae (5%) during the middle two weeks of October, 1966. Thereafter no Beauveria was seen in the field. Identification was made according to the key of Barnett (1955) and with reference to Madelin (1963). Identification was confirmed by comparison with a type specimen obtained from the Commonwealth Mycological Institute. Diseases caused by Beauveria were reviewed by Madelin (op. cit.). A pathogenicity test of Beauveria bassiana for Zygaena larvae was made in the laboratory. Three series of ten, 9 cm. diameter petri dishes each containing six third instar larvae were treated as follows:- First series - into each dish a segment of malt agar, 1cm. by 5 cm. long, bearing a heavy growth of fruiting Beauveria. Second series - a heavy suspension of Beauveria conidia in water was sprayed into each dish and momentarily on each larva.

Third series (controls) - each dish received a 1 cm. x 5 cm. segment of sterile malt agar, and was sprayed, including the larvae, with distilled water. After treatment the dishes were closed and kept at laboratory temperature (18° - 21°C). Lotus shoots were given as food.

Results - the whole fungus produced 86% mortality in 240 hours and is evidently highly pathogenic for Zygaena larvae. The conidial suspension appeared to show a lag in infection time, possibly the germination of the conidia was affected by the treatment. By 240 hours the conidial suspension had killed 64% of the test larvae. The control larvae began to die after 144 hours, mortality reaching 34% by 240 hours. These larvae died as a result of drowning in the droplets of water left after spraying, and this may have effected the delay noted in the death of larvae treated with the suspension of conidia, since mortality of these also remained low (6%) until after 144 hours. In contrast larvae exposed to the whole fungus showed a steadily increasing mortality. The results are shown graphically in Figure 2. These results are similar to those obtained by Brooks and Raun (1965), who found high pathogenicity of B. bassiana when tested against larvae of Ostrinia nubilialis (Hubner) (Pyralididae). These workers also made a field collection of dead insect pests of corn and found evidence of fungal infection in 14%. Many of the fungi isolated were found to be only saprophytic.

At Dungeness, Beauveria attacked only hibernating larvae, killing 4 - 5%. Beauveria infection of hibernating larvae was also found by Jaques and MacLellan (1965) in a study of overwintering codling moth,

Figure 2. Pathogenicity of preparations of Beauveria bassiana for Zygaena larvae.



Carpocapsa pomonella (L.) (Eucosmidae). These workers found from 4.1% to 0.5% mortality caused by the fungus.

No fungus, other than Beauveria bassiana was found as a primary pathogen in Zygaena larvae. It was noted, however, that the emergence wounds of Apanteles were rarely lethal, but that death of the host was usually caused by the infection which, in the field, inevitably followed. Zygaena larvae could be kept, in conditions of strict hygiene, for days or even weeks after the emergence of their Apanteles parasites, although all eventually died as a result of wound infections. In the field, the fungus most often encountered in infected wounds was Verticillium sp.

In chronological order, the factors causing mortality in the Zygaena population on the Dungeness study area were as follows:-

October 1966 - Beauveria bassiana (Balsamo) Vuillemin caused mortality of the hibernating larvae. Mortality due to this fungus was estimated twice, the percentages obtained were approximately 4% and approximately 5%. It was demonstrated in the laboratory that Beauveria was carried by the isopod Philoscia muscorum (Scopoli), which was common on the study area. No other microbial disease was found causing mortality of Zygaena larvae, in the field.

November 1966 - January 1967. Flooding of study area, 74% of the hibernating larvae killed.

March 1967 - Mortality of Zygaena larvae due to emergence wounds of Apanteles zygaenarum Marshall. Estimated twice - mortality figures were 7.5% and 10%.

July 1967 - Eggs of Zygaena parasitised by Telenomus sp. to produce 95% mortality. As a result, larval numbers in late July and early August could not be estimated, the numbers being too low to allow counting.

ISOLATION AND IDENTIFICATION OF THE BACTERIAL FLORA OF ZYGAENA LARVAE.

Methods of Collection.

During visits to Dungeness, a sample of larvae was collected from an area close to, but not on, experimental Plot 4. Collections were made at approximately monthly intervals during the Winter, when the larvae were in hibernation, and at weekly intervals during the Summer. Larvae from Thursley Common, Surrey, were collected at approximately monthly intervals; the sample from Cornwall and the Isles of Scilly was a single consignment

Collected larvae were placed in polythene bags, together with a few sprigs of Lotus corniculatus, and taken to the laboratory. Normally 12 larvae from each sample were taken at random from the polythene bags and used for bacteriological studies. With the exception of the sample from Cornwall and the Isles of Scilly, larvae for bacteriological study were processed within 18 hours of collection.

Surface Sterilisation Procedure.

It is evident that the surface sterilisation of the larvae is an essential pre-requisite to aseptic dissection. The result required in this case was to be able to isolate the bacterial flora of healthy larvae. An ideal surface sterilisation treatment would have three qualities. The treatment must be effective, reliably achieving surface sterility. The agent used must be non-persistent and easily removed from the larval integument so that there is no risk of ingress during dissection. The

treatment should not be lethal to larvae, thus avoiding post-mortem changes. Wittig (1963) reviewed the several treatments reported in the literature. All these, to give reliable results, were lethal to larvae. In addition many used persistent bacteriocides which even at great dilution were often bacteriostatic. Most treatments used ethyl alcohol as the bacteriocide, often followed by burning off the excess alcohol. Many insects will withstand immersion in ethyl alcohol, followed by rinsing in sterile water, but this treatment does not achieve surface sterility with any degree of reliability. Martignoni and Milstead (1960) also found ethyl alcohol an unsatisfactory agent for this purpose. Angus (1952), working with the eastern hemlock looper, found Hyamine 1622* an effective bacteriocide for surface sterilisation, and Martignoni and Milstead (1960) also found that this compound, with agitation, would give a consistently high proportion of surface sterilised larvae. The low concentration required, and the mild detergent action of the solution indicated that a non-lethal treatment, based on Hyamine, might be developed. Trials with field-collected third instar Zygaena larvae showed Hyamine to be non-toxic at a concentration of 0.2%, and effective as a surface sterilant in about half the larvae treated. Microscopic examination of the larvae immersed in the Hyamine solution showed many air-bubbles trapped against the body wall by the numerous tufts of setae. Evidently some increase in surfactant activity was necessary to wet the larvae thoroughly. Ethyl alcohol was finally chosen to pre-treat the larvae; its surface

*Hyamine 1622 is diisobutylphenoxyethoxyethyldimethylbenzyl ammonium chloride monohydrate.

tension is low, also it is moderately germicidal. Larvae immersed in 95% ethyl alcohol for two seconds and rapidly transferred to the Hyamine solution rarely had air trapped against the body wall or among the setae. immersion for two seconds in 95% ethyl alcohol, followed by agitation for five minutes in 0.2% Hyamine solution, and two, five minute rinses in sterile distilled water produced 80% of the test larvae surface sterile. All larvae were alive (responded to tactile stimuli) after this treatment. Many of the bacteria surviving this treatment were species of Bacillus, and were possibly present on the larvae as spores. Because larvae were processed to completion before the surface sterility tests could be read, it was desirable to reduce the contamination, and therefore rejection, rate as much as possible. The use of 1% sodium hypochlorite solution, after the ethyl alcohol and Hyamine, gave excellent results with a negligible contamination level. It was now necessary to remove all traces of sodium hypochlorite from the larva before it was dissected. This was achieved with a solution of 10% sodium thiosulphite. The final rinsing water gave no opacity when 0.2N silver nitrate solution was added, indicating the absence of chlorine. The surface sterilisation treatment was therefore standardised as follows:-

1. 95% ethyl alcohol - 2 seconds
2. 0.2% Hyamine 1622 - 5 minutes with agitation
3. 1% sodium hypochlorite - 5 minutes with agitation
4. sterile 10% sodium thiosulphite - 5 minutes with agitation
5, 6. sterile distilled water - 2 rinses of 5 minutes each, with agitation.

The treatment was carried out in 1 oz. McCartney vials, the larvae transferred from one vial to another using sterile forceps or sterile spatula. Agitation was standardised so that each vial received 8 - 10, 1 cm. strokes per second for five minutes.

Surface sterility was proved by gently pressing and rolling the treated larvae on plates of nutrient agar identical with those used for initial isolation of internal bacteria. The absence of visible colonies on the nutrient agar plates after incubation at 27.5°C for seven days was taken as proof of surface sterility.

To check that there was no carry-over of bacteriocidal or bacteriostatic substances into the dissection, the final rinse water was tested for inhibitory action with a wide range of bacteria. Both broth and plate- inhibition tests were made, the nutrient agar plates again being identical with those used for initial isolation. No inhibition, by either test, could be demonstrated using the following bacteria:- Pseudomonas eisenbergii Migula (= Ps. non-liquefaciens Begey et al.), Agrobacterium tumefaciens (Smith and Townsend) Conn., Escherichia coli (Migula) Castellani and Chalmers, Aerobacter cloacae (Jordan) Begey et al., Erwinia aroideae (Townsend) Holland, Proteus vulgaris Hauser, Staphylococcus epidermidis (Winslow and Winslow) Evans, Bacillus cereus var. mycoides Smith, Gordon and Clark.

Aseptic Dissection and Initial Isolation of Internal Bacterial Flora.

It is as important to determine the origin within the insect of any bacterium isolated as it is to know its identity. Thus Steinhaus (1941) and Stevenson (1966) made separate isolations for different regions of the alimentary canal and other origins. Unfortunately many other workers have limited the value of their work treating the insect body as a single bacterial habitat. These workers, for example Bucher and Stephens (1959), Port and Foster (1965), McWhirter and Scali (1966), McLaughlin et al. (1966) all triturated their insects so that a single homogenate was the source of all their isolates, from each insect. The results so gained provide valuable information on the identity of the bacteria present, but no information on their distribution. Most of these studies have been concerned with the occurrence of pathogenic bacteria in insects, where a knowledge of bacterial distribution is most desirable, since many potential pathogens must be in a particular environment within the host in order to cause disease. It was decided therefore to make separate isolations from the fore, mid and hind gut, also from the haemolymph. Each larva was transferred using flamed and cooled forceps from the nutrient agar plate serving as the check on surface sterility to a flame sterilised dissecting dish 3 cm. in diameter. Using instruments flame sterilised and cooled immediately before use, the larval integument was lifted to one side of the dorsal mid-line of the third or fourth abdominal segments, and a small incision made. Gentle pressure on the larva forced out a drop of haemolymph, which was collected in a platinum hoop and streaked out on a quarter of a divided nutrient agar plate. This was repeated in duplicate for each medium used. After sampling the haemolymph, the

larva was opened by longitudinal incision through the body wall towards the anterior and posterior ends. Great care was taken to avoid the gut wall, and any larvae showing, or suspected of having, damage to the gut wall were discarded. The gut, still attached at the oral and anal ends, was gently moved out so as to lie in an arc beside the larva. The fore, mid and hind gut are easily distinguishable, and a small incision was made in the gut wall at approximately the mid point of each region. Each incision was made with a separately sterilised scalpel to eliminate any possibility of transfer of bacteria from one region of the gut to another. For this reason also, the dissections were made 'dry' - that is, with no liquid in the dissection dish.

Samples of fore, mid and hind gut were placed on separate quadrats of a divided nutrient agar plate, and streaked out. As with the haemolymph, This was repeated in duplicate for each medium used.

The earlier isolations were made in triplicate, one set of plates being incubated aerobically, another in an atmosphere of hydrogen in a McIntosh and Fildes pattern anaerobic jar with Lucas indicator, and the third set in a carbon dioxide enriched atmosphere. Although many bacteria grew faster in the carbon dioxide enriched atmosphere, no greater number of isolates resulted from this set than from those plates incubated aerobically. In view of this, and the limited incubator space available, this method of culture was eventually abandoned.

The earlier isolations were made on three media, so that the maximum recovery of bacteria present could be made. The media were nutrient

agar (Oxoid No. 2), 10% horse blood, and chocolate agar. With the exception of the streptococci, which are fastidious in their growth requirements, (Tittsler et al., 1952), all bacteria grew well on nutrient agar. When brain heart infusion agar was used, all bacteria, including the streptococci, which were isolated on the three original media, grew well and usually produced visible colonies within 48 hours at 27.5°C. Consequently brain heart infusion agar alone was used for all primary isolations. Duplicate samples were streaked out on this medium and incubated aerobically and under hydrogen at 27.5°C. Plates were examined on each of seven successive days, by which time it was assumed that all bacteria present and able to grow would have formed visible colonies. All bacterial colonies having different appearances were isolated in pure culture on brain heart infusion agar slopes and stored at 4°C.

IDENTIFICATION OF BACTERIAL ISOLATES

Introduction.

The medium chosen for primary isolation of bacteria gave a high recovery rate of bacteria present in the specimen. That a single, isolated colony is in fact a mixture of species is much less likely on a non-selective medium such as the one used, compared with colonies growing on selective or inhibitory media. Characterization tests were only commenced on isolates known to be pure cultures. To minimise the possibility of selecting a mutant within the pure culture, all subcultures were made from a sweep of many colonies, or by collecting bacteria from many places on the maintenance slope.

Identification of the cultures was made following according to the scheme proposed by Cowan and Steel (1966). Isolates designated as within the genus Bacillus by this scheme were identified to species following the monograph of Smith, Gordon and Clark (1952). More specialised works were also consulted for the identification of Lactobacillaceae. The scheme adopted was essentially that of Sharpe, Fryer and Smith (1966), but there is no fully satisfactory key for streptococci. That major taxonomic work of Breed, Murray and Smith (1957) does not list all the Streptococcus species now recognised and must be considered obsolete with respect to the group. Reference to specialised papers on particular groups of streptococci will be made in the discussion of results.

Methods.

Primary characterisation

Each isolate was examined as follows:-

1. Gram reaction - Lillies (1928) modification was used.

2. Morphology - taken from the Gram stain, or from smears stained with Loefflers methylene blue.

3. Motility - a drop of an overnight culture grown in brain heart infusion with 0.3% glucose added was examined under phase-contrast. Motile strains were stained by Leiffsons (1951) method.

4. Growth in air - on Brain heart infusion agar.

5. Catalase - the organism was grown on a brain heart or nutrient agar slope, and 1 ml. of 3% hydrogen peroxide added. Bubbles within 5 minutes indicated a catalase positive organism.

6. Oxidase activity - Kovac's (1956) method was used, 0.1% ascorbic acid was added to the reagent to retard autoxidation (Steel, 1962).

7. Production of acid from glucose - 1% glucose in peptone water was used, with bromcresol purple as indicator. A yellow colouration (pH 5.0) was read as positive.

8. Oxidation - fermentation test. Hugh and Leifson's (1953) medium was used, the carbohydrate used was glucose.

9. Production of spores - detection of spores was by either examination under phase contrast of wet preparations, or by the use of Schaeffer and Fulton's (1933) spore stain. Heat-resistance tests (80°C for 10 minutes) were not used. Several strains of Bacillus were isolated which could not

be induced to produce spores. Such asporogenous strains are well known, but their inclusion in the genus Bacillus would, strictly, necessitate the demonstration of spore production. A few asporogenous strains were restored to the sporing state by subculturing on Lepper and Martin's (1929) modification of Robertson's cooked meat medium, with the vial sloped and the cap only loosely screwed on.

The nine tests described were sufficient to place each isolate within a group of genera, or in some cases into a single genus. Secondary tests made of each group allocated each isolate to a species or species-group. Certain secondary tests were of value in the identification of species in most of the genera found. These tests were accordingly made on all isolates, and are as follows:

Methyl red test- each isolate was grown in glucose-phosphate medium at 30°C for 5 days, and two drops added of 0.04% w/v methyl red solution in 40% ethanol.

Voges-Proskauer reaction - the method used was that of Baritt (1936), the reagents being added to the vial after completion of the methyl red test.

Reduction of nitrates - 0.1% potassium nitrate broth was inoculated and incubated at 32°C for 2 - 3 days. To each vial was then added 1 ml. of 0.8% sulphanilic acid in 5N acetic acid, followed by 1 ml. of 0.5% α-naphylamine in 5N acetic acid. A red colouration was taken to indicate the presence of nitrite. A sample of each batch of medium was tested for the absence of nitrite before inoculation. Powdered zinc was added to those vials not giving a positive reaction for nitrite. Absence of a red

colour after the addition of the zinc was taken to indicate that the organism had reduced the nitrite further.

Haemolysis - tests were made on plates of layered, 10% defibrinated horse blood agar. In accordance with modern convention, Brown's (1919) terms of description were used.

Litmus milk - this test was mainly included to compare results with those in the literature. The medium is complex, and the possibility that different syntheses may result in a similar appearance of the medium must be borne in mind.

Production of acid from mannitol, lactose, and arabinose, in each case, and for all carbohydrate studies, 1% of the carbohydrate in peptone water was used, with bromcresol purple as indicator. Durham's (1898) tubes were included to detect gas production.

Gelatin hydrolysis - stab cultures in nutrient gelatin were examined for liquefaction daily for four weeks after incubation at 22°C, also for failure to resolidify after incubation at 32°C.

Many additional biochemical tests were required within each genus or group of genera for the identification of species, and these are described, where necessary, in the following pages.

Identification of Streptococci.

All isolates with the following results from the primary characterisation tests were considered probable streptococci. Gram-positive, homofermentative cocci in pairs or chains, catalase negative, non-sporing. Facultatively anaerobic.

The following tests were made on each isolate.

Growth at 10°C, and at 45°C.

Growth at pH 9.6, and in 6.5% sodium chloride.

Growth in 0.1% methylene blue milk.

Survival at 60°C for 30 minutes.

Arginine hydrolysis - the medium of Niven et al (1942) was used, the production of ammonia was detected by Nessler's reagent. These tests, with the haemolysis test, from the basis of the criteria of Sherman (1937), which divided the streptococci into four groups, enterococcus, lactic, viridans, and pyogenic. Further tests were made as follows.

Hippurate hydrolysis - the method described by Hare and Colebrook (1934) was used.

Tellurite tolerance - indicated by black colonies on Skadhauge's (1950) glucose yeast extract agar containing 0.04% potassium tellurite.

Tetrazolium reduction - the solid medium described by Barnes (1956), containing triphenyltetrazolium chloride at pH 6.0, was used. Deep red colonies were recorded as positive.

Fermentation reactions - the following substrates were tested, their selection influenced by the keys of Sharpe, Fryer and Smith (1966) and Deibel, Lake and Niven (1963), - arabinose, glucose, lactose, maltose melibose, melezitose, raffinose, glycerol, mannitol, and sorbitol.

In addition to the physiological tests described, streptococci were also grouped serologically.

Serological Grouping of Streptococci.

The grouping of streptococci by means of the precipitin reaction between an extract of the streptococcus and group specific antisera is now a standard procedure. Antisera of proven specificity are available, but the value of the precipitin reaction as a diagnotic aid is dependent on the degree of cross-reaction which may occur. The method of extraction is not standardised, and it was necessary to select the method which would yield extracts showing fewest cross-reactions between sera This was particularly important because physiological tests indicated that most streptococcus isolates were in the enterococcus group described by Sherman (1937), and would therefore probably fall in the serological group D. The grouping of these streptococci was reported by Williams (1958) and Cowan and Steel (1966) as being particularly difficult. Three methods of extraction have been described. Lancefield (1933) first differentiated streptococci serologically into a number of groups by means of extracts of the cells prepared with hot hydrochloric acid. Fuller (1938) described a more complex method of extraction using formamide as the solvent. The formamide extracts gave fewer cross-reactions than those obtained by Lancefield's method, but Fuller did not test group D streptococci by his routine method, and large-scale preparations of group D extracts gave poor results, due largely to unsatisfactory antiserum. A third method was described by Maxted (1948) using a lytic enzyme from Streptomyces albus. This method, although satisfactory for groups A, C, and G, could not be used because some strains of group D resist the lytic action of the enzyme.

Consequently all the streptococcus isolates were extracted by the formamide method only, as this would give the most reliable results. This conclusion is in agreement with Williams (1958). Formamide extracts were set up against 'Wellcome' group-specific antisera of groups A, B, C, D, F, N, and H, in capillary pipettes for the precipitin ring test, using the automatic multiple dispenser described by Weitz (1957).

Identification of Bacillus species.

Isolates found in the primary characterisation tests to be rod-shaped bacteria, Gram-positive in young cultures, catalase positive, aerobic or facultatively anaerobic, typically motile, and to produce spores were designated as Bacillus species.

Further identification was made following exactly the key of Smith Gordon and Clark (1952). The authors give full details of the media and methods used; these details will not be repeated here.

Identification of the Micrococcaceae.

Isolates placed in this family had the following characteristics. Gram-positive cocii in pairs or clusters, catalase-positive, non-sporing, aerobic or facultatively anaerobic. Further identification was made following the key of Baird-Parker (1966). Phosphatase production was tested by the method of Barker and Kuper (1951).

Identification of Miscellaneous Bacteria.

Isolates not apparently falling into one of the families described earlier were identified according to the scheme proposed by Cowan and Steel (1966). For certain tests there are different methods described. For

these tests the methods used are described below.

Indole production - each isolate was grown on peptone water for 72 hours at 32°C. 0.5 ml. of Kovac's (1928) reagent was then added to each vial. A red colouration in the reagent layer indicated the presence of indole.

Citrate utilisation - Koser's (1923) medium was used, with Simmons (1926) modification.

Urease activity - was tested using the medium of Christensen (1946).

RESULTS

The haemolymph was in almost every case sterile. Isolates from the alimentary canal were made from the fore, mid and hind gut during the earlier part of this work, but it was found that the fore-gut flora was essentially that of the incoming food, which had already been analysed. It was decided therefore to concentrate on the mid-gut flora, since in this region the observed selection of bacteria occurred. Consequently all isolates described are from the larval mid-gut.

The identification, number and source of bacterial isolates from Zygaena larvae are summarised in the following tables.

D denotes Dungeness larvae.

W denotes Western larvae - from Cornwall and the Isles of Scilly.

T denotes larvae from Thursley Common.

Notes on Nomenclature.

The Micrococcaceae was divided into the genera Staphylococcus and Micrococcus according to the method proposed by the International Subcommittee on Staphylococci and Micrococci (1965). Further classification was made into subgroups according to the scheme proposed by Baird-Parker (1966).

A satisfactory scheme for classifying the Streptococceae is still emerging. The species Streptococcus faecium was described by Orla-Jensen (1919), was discounted by Sherman (1937) and restated as valid by Gunsalus (1947). Further evidence from Skadhauge (1950), Shattock (1955) and Barnes (1956) also supported the distinction between S. faecalis and S. faecium. The distinguishing tests recommended by those authors were

included in the present study. The recognition of S.faecium as a species gives cause to examine the validity of the closely related species S. durans, described by Sherman and Wing (1937). In their recent classification, Sharpe, Fryer and Smith (1966) list S. durans as a separate species, although Deibel, Lake and Niven (1963) were of the opinion that S. durans would be more logically considered a variety of S. faecium. In the results presented here, S. durans and S. faecium are listed separately in the tables, but S. durans is combined with S. faecium in the synopsis, as are S. faecalis and its varieties.

For the remaining bacteria the names used are those given by Breed, Murray and Smith (1957), the most widely accepted taxonomic work. The key for identification of the Corynebacteriaceae given by Breed et al (1957) uses habitat relationships because comparative studies of groups within this family are lacking. The authors are, of course, well aware of this shortcoming (Breed et al. (1957) p. 579). The identification of species of Corynebacterium from new sources is therefore difficult. The specific names assigned are those which seemed most logical and gave the best fit to the published descriptions. Departure from the published description is appended to each species, where necessary.

Micrococcaceae

Group I - Staphylococcus Rosenbach

Subgroup (Baird-Parker, 1966)	Number of isolates
I	D2, W0, T0
II	0
III	D1, W0, T0
IV	D2, W0, T0
V	D3, W0, T0
VI	D13, W1, T0
	21 1 0

Group II - Micrococcus Cohn

Subgroup (Baird-Parker, 1966)	Number of isolates
1	0
2	0
3	0
4	0
5	D3, W1, T0
6	D2, W0, T0
7	D5, W1, T1
8	D1, W0, T0
	11 2 1

Lactobacillaceae - Streptococcus Rosenbach

All isolates tested gave positive reaction to Group D antisera only.

	Number of isolates
Streptococcus faecalis Andrewes and Horder	D29, WO, TO
S. faecalis var. zymogenes (MacCallum and Hastings)Mattick	D8, WO, TO
S. faecalis var. liquefaciens (Stenberg, emend. Orla-Jensen) Mattick	D1, WO, TO
S. faecium Orla-Jensen	D28, W2, TO
S. durans Sherman and Wing	D3, WO, TO
S. bovis Orla-Jensen, emend. Sherman	D3, WO, TO
	135 2 0

Two strains of S. faecalis were motile.

Lactobacillaceae - Lactobacillus Beijerinck

Lactobacillus casei (Orla-Jensen) Holland, one isolate.

L. plantarum (Orla-Jensen) Holland, three isolates.

All isolates of L. plantarum were from larvae from Dungeness, the isolate of L. casei was from the larva from St. Martins, Isles of Scilly.

Corynebacteriaceae

Corynebacterium xerosis Lehmann and Neumann. Differs from the description given in Breed et al (1957) in that there was a slightly alkaline reaction in litmus milk.

Corynebacterium bovis Bergey et al. As in Breed et al. (1957) but agar colonies were moist.

Corynebacterium humiferum Seliskar.

Corynebacterium michiganense (Erw. Smith) Jensen. Differed from the description given in Breed et al (1957) in that this isolate was more salt tolerant and grew in three per cent salt well, but poorly in five per cent.

There was a single isolate of each species, all from larvae from Dungeness.

Bacillaceae

	D.	W.	T.
Group 1			
Bacillus megaterium de Bary.	2	4	0
B. cereus Frankland and Frankland.	4	1	0
B. cereus var. mycoides Smith, Gordon and Clark.	0	0	2
B. licheniformis (Weigmann) Gibson.	1	1	0
B. subtilis Cohn, emend. Prazmowski.	3	1	0
B. pumilus Gottheil.	3.	0	0
B. coagulans Hammer.	1	0	0
B. lentus Gibson.	6	0	1
Group 2			
B. polymyxa (Prazmowski) Migula	2	0	0
B. circulans Gordon, emend. Ford.	4	2	0
B. alvei Cheshire and Cheyne.	1	0	1
B. laterosporus Laubach.	0	1	0
B. pulvifaciens Katznelson.	1	0	0
B. brevis (Flugge) Migula, emend, Ford.	1	3	0
	29	13	4

No Group 3 Bacilli isolated.

Miscellaneous bacteria

Achromobacteraceae - Alcaligenes Castellani and Chalmers

A. faecalis Castellani and Chalmers - one isolate from Dungeness.

Acinetobacter Brisou and Prevot

A. eurydice Brisou and Prevot - one isolate from Thursley.

A. delmarvae Brisou and Prevot - one isolate from Hayle, Cornwall.

Enterobacteriaceae - Hafnia Moller

H. alvei Moller - one isolate from Hayle, Cornwall.

Serratia Bizio

S. marcescens Bizio - two isolates, both achromogenic strains, one from St. Martins, Isles of Scilly, one from Hayle, Cornwall.

Proteus Hauser

Providence group A - two isolates, both from Hayle, Cornwall.

It has been reported - Barrett,(1897), South (1948) that Zygaena larvae may take two years to develop into adults. A few of these larvae were found at Dungeness - easily identified by their straw colour and hibernation position at the bottom of the vegetation. Twelve of these larvae were examined by the usual procedures and the isolated bacteria identified. The hibernating larvae contained micrococci, staphylococci and streptococci but no Bacillus species. This might have been due to the elimination of the vegetative Bacillus cells, following germination of spores.

Table 9.

Synopsis of isolates from Zygaena larvae.

	Dungeness			Cornwall			Thursley		
	a	b	c	a	b	c	a	b	c
Total examined	209	161	70	26	9	4	8	60	26
Micrococcus	11	9	6	2	1	11	1	1	1.7
Staphylococcus	21	16	10	1	1	11	0	0	0
Micrococcaceae totals	32	25	16	3	2	22	1	1	1.7
Streptococcus faecalis (+vars)	101	74	46	0	0	0	0	0	0
Streptococcus faecium + S. durans	31	19	12	2	2	22	0	0	0
S. faecalis + S. faecium	?	7	4						
Streptococcus bovis	3	2	1	0	0	0	0	0	0
S. bovis + S. faecalis	—	1	0.6						
Streptococceae totals	135	103	64	2	2	22	0	0	0
Bacillus species	29	15	9	13	5	55	4	4	6
Miscellaneous Gram-positive	8	8	5	1	1	11	0	0	0
Total Gram-positive	203	122	76	19	7	77	5	5	8
Miscellaneous Gram-negative	1	1	0.6	6	6	66	1	1	1.7
Bacteria	204	123	76	25	7	77	6	6	10
Yeasts	5	5	3	1	1	11	2	2	3
Sterile	-	38	24	-	2	22	-	52	87

a = Number of isolates b = Number of larvae
c = % of larvae

THE BACTERIAL FLORA OF THE LEAVES OF LOTUS CORNICULATUS

Isolation technique.

In the field, shoots of L. corniculatus were clipped using flame-sterilised scissors, and placed in McCartney vials of brain heart infusion. At the laboratory, the leaf surfaces were scrubbed with a sterile platinum loop whilst still immersed in the infusion. After incubation for 12 hours at 27.5°C, the infusion was plated out on brain heart infusion agar. Isolations for anaerobes were made similarly on Lepper and Martin's (1929) modification of Robertsons cooked meat medium, and the plates incubated in an atmosphere of hydrogen in a McIntosh and Fildes pattern anaerobic jar. Ten aerobic and ten anaerobic samples were taken from both Dungeness and Thursley Common in May, when Zygaena larvae are actively feeding. All bacterial colonies growing on the plates after incubation at 27.5°C for seven days were examined carefully, and those appearing or suspected of being different were isolated on to brain heart infusion agar and stored at 4°C.

Identification of bacterial isolates.

The identification schedules used were those followed for the identification of the bacteria from Zygaena larvae. Time did not permit complete characterisation of the isolate of Pseudomonas, (Breed et al (1957) list 149 species), and this is therefore listed here as Pseudomonas sp. The rules of nomenclature adopted and the limitations of the classification used are as described for the isolates from Zygaena larvae.

All the species listed were isolated regularly from Lotus leaves, and are considered members of the normal epiphytic flora.

Bacteria isolated from the leaves of Lotus corniculatus at Dungeness.

Pseudomonadaceae

Aeromonas liquefaciens Beijerinck) Kluyver and von Niel

Enterobacteriaceae

Hafnia alvei Moller

Serratia marcescens Bizio - achromogenic strains only.

Aerobacter aerogenes (Kruse) Beijerinck

Micrococcaceae

Staphylococcus subgroup VI Baird-Parker

Lactobacillaceae

Streptococcus faecalis Andrewes and Horder

Streptococcus faecium Orla-Jensen

Bacillaceae

Bacillus cereus Frankland and Frankland

Bacillus brevis Migula, emend. Ford

Bacteria isolated from leaves of Lotus corniculatus at Thursley Common.

Pseudomonadaceae

Pseudomonas sp.

Aeromonas liquefaciens (Beijerinck)Kluyer and van Niel

Enterobacteriaceae

Aerobacter aerogenes (Kruse) Beijerinck

Lactobacillaceae

Streptococcus faecalis Andrewes and Horder

Lactobacillus casei (Orla-Jensen) Holland

Bacillaceae

Bacillus megaterium de Bary

Bacillus cereus Frankland and Frankland

Bacillus licheniformis (Weigmann) Chester, emend. Gibson.

FLUORESCENT ANTIBODY TRACING

The finding of streptococci in most larvae at Dungeness raised the question of their possible role in the physiology of the larvae. It was decided to attempt to rear larvae aseptically and then give known floras, and to investigate the possibility of finding a rapid, specific method of identifying streptococci from large samples of larvae. The fluorescent antibody technique is not only specific, it also provides information on numbers of bacteria present, and gives accurate location of the bacteria within the host. An attempt was therefore made to develop a fluorescent antibody technique suitable for use with insect tissue.

Fluorescent Antibody Tracing.

Preparation of Conjugate.

Strain Z6 of Streptococcus faecalis was grown on Oxoid brain heart infusion broth at 25°C for 72 hours. The cells were collected by centrifugation at 5000 r.p.m. for 20 minutes and rinsed three times in sterile Ringer's solution. The Ringers' solution was also used for the final suspension. The bacteria were killed by exposure to a temperature of 60°C for 1 hour. The suspension of bacteria was counted in a Helber chamber with Thoma ruling, and appropriate dilutions made with Ringers' solution so that the final cell count was approximately 10^8 cells per ml., the concentration recommended by Evans (1957). The final count (average of ten counts) was 1.2×10^8 cells per ml. The suspension was aseptically dispersed into sterile vials with serum caps and stored at 4°C. The vaccine was tested for sterility after dispensing and at intervals

during the immunisation schedule, by withdrawing 0.25 ml. with sterile hypodermic syringe and culturing the sample in 5 ml. of brain heart infusion. Because the immunisation schedule was to commence immediately, no preservative was added to the vaccine.

An adult healthy rabbit bled one week previously to provide normal, reference serum, was given the following immunisation schedule. No adjuvant was used.

Day 1. 0.58×10^8 cells (calculated by volume of standardised vaccine) intramuscular.

Day 3. 0.22×10^8 cells (" " " " " ") intravenous.

Day 5. 0.58×10^8 cells (" " " " " ") intravenous

Alternate days to Day 15, as for Day 5.

Samples of blood were taken from the rabbit at intervals, and the serum tested for antibody activity by the microscopic slide agglutination test. A satisfactory titer was indicated on Day 15, and a 25 ml. sample of blood was taken on Day 17, and the serum separated. This was either used immediately for preparation of the conjugate, or freeze-dried and sealed in 5 ml. ampoules. The method of conjugation used was broadly similar to that described by Holborow (1964).

The fresh or reconstituted antiserum was cooled in an icebath and an equal volume of an ice-cold saturated solution of ammonium sulphate

was added. The mixture was stirred thoroughly, and then allowed to stand for 1 hour at 0°C, after which it was centrifuged at 0°C, 4,200 r.p.m. for 45 minutes. The precipitated globulin was redissolved in a minimum volume of buffered physiological saline at pH7.0, and dialysed with magnetic stirring overnight at 4°C against several changes of the buffered saline. The dialysed globulin was adjusted to pH 9.0 by the addition of carbonate- bicarbonate buffer, and the fluorochrome added. Fluorescein isothiocyanate was added at the rate of 3 mg. fluorochrome per ml. of serum; lissamine rhodamine B (RB 200) was added at the rate of 0.1 ml. of the sulphonyl chloride (prepared according to Fothergill (1964)) per ml. of serum. The conjugation was made at 0°C, and with constant stirring for 1 hour with RB 200, and overnight with fluorescein isothiocyanate. The labelled proteins were separated from unreacted fluorochrome and fluorescent compounds not attached to protein by passing the conjugate through a gel filtration column 20 cm. x 1.5 cm. diameter of cross-linked dextron (Sephadex G-25, water regain 2.4), at a flow rate of 60 ml. per hour. Buffered saline at pH 7.0 was used as the eluant, and the fast moving band collected for use. The purified conjugate was stored at 4°C.

Sectioning of Zygaena larvae.

It was decided to attempt to cut frozen sections of the larvae because this method avoided any drastic chemical change and would therefore alter least any antigenic properties of bacteria in the sections. It is known (Smith, 1961) that glycerol has a protective effect on frozen tissues, reducing the degree of cell disruption which occurs on thawing. Third

instar larvae were quickly anaesthetised with carbon dioxide and then immersed in 24% aqueous glycerol at 0°C overnight. Other larvae were immersed in the glycerol solution for only two hours, also at 0°C

A Peltier Effect wooled microtome stage was cooled with layers of distilled water until a bed of ice 2 mm. thick had been built up. Distilled water was then added to the edges of the ice with a fine pipette, so that a cup of ice was formed. The cup was lined with a 1 mm. thick layer of frozen 3% aqueous glycerol, then 6%, 12% and finally 24%. A gradient of glycerol concentration was thus obtained. The Zygaena larva was placed in the centre of the cup, surrounded by 24% glycerol, and then frozen.

Sections were cut on a rotary microtome, using a flat-ground, 17° knife blade, inclined towards the stage at an angle of 4°. The knife was cooled by means of a copper heat-sink, made from a flattened piece of water pipe bent through a right angle, and with the free end immersed in a vacuum flask of liquid nitrogen. Cut sections formed a "ribbon" if cut at 10μ, but only with difficulty if cut at 6μ. The critical factor seemed to be the speed at which the stage passed the knife. The ribbon of sections was handled with drinking straws kept cool by occasional immersion in the liquid nitrogen. The ribbons were carefully placed on to slides previously kept with powdered carbon dioxide in a vacuum flask, and the slide plus sections allowed to thaw slowly.

On microscopic examination of the sections it was found that a redistribution of gut contents had occurred. It was confirmed by

observation that this took place during the thawing process, when different areas of the section thawed at different rates. The resulting liquid flowed, often rapidly, from one part of the section to another and gut contents were frequently seen to be translocated in this way. It was found that rapid thawing, or thawing progressively from one end of the slide to the other would not avoid this difficulty, and so it was decided to use wax-embedded sections. These were prepared in the classical way.

The conjugate was found to be specific for streptococci only, other bacteria remained unstained.

The larvae of both species of Zygaena at Dungeness usually contained streptococci in the alimentary canal. The great reduction of the population as a result of egg parasitism by Telenomus made it impossible to collect Dungeness larvae for fluorescent antibody work. Larvae from Thursley Common were sectioned and the sections allowed to equilibrate at pH 8.0. The fluorescein isothiocyanate conjugate was added and the slides were incubated for one hour at 35°C. On examination with ultraviolet-blue illumination, it was discovered that the autofluorescence of the insect tissue was of the same wavelength as the emission of the conjugate. This difficulty was overcome by pretreating the sections with normal bovine serum conjugated with lissamine rhodamine B.

It was found that the larvae from Thursley Common, although containing many bacteria, did not contain any which reacted with the conjugate. Thus no streptococci were located in these larvae, a finding in accordance with those obtained by conventional bacteriology.

The method has been described here in the hope that it may be of value to others making similar investigations.

ASEPTIC REARING EXPERIMENTS

The occurrence in Zygaena larvae from Dungeness of Group D streptococci, and their absence in larvae from Thursley Common was reported earlier. In an attempt to elucidate the role played by these bacteria in the physiological processes of the host it decided to test the effect of a) no bacteria, and b) known bacterial floras on the development of the larvae. For these studies it was necessary to rear larvae aseptically. Bacteria probably could have been eliminated from the larval gut contents by appropriate chemotherapy, but the results obtained could have been subject to side-effects which such treatment might have produced. Earlier work with locusts (Pinnock unpublished) had shown that it was possible to surface sterilise insect eggs without causing major increase in mortality. Newly-emerged adults of both species of Zygaena readily mated in the laboratory, and the female moths could be easily induced to lay their eggs on polythene sheets placed in their cages. For surface sterilisation, the eggs could be easily prised away from the polythene sheet with a fine probe. A single layer of eggs, after exposure to the radiation from a Hanovia Chromatolite for two minutes at a distance of 9 cm. from the tube, still had viable bacteria adhering to them. A chemical method of surface sterilisation was therefore tried. The newly-laid eggs were placed into vials of 0.33% sodium hypochlorite, 5.5% sodium chloride solution and the vials rocked on a flask shaker for 5 minutes at 4, 1 cm. strokes per second. The sodium hypochlorite/sodium chloride solution was removed by suction using a sterile Pasteur pipette, and sterile, 10% sodium

thiosulphite solution added to the vial aseptically. The vial was gently agitated as before and then the sodium thiosulphite removed aseptically and replaced by sterile distilled water. After further gentle agitation a second rinse was given in sterile distilled water, also with agitation, and then the eggs were transfered using aseptic techniques to duplicate plates of brain heart infusion agar, 10% horse blood agar and chocolate agar. One set of plates was incubated aerobically at 27.5°C, the second set under hydrogen in a McIntosh and Fildes' pattern anaerobic jar, also at 27.5°C. Eggs thus treated were sterile, and yielded no bacteria when macerated and plated out as before. It was concluded that the method described was efficient for the surface sterilisation of Zygaena eggs, and that the eggs were normally sterile internally. Undamaged eggs, surface sterilised as described, hatched in 5 - 6 days.

A sterile diet was now required. Modifications of two published diets were tried, that of Ignoffo (1963) and of David and Gardiner (1965). In both diets the leaf stock used was of Lotus corniculatus, and in both diets the methyl-p-hydroxybenzoate and aureomycin were omitted, as these might have had adverse effects on the bacteria it was hoped later to incorporate. Thermostable ingredients were sterilised separately by autoclaving at 10 lbs/in^2 (115.5°C) for 20 minutes. Other ingredients were sterilised by filtration. Sterile leaf stock was obtained by surface sterilising fresh Lotus with 1% sodium hypochlorite solution, draining, neutralising the excess chlorine with sterile 10% sodium thiosulphite solution, draining again and finally rinsing three times in sterile

distilled water. Sterility checks were made on the final rinse water and on samples of the Lotus. The stainless steel container of a Waring Blendor was autoclaved at 15 16/in^2 (121°C) for 15 minutes, and then cooled by placing in a deep-freeze cabinet. The sterilised Lotus was added aseptically to the Blendor container, together with a small quantity of the final rinse water. The Lotus was macerated in the Blendor for ten minutes, and then the resulting homogenate was transfered to a sterile flask and vacuum-dried.

The sterile media were dispensed as slopes in 100 ml. Ehrlenmeyer flasks, and the flasks closed with cotton wool plugs covered with aluminium foil. The plugs would allow gaseous exchange and the aluminium foil could be adjusted to control water-loss due to evaporation. As they were prepared, 10 - 12 undamaged, surface-sterilised Zygaena eggs were added to each flask. The flasks were then placed in a Fison's climatic cabinet with the following programme:-

Environment A - 12 hours, full illumination, temperature 20 ± 2°C, relative humidity 90%.

Environment B - 12 hours, darkness, temperature 15 ± 2°C, relative humidity 90%. The relative humidity within the flasks was at all times close to 100%. The cabinets were maintained at high humidity to avoid excessive water loss from the flasks by evaporation.

Although marks on the media indicated that the newly hatched larvae had bitten into them, the larvae failed to maintain continual feeding, and eventually died, presumably of starvation.

Both media were again tried, but this time the quantity of Lotus leaf stock in each was doubled. Again the larvae failed to feed. The media were tried a third time, this time without the sterilisation procedures, and with the methyl-p-hydroxybenzoate incorporated to control fungal growth. As before, the larvae failed to maintain feeding. It was evident that considerable research would be required, for which there was not time, before a semisynthetic, sterilisable diet suitable for Zygaena larvae could be devised.

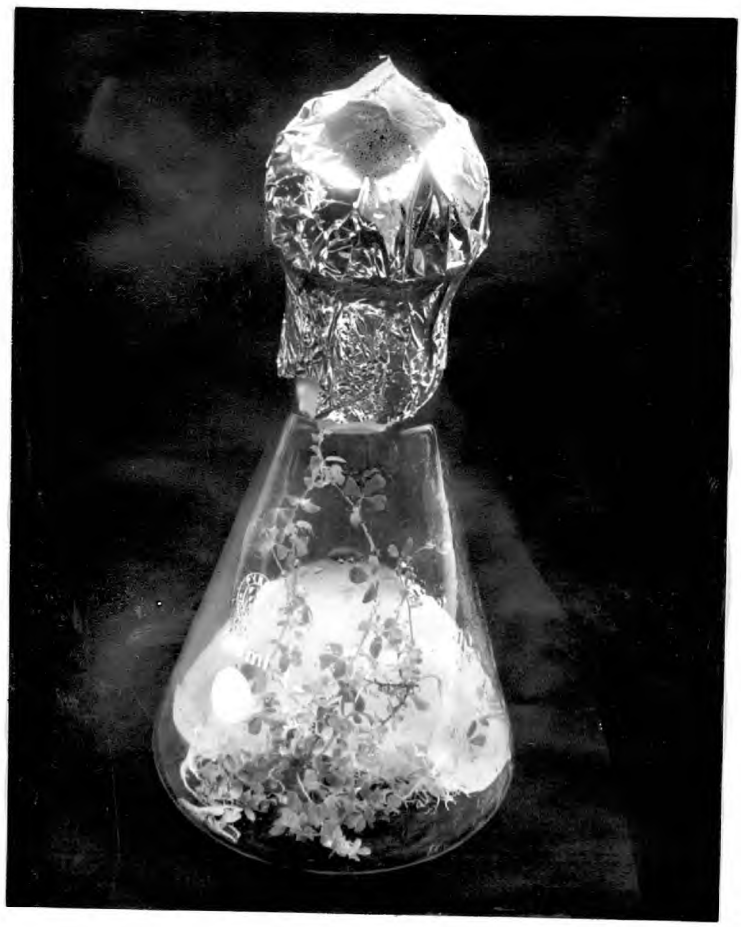
A fresh approach was required, and so it was decided to investigate the possibility of providing sterile, natural food for these larvae. To provide food through the Winter, Lotus would have to be grown in a glasshouse, because under natural conditions each plant "dies back" to a few tiny, dormant shoots which would provide little food, and require much labour to gather. The sterilisation of the Lotus was exceedingly laborious, so also were the aseptic handling and dispensing techniques which would be required. Lotus was relatively easy to grow, but difficult to sterilise and handle afterwards. An attempt was therefore made to grow Lotus corniculatus under aseptic conditions, for the seeds could probably be surface sterilised in much the same way as Zygaena eggs, and all that would be required was a suitable, sterilisable hydroponic solution. It was found that the Lotus seeds could be surface sterilised by vigorous agitation for 20 minutes in 1% sodium hypochlorite solution, followed by sterile 10% sodium thiosulphite solution for 20 minutes, also with vigorous agitation, and finally in two rinses of sterile, distilled water. The

seeds were checked for surface sterility by the same method as used for Zygaena eggs. The hydroponic solution used was that of Hoagland and Arnon (1950) to which 1.5% agar was added thus producing a hydroponic gel. The medium was dispersed into Ehrlenmeyer flasks, sterilised by autoclaving at 10 lbs./in^2 (115.5°C) for 20 minutes, and the flasks allowed to cool at an angle, thus producing a 'slope' of the gelled medium. Five or six sterile Lotus seeds were added to the medium in each flask, and the flasks were placed in the Fison's climatic cabinet with the same programme as before. Most (86%) of the seeds germinated, and almost all of these grew into vigorous Lotus plants (Plate 1).

It was now nearing the end of the egg-laying season for Zygaena. Great efforts were made to obtain as many eggs as possible, these were surface sterilised and checked for sterility as described, and introduced aseptically into the flasks containing the sterile Lotus plants. A few of the eggs did not hatch, but after five days 20 flasks contained a total of 160 first-instar larvae. The young larvae were very active and soon climbed the Lotus plants and began to feed. It appeared that an aseptic system had at last been achieved.

This success was short-lived, for the following weekend a refrigerant pipe on the climatic cabinet fractured, probably as a result of severe strain during installation, and so allowed the escape of the refrigerant from the light-box cooling system. The heat from the many lights soon caused the cabinet temperature to rise, and although the heaters were controlled by a high-temperature safety cut-out, the lights were not.

Plate 1.



Aseptic culture of Lotus corniculatus , growing on gelled Hoagland and Arnon medium. x 0.5

Consequently the cabinet temperature rose until equilibrium was reached. This temperature was almost 50°C, and was maintained for the two, 12-hour periods that the lights were on during the weekend. All the Zygaena larvae were killed.

Because no more Zygaena eggs were available, attention was turned again to the possibility of sterilising larvae. As explained earlier, the possible residual and side-effects of chemical treatments made this method undesirable, and so the possibility of using radiation was investigated. Long exposure to the ultra violet sources available, a Hanovia Chromatolite and a very high energy mercury vapour lamp (Osram HBO 200), proved irritant to the larvae and did not kill all the bacteria present in the gut. Larvae were also placed in the University of London reactor, as close to the core as temperature would permit, after the reactor had been running at full power. It was hoped that the radiation during decay would be sufficient to sterilise the larvae. Although this treatment killed vegetative bacteria, spores remained viable. The author is grateful to the Superintendent of the reactor for permission to conduct these trials, and to Mr. W. Alston for advice.

In view of these results, further work on the aseptic rearing of Zygaena was abandoned.

INHIBITION EXPERIMENTS

Comparison of the bacterial floras of the leaves of Lotus corniculatus and of the Zygaena larvae feeding on the Lotus showed that there was, apparently, selective inhibition within the larvae of the Gram-negative bacteria. (Families Pseudomonadaceae and Enterobacteriaceae.) Preliminary experiments were made in an attempt to discover the mechanism by which this apparent inhibition occurred.

The bacteria could possibly have been inhibited by :-

i. A chemical compound or mixture of compounds released from the Lotus by either the chewing or digestion process of the larva.

ii. Chemical compounds or enzymes released by the larva into the gut.

iii. Antibiotics or bacteriocines produced by antagonistic bacteria within the gut.

Lotus extract inhibition test.

Fresh shoots of Lotus corniculatus were washed three times in sterile distilled water with vigorous agitation, and then homogenised in a Waring Blendor. 5 gms. fresh weight of Lotus was homogenised in 25 ml. of sterile, distilled water. All these operations were done at 0° - 4°C.

Nutrient agar plates were seeded with the test bacteria, two plates for each bacterium tested. Each plate was then "ditched", that is, a diametrical strip of agar aseptically removed. The plates were now divided into two halves. One half was left as control, the other half of each plate was cut several times with a sterile 8 mm. diameter cork-borer, thus making 'wells' in the agar. Each well was filled with Lotus

extract, and the plates incubated at 27.5°C. Lotus from both Dungeness and Thursley Common was tested.

The bacteria tested were as follows:- Pseudomonas eisenbergii Migula, Aerobacter cloacae (Gordon) Bergey et al., Proteus vulgaris Hauser, Erwinia aroideae (Townsend) Holland.

No inhibition was noted for any of the strains tested, this would indicate that no inhibitory substances would be released from Lotus simply by the chewing of Zygaena larvae.

To test whether inhibitory substances were released within the larval gut, nutrient agar plates were seeded as before, but this time the gut of an activety-feeding Zygaena larvae was placed in the centre of each plate. The isolated gut was then slit longitudinally, so releasing its contents on to the seeded agar.

As before, no inhibition of any of the strains tested was noted. This would indicate that no inhibitory substances were released in the gut during digestion of the Lotus, although the gut contents were inevitably diluted in a test of this sort.

That inhibitory substances can occur in plant extracts or in insect gut contents was shown by Masera (1954) and Kushner and Harvey (1960).

It was reported by Jones et al. (1962) that Zygaena larvae feed on the strains of Lotus corniculatus which contain cyanogenic glucosides. These workers also reported the finding of hydrocyanic acid within the tissues of all stages of Zygaena filipendulae and Z. lonicerae. It is well known (e.g. Cowan and Steel, 1966) that certain species of bacteria in

the families Pseudomonadaceae and Enterobacteriaceae are inhibited by relatively low concentrations of cyanide. Inhibition tests were therefore made with various concentrations of cyanide, by the following methods.

Nutrient agar plates were seeded with the test strains of bacteria as before, and a central well and six peripheral wells 1 cm. in diameter were cut into the seeded agar with a sterilised cork-borer. Lotus extract was put into the central well, and various concentrations of cyanide into the peripheral wells, The wells were approximately 2 cm. apart. The concentrations of cyanide used, (as potassium cyanide solution) were:-1M x 10^{-2}, 1M x 10^{-3}, 1M x 10^{-4}, 1M x 10^{-5}, 1M x 10^{-6}, and 1M x 10^{-7}.

Parallel experiments were made, with the cyanide impregnated in the various concentrations on blank 'Oxoid' Multodiscs.

The bacteria tested were, with the exception of Ps. eisenbergii and A. cloacae the strains earlier isolated from the leaves of Lotus corniculatus:- Pseudomonas eisenbergii Migula, Aeromonas liquefaciens (Beijerinck) Kluyer and van Niel, Aerobacter aerogenes (Kruse) Beijerinck, Aerobacter cloacae (Gordon) Bergey et al., Hafnia alvei Moller, Serratia marcescens Bizio.

No cyanide inhibition was noted for any of the strains tested, either with, or without the presence of either Lotus extract.

Measurement of Gut pH of Zygaena larvae.

In vitro studies had shown no inhibition of Gram-negative bacteria by either Lotus extract, or cyanide concentration, or both. Several workers, for example Pringle Jameson (1922), Waterhouse (1949), Heimpel

(1955), Angus (1956) and Raun et al. (1966) had reported alkaline gut contents in Lepidopterous larvae. An attempt was made to measure the pH of the gut contents of Zygaena, so that in vitro studies could be made in conditions more closely approximating to those found in vivo.

Caldwell (1954) described the construction of micro-electrodes for pH determination which, modified, would have been suitable for this study. Due to lack of time, this method could not be adopted and chemical indicators were therefore used.

Method.

A glass capillary pipette was drawn to give an approximately constant bore of about 0.8 mm. diameter over a length of 3 cm. from the tip, and the tip flame-polished. The capillary was softened in the pilot flame of a bunsen burner and a small bulb was blown, about 4 mm. in diameter, and at about 1 cm. from the flame-polished end of the tube. Various indicator solutions were made up according to Cohen (1957) and a droplet of indicator solution was taken up into the glass bulb in the capillary pipette.

Zygaena larvae were anaesthetised with chloroform vapour and quickly transferred to a piece of filter paper, moistened with chloroform, on the stage of a dissecting microscope. The gut was exposed by a longitudinal incision through the body wall, and gently raised with a section-lifter. haemolymph was removed from the gut wall by means of an absorbent cotton-wool pad, and then a small incision was made to enable the tip of the capillary pipette to enter the lumen of the gut. A droplet of the gut

contents was taken up, and mixed with the indicator in the glass bulb of the pipette. The colour of the indicator was compared to that of controls of known pH. 30 larvae were tested, 10 each for foregut, midgut and hindgut pH. The pH of the haemolymph was also measured.

Result.

Average of 10 determinations = Foregut - pH 6.8
Midgut - pH 9.7
Hindgut - pH 7.2
Haemolymph - pH 6.8.

Bacterial Inhibition Tests using alkaline media.

To test the effect of alkaline conditions on the growth of bacteria from Lotus leaves, nutrient agar which was known to support growth at pH 7.2, was adjusted to pH 9.6 before pouring by the addition of sodium hydroxide. Each isolate from Lotus was streaked out and the plates incubated at 27.5°C.

Results.

It was found that Lactobacillus casei was completely inhibited, so also were one strain each of Bacillus cereus and Streptococcus faecium. All other isolates grew, although Bacillus brevis and one strain of Streptococcus faecalis grew poorly.

Oxidation-reduction potential of the larval midgut.

The high midgut pH of Zygaena larvae may have also acted in a selective manner on the bacterial flora due to its influence on the redox potential. Using Hutchingson's formula, at 18°C:-

$$Eh = 1.234 - 0.058\ pH + 0.0145 \log p\ Oxygen.$$

If an oxygen concentration is assumed of 20% saturation (probably a high estimate) then the reduction in Eh when the pH is raised from 7.0 to 9.6 is 151mV. If to this situation we add the further reduction in potential due to the metabolism of bacteria (Frobisher, 1963) it would seem likely that the conditions in the larvae midgut are reducing. The presence of Streptococcus faecalis and Streptococcus faecium would tend to lower the Eh. value even further, for these are strongly reducing organisms (Barnes, 1956).

Time did not permit direct measurements of the Eh value of the larval midgut, but to obtain some indication of the effect of a reduction in hydrogen acceptors on the bacteria isolated, inhibition plates were set up as before at pH 9.6, and incubated in an atmosphere of hydrogen. A McIntosh and Fildes pattern anaerobic jar was used.

Result.

Culture at pH 9.6 in the absence of gaseous oxygen as a hydrogen-acceptor resulted in the complete inhibition of the following bacteria:- Pseudomonas sp., Aeromonas liquefaciens (Beijerinck) Kluyver and van Niel, Serratia marcescens Bizio, Aerobacter aerogenes (Kruse) Beijerinck, Lactobacillus casei (Orla-Jensen) Holland.

The selective elimination of certain bacteria (the only exception was Hafnia alvei) from the flora found on Lotus to produce the flora found in the larval midgut was therefore possible, when certain environmental conditions were used. The high pH used was known to exist in the midgut, and it was most probable that reducing conditions also occurred.

Bacterial Antagonism.

It was possible that production of antibiotics by certain bacteria within the midgut of Zygaena larvae might also cause the selective elimination that had apparently occurred. Bacillus species, known to occur in the larval midgut, were also known to produce a variety of lysins (Satomura et al. (1957) and Richmond, 1959) polypeptide antibiotics (for examples see Umbreit (1962)) although these are more active against Gram-positive rather than Gram-negative organisms (Newton, 1956). In Dungeness larvae, a high incidence of enterococci was found. Work by Brock et al. (1963) has shown this group of organisms produce at least five types of bacteriocine.

It has been shown that environmental conditions might account for the elimination of Lotus bacteria not found in Zygaena, with the exception of Hafnia alvei. Plate inhibition tests were carried out in an attempt to show whether bacterial antagonism operated against any Lotus bacteria, and against H. alvei in particular.

Streak plate of Lotus bacteria or nutrient agar at pH 9.6 were counter-streaked with representative strains of Zygaena bacteria. Two sets of plates were inoculated, one set incubated aerobically, the other

under hydrogen, both at 27.5°C.

Result.

No increased inhibition, due to possible antibiotic substances was noted.

Discussion.

Possible causes of the selective elimination of bacteria in the larval midgut.

The bacteria found in Zygaena larvae must, under normal conditions, have been ingested during feeding, since the eggs were found to be laid free of internal bacteria. It was to be expected, therefore, that the larval flora would correspond closely with the Lotus flora, and this was found to be the case.

A wide range of bacteria was isolated from the leaves of Lotus corniculatus. Gibson et al. (1958) and Stout (1960) also found many types of bacteria in epiphytic floras. The occurrence of enterococci, found om Lotus, was once considered evidence of possible faecal contamination (e.g. Wilson and Miles, 1964) but work by Mundt et al. (1958) has shown that these organisms are widely distributed in nature and form part of the normal epiphytic flora of many plants, where they may multiply (Mundt et al. (1962). It is to be expected that enterococci, which can grow in media of very high pH (Sherman, 1937), would survive in the alkaline conditions of the larval midgut, or even multiply there (Bucher, 1963). Staphylococci are also able to grow in alkaline conditions, and, in general, Gram-positive organisms are more alkaline-tolerant than Gram-

negative (Umbreit, 1962). They are also less susceptible to proteolytic enzymes in general, and so would have selective advantage over Gram-negative organisms in the larval midgut. Bacillus species, in addition to the attributes of Gram-positive bacteria, also produce exceedingly resistant endospores.

The elimination of Gram-negative bacteria in the larval midgut has been shown to occur. The data presented indicate that the factors involved are:-

i. High pH. Certain bacteria were shown to be eliminated in in vitro experiments at pH 9.6. Wood (1961) has shown that certain bacterial enzymes are inactivated at high pH values, and that profound changes in carbohydrate and organic acid metabolism, with corresponding changes in end-products, occur under these conditions. All the organisms eliminated were in isolation; the lack of antagonism at pH 7.2 and at pH 9.6 would indicate that no increase in antimicrobial activity occurred in the surviving bacteria as a result of different metabolite production.

ii. Low redox-potential. The redox-potential in the larval midgut could not be measured, but it is known that the microflora consisted of many strongly-reducing bacteria. In vitro culture at pH 9.6 and under one atmosphere of hydrogen eliminated all Gram-negative bacteria except Hafnia alvei. It is not known how nearly these conditions approximate to those occurring in the gut, but the results agree closely with the situation as shown by analysis of isolates.

iii. Antibiosis. No antibiotic or lytic activity was recorded in in vitro experiments at either pH 7.2 or pH 9.6, in air or under hydrogen. Certainly antibiotics need not occur for the observed suppression of Gram- negative bacteria to take place in the larval midgut, but no proof has been advanced that antibiotics did not occur. Such proof would be most difficult to provide. All that may be said is that physical conditions likely to occur in the larval midgut can, in vitro, bring about the change observed in vivo.

These findings are not in agreement with the opinion of McWhirter and Scali (1966) who propose bacterial antagonism as the main factor in selection of gut bacteria in larvae of Maniola jurtina, but entirely fail to prove that any antagonism occurs. They also suggest that their approach is "a highly economic method of search for antagonistic systems". It is the writer's opinion that they should consider insect/bacteria antagonism as a possible explanation of the phenomena they observe, as any antagonism between bacteria would surely be secondary or dependent on this.

DISCUSSION

Significance of the midgut flora of Zygaena larvae.

Although the epiphytic microflora of Lotus corniculatus was similar at the two study area, marked differences were found when the bacterial floras of the midgut of Zygaena larvae from Dungeness and Thursley Common were compared. 90% of the larvae from Thursley Common were apparently free of any internal bacteria, there being no difference in the proportion of these 'sterile' larvae throughout the season. In contrast, at Dungeness only 24% of the larvae examined were sterile, the majority (70%) of these occurring during the hibernation period when it might be expected that the gut contents would tend to be self-sterilising due to the lack of bacterial inocula and/or fresh substrate. Since Lotus leaves were found to have many bacteria on them, the occurrence of large numbers of sterile larvae from Thursley is remarkable. Experiments indicated that the Gram-negative bacteria from the Lotus would be inhibited by the high pH and reducing conditions of the larval midgut, and thus the flora of Dungeness larvae could be largely explained. In vitro antagonism and inhibition tests failed to demonstrate any suppression of streptococci, yet these were entirely absent from Thursley larvae, although shown to occur on the Lotus there. Kushner and Harvey (1960) found antibacterial substances in extractions of leaves of trees, and showed that the pH of extraction influenced the antibacterial activity. No such activity could be shown in extracts of Lotus from either locality at pH 9.6, nor could any difference in midgut pH be demonstrated by the method used for larvae

from the two colonies. The absence of streptococci, and high incidence of sterile larvae from Thursley Common remains unexplained.

It is of interest to note that Masera (1954) reported finding that 43% of the larvae of a culture of Bombyx mori were sterile.

The following groups of bacteria were frequently isolated from the midgut of Zygaena larvae at Dungeness: Genus Streptococcus, the family Micrococcaceae, and the genera Bacillus and Corynebacterium. The significance and possible pathogenicity of each of these groups will be discussed in turn.

The occurrence of streptococci in healthy Dungeness larvae is in accord with the findings of several workers who consider these organisms normal inhabitants of the gut of phytophagous insects. Steinhaus (1941) isolated Streptococcus faecalis from the gut of larvae of Thyridopteryx ephemeraeformis (Haworth) (Psychidae) and Hyphantria cunea (Drury) Arctiidae). Enterococci were noted by Shyamala et al. (1960) and Masera (1954) in larvae of Bombyx mori L. (Bombycidae) and by Eaves and Mundt (1960) in larvae of Hyphantria cunea (Drury)(Arctiidae), Thyridopteryx ephemeraeformis (Haworth) (Psychidae) and Heliothis zea (Boddie) (Noctuidae). McLaughlin (1962), found enterococci the most common bacteria in larvae of Pseudaletia unipuncta (Haworth) (Noctuidae) when reared at 25.6°C. Larvae reared at higher temperatures had fewer enterococci. There was no correlation of the occurrence of enterococci with combinations of other bacteria. Bucher (1963) found that Streptococcus faecalis multiplied in the gut of Galleria mellonella (L.) (Galleriidae) during prepupal and pupal stages, from a small flora remaining in the larval

gut prior to spinning the cocoon. Streptococci have also been reported by Cosenza and Lewis (1965) from larvae of Porthetria dispar (L.) (Lymantriidae), by McWhirter and Scali (1966) from larvae of Maniola jurtina L. (Satyridae), and by Bucher (1967) from larvae of Protoparce sexta (Johanssen) and Protoparce quinquemaculata (Haworth), (Sphingidae).

Most work on the bacterial flora of lepidopterous larvae has been concerned with diseased insects. Often the investigators were apparently unaware of the common occurrence of streptococci in larval gut contents, and erroneously ascribe the disease they were studying to these organisms. The most notable of these diseases is 'gattine' of the silkworm, Bombyx mori. Bacteria very similar or identical with streptococci were isolated from diseased larvae by Pasteur (1870), Krassilschtschik (1896), Sawamura (1902), Sasaki (1910), and many others. Paillot (1930a, b) was the first to demonstrate that gattine was caused by a virus, the streptococci being secondary invaders.

Earlier workers commonly ascribed what are now known as virus diseases to bacteria. Other examples are silkworm "jaundice", "Wipfelkrankheit" of Lymantria monacha L. and "wilt" of Porthetria dispar (L.), all now known to be polyhedroses.

Considerable confusion and much synonymy occurred because most of the earlier works assigned new species to the isolates. An attempt to correlate his identifications with those of earlier Japanese workers was made by Pringle Jameson (1922), and Paillot (1928) found that the Streptococcus pastorianus of Krassilschtschik was identical with the

Streptococcus bombycis of Pasteur. The first identification of silkworm streptococci by modern techniques was made by Seelemann (1942) who identified Streptococcus bombycis as an enterococcus in the serological group D. A critical examination of six "species" placed by the early workers in three genera, was made by Lysenko (1958). He found that all of these were similar to Streptococcus faecalis or S. faecium. These streptococci were lethal to silkworms only in very large doses (probably 2×10^6) by intrahaemocoelic injection, or by feeding them leaves coated with a very heavy suspension of the bacteria. The huge doses necessary to produce disease would not be ingested by the silkworms under natural conditions, and predisposing factors would be necessary before the streptococci would cause overt disease. There was little difference in pathogenicity between streptococci isolated from diseased silkworms and those from non-insect sources. The common occurrence of Streptococcus faecalis and S. faecium in Zygaena larvae, and the complete absence of any bacterial disease, would indicate that the situation is the same in this case.

Some strains of Streptococcus faecalis isolated from Zygaena larvae were pigmented and motile. The production of pigment may be influenced by the presence of metal ions (Jones et al. 1963). Motile streptococci are regarded as bacteriological curiosities, although the writer suspects that they are more common than the literature would indicate. Motile streptococci were described by Flatzek (1919), Schieblich (1932), Pownall (1935), Koblmuller (1935), Sherman (1938), Levenson (1938), Auerbach and Felsenfeld (1948), Graudal (1952, 1957) and Cowan and Steel

(1966).

Consenza and Lewis (1965) claimed to have made the first isolation of motile streptococci from insects. Steinhaus (1946) refers to an earlier description of these organisms, isolated from larvae of Thaumatopoea pityocampa by Dufrenoy (1919). Stevenson (1966a, b) isolated motile streptococci from the alimentary canal of laboratory-reared locusts. They do not, prima facie, appear to be more pathogenic than non-motile strains.

The status of the Micrococcaceae as insect pathogens is uncertain. It is known (Cameron 1934; Briggs 1958) that large doses of micrococci and staphylococci will kill lepidopterous larvae when injected into the haemocoele. Steinhaus (1946) lists 12 "species" of Micrococcus isolated by several workers from lepidopterous larvae. The poor descriptions and erection of new species (e.g. Micrococcus galleriae from Galleria mellonella, M. pieridis from Pieris rapae) makes comparison with species now recognised virtually impossible. It seems possible that the micrococci isolated by the early workers were secondary invaders, and that some predisposing factor was overlooked. Thus the 'wilt' of larvae of Danaus plexippus (L.) (Danaidae) thought by Brown (1927) to be caused by "micrococcus flaccidifex danai" was probably a virus disease. Fungal disease also predisposes insect larvae to micrococcal infection (Vago 1959). Working with larvae of Bombyx mori, Cappelato and Narpozzi (1960) found that Micrococcus lysodeikticus Fleming and Sarcina flava de Bary were inhibited by haemolymph at a titer of 1:250. This would also seem

to indicate that predisposing factors are necessary before micrococcal infections can occur. These considerations, and the finding of many micrococci in healthy Zygaena larvae make it seem unlikely that these organisms are primarily pathogenic. This opinion is in agreement with Steinhaus (1949) and Bucher (1967).

Bacillus species were frequently found in Zygaena larvae, but as few vegetative cells of Bacilli were seen in stained preparations of midgut contents, it is likely that the bacteria occurred as spores, and that these germinated on the isolation media later. Certain Bacillus species, notably B. thuringiensis Berliner and its varieties, are highly pathogenic for lepidopterous larvae. B. cereus, found in Zygaena larvae, is reported to be pathogenic for a range of lepidopterous larvae by Heimpel and Angus (1963). A possible explanation of the apparent lack of disease in Zygaena larvae with B. cereus may be found in work by Heimpel (1955), who showed a correlation of pathogenicity of various strains of the bacteria with the production of phospholipase C. He also found that the optimum pH for phospholipase C activity was 6.6 to 7.4 and that at higher pH values the action of the enzyme was limited or inhibited. It would appear therefore that in Zygaena the highly alkaline (pH 9.7) midgut contents may prevent germination of some Bacillus spores, and cause any phospholipase that is produced to be inactivated. All the other Bacillus species found are considered to be saprophytes only, although B. alvei would seem to be a secondary invader in American Foulbrood disease of larval bees (Heimpel and Angus 1963).

Four species of Corynebacterium were found in Zygaena larvae. Little mention is made in the literature of the association of this genus with insects. Earlier work, reviewed by Steinhaus (1946), did show that lepidopterous larvae were apparently immune to moderate doses of C. diphtheriae (Flugge) Lehmann and Neumann. Only four isolates of Corynebacterium were found in the present study, its occurrence in Zygaena larvae may therefore be said to be rare. It was never found on Lotus leaves. In the absence of definitive work, the status of Corynebacterium species as insect pathogen s must remain doubtful.

The remaining bacteria isolated from Zygaena larvae are listed under "Miscellaneous Bacteria" (Table 9), very few isolates were made of any of these, with one exception all were regarded as non-pathogenic or of doubtful pathogenicity (Bucher 1967). The exception is Serratiamarcescens, found in two isolates only. Bucher (1963) lists many lepidopterous larvae which are reported to be susceptible to S. marcescens. Relatively massive doses (at least 10^5) must be fed to the larvae before disease is caused, the larvae not showing symptoms until the bacteria invade the haemolymph.

The absence of any disease in which bacteria would be implicated, and the information from the literature indicate that the bacteria isolated from the midgut of Zygaena larvae are harmless, the evidence being that they are fortuitous contaminants only.

It was intended to include in the present work an investigation into the possible beneficial effects on the larvae of their intestinal microflora. The destruction, due to an equipment fault, of the aseptic cultures of Zygaena larvae prevented further progress of this part of the

work. There are few references to investigations of this sort, due largely to the technical difficulties involved in achieving aseptic cultures (Dougherty 1959). Aseptic culture of Galleria mellonella was claimed by Rybicki (1952). She found that aseptic wax moth larvae could not assimilate beeswax, and that bacteria isolated from normal larvae could decompose the wax in vitro. Later work on G. mellonella by Waterhouse (1959) showed that aseptic larvae were able to digest some, but not all, beeswax constituents.

Shyamala et al. (1960) found that administration of chloromycetin to larvae of Bombyx mori resulted in an increase in assimilation of food, a faster growth rate and higher fecundity. They point out that these effects are not due entirely to the observed suppression of the intestinal microflora, as the growth stimulant p-nitrobenzaldehyde is formed as a degradation product of chloromycetin. However, their work does give the impression that the intestinal microflora is, to some extent, in competition with the host.

The presence of bacteria would not seem essential for the normal development of Zygaena larvae, since many were found sterile, although it is not known for what length of time the sterile larvae had been without internal bacteria. Masera (1954) found 43% of the examined larvae of Bombyx mori had sterile intestines, and considered that bacteria, when they occurred, were not important in the alimentary physiology. The data for Zygaena larvae would indicate that the midgut bacteria are fortuitous contaminants.

VIRUS DISEASE

The occurrence of overt nuclear polyhedrosis was noted in E. chrysorrhoea at Canvey Island, but not at Dungeness. No disease was noted in the Dungeness colony of Malocosoma neustria, but it was found that these larvae, when stressed by high temperature (28°C) all developed nuclear polyhedrosis. The virus was evidently within the population, but in a latent form. It was noted that the M. neustria colony was small (p.10). Wellington (1962) has shown the significance of population density and quality in the maintenance of nuclear polyhedrosis in Malacosoma pluviale (Dyar). At low densities even less vigorous broods of M. pluviale were less liable to develop overt disease. The lack of overt disease among the small M. neustria colony at Dungeness, and the finding of the latent virus by stress-induction would indicate a similar situation.

Zygaena larvae, collected in the field, were subjected to various stresses in an attempt to induce overt virus disease. In his review, Aruga (1963) refers to the effects of crowding and temperature extremes as possible factors in the induction of overt symptoms from latent infections. Crowding and various temperature changes were tried on groups of 30 third and fourth instar larvae.

Crowding.

The 30 larvae were placed in a plastic container approximately four times the total volume of the larvae.

Temperature.

A set of three plastic dishes, each 10.5 cm. diameter and 4.5 cm. high, and each containing ten larvae was given the following temperature changes:-

Set 1. 12 hours at 25°C, then 12 hours at 4°C. Repeated for five days.

Set 2. 12 hours at 30°C, then 12 hours at 4°C. Repeated for five days.

Set 3. 12 hours at 35°C, 12 hours at 4°C. Repeated for five days.

Set 4. (2 dishes only) - Control at room temperature, 20 - 21°C.

All dishes received several shoots of Lotus, so that it was possible for the larvae to feed. The Lotus shoots were replaced daily.

Result.

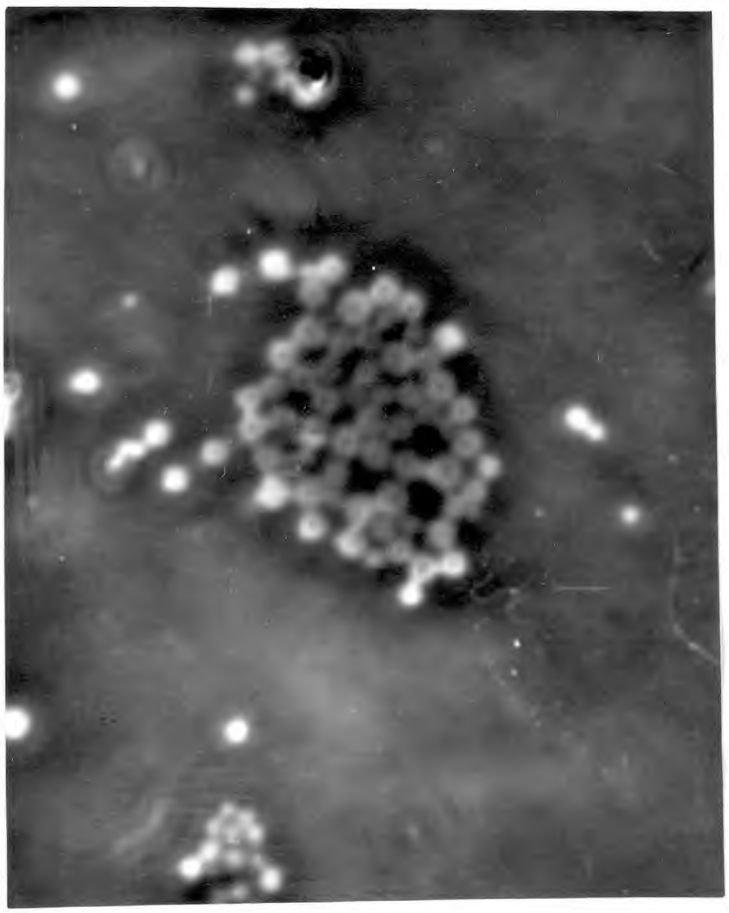
No mortality was observed in any of the test or control dishes. It was therefore concluded that the Zygaena population at Dungeness was free of easily-induced virus disease.

A possible nuclear polyhedrosis of Zygaena filipendulae.

It was usual, during aseptic dissection of larvae for bacteriological work, to make smears of various tissues and organs as a check on the isolation efficiency. It was noted that two, third instar larvae of Z. filipendulae appeared to contain low numbers of polyhedra in the haemolymph. Both larvae had appeared to be of normal appearance and behaviour. The slides were stained with Giemsa's solution according to the method of Smith (1967), and the polyhedra did not take up the stain, a characteristic of nuclear polyhedra. The polyhedra, photographed under anoptral phase contrast, are shown in Plate 2 .

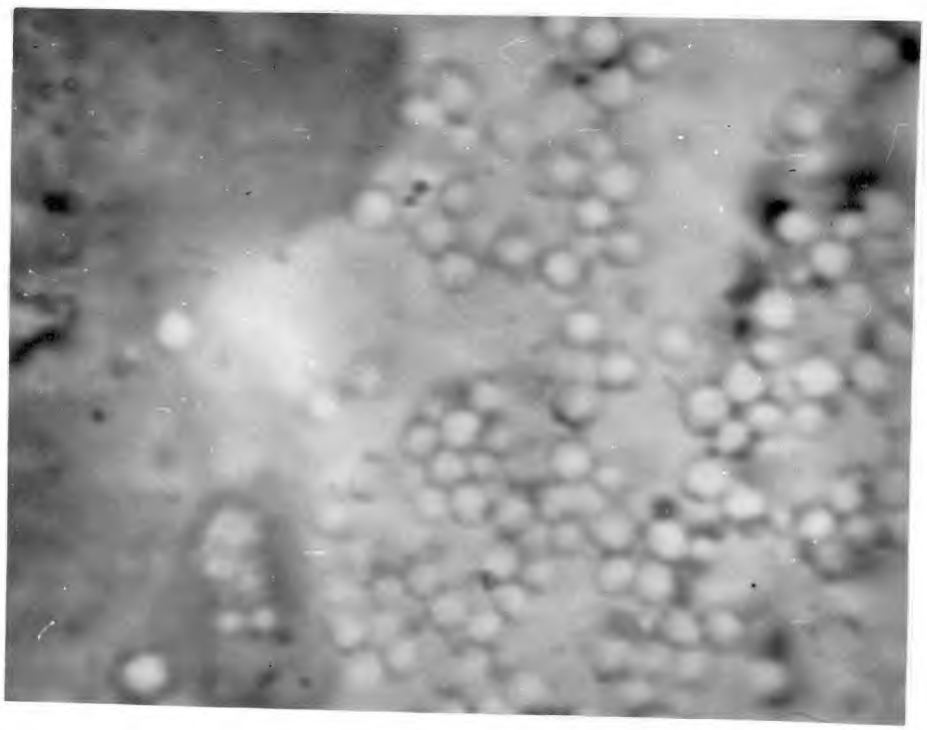
It would have been desirable to examine the polyhedra by electron microscopy; this could not be done, partly due to acute lack of time, partly because all existing material was fixed on slides. Although searched for, additional polyhedra were never found. If confirmed, this would be the first report of polyhedrosis disease of the Zygaenidae. The only other virus disease known in this family is a granulosis of Harrisina brillians B. and McD. (Hughes (1957), Martignoni and Langston (1960)).

Plate 2.



Haemolymph smear from a larva of Zygaena filipendulae showing suspected inclusion bodies of nuclear polyhedrosis. Unstained preparation under phase contrast (anoptral) illumination. x 2000

Plate 3.



Haemolymph smear from a larva of Euproctis chrysorrhoea showing inclusion bodies of nuclear polyhedrosis.
Giemsa stain. x 2000

APPENDIX

Bacterial Flora of Euproctis chrysorrhoea.

Nests of overwintering larvae, collected at Dungeness, were carefully dissected in the laboratory and the young larvae separated. These were surface sterilised in the manner already described for Zygaena and aseptically dissected. All were sterile.

Fourth instar larvae, feeding on Rubus sp. contained many bacteria, but relatively few species. These are listed in Table 10.

Diseased fourth instar larvae were found in small numbers in the Canvey Island colony. In most cases the disease was a nuclear polyhedrosis (Plate 3). A few larvae were apparently free from virus and seemed to be suffering from diarrhoea. On investigation these larvae were found to contain many bacteria in the alimentary canal that were not found in healthy larvae. These bacteria were Acinetobacter delmarvae and Aerobacter aerogenes. Further work on E. chrysorrhoea was prevented, as a result of the allergic responses of the writer, therefore the pathogenicity and epizootiology of A. delmarvae and A. aerogenes remains unknown. (In his review, Bucher (1963) lists A. aerogenes as a potential pathogen of insects. Acinetobacter (= Achromobacter) eurydice, closely related to A. delmarvae, is implicated as a cause of European Foulbrood of hive bees). Many, -approximately 20%, of 'healthy' larvae at Canvey Island were found to contain what appeared to be spores of microsporidia.

Appendix Table 10

Bacteria isolated from healthy fourth instar larvae of

Euproctis chrysorrhoea.

Achromobacteraceae

Acinetobacter eurydice Brisou and Prevot (from midgut and hindgut)

Micrococcaceae

Staphylococcus subgroup VI Baird-Parker (from foregut and midgut)

Bacillaceae

Bacillus megaterium de Bary (from foregut)

Bacillus cereus Frankland and Frankland(from midgut)

Bacillus licheniformis (Weigmann) Chester, amend Gibson (from midgut)

Bacillus pumilus Gottheil (from midgut)

Bacillus firmus Werner (from foregut)

Bacillus lentus Gibson (from midgut)

Appendix Table

Bacteria isolated only from diseased fourth instar larvae of

Euprotus chrysorrhoea.

Achromobacteraceae

Acinetobacter delmarvae Brisou and Prevot

Enterobacteriaceae

Aerobacter aerogenes (Kruse) Beijerinck

SUMMARY

1. A study was made of a population of *Zygaena filipendulae* and *Z. lonicerae* at Dungeness, Kent. Population estimates were made during all developmental stages of the moth, and it was found that young and hibernating larvae were apparently aggregated in the field, their dispersion being adequately described by the negative binomial distribution.

2. In the laboratory, female moths laid, on average, 60 - 108 eggs each, with, apparently, little specificity of oviposition site. Field observations indicated that the moths selected leaves as oviposition sites by their physical characteristics, notably height, size, and type of surface. Oviposition was never recorded on the food plant.

3. In the field, larvae seemed to feed only on *Lotus corniculatus* and *L. uliginosus*, and this observation was confirmed by laboratory experiment.

4. Because the moths do not lay their eggs on the food plants, the survival of the newly-hatched larvae appeared to be largely dependent on the distribution of *Lotus* in the vicinity. Observations in the field indicated a very much higher survival rate of young larvae in areas where *Lotus* was abundant, compared with those areas where the food plant was rare or absent.

5. During the season 1966-67, the numbers of larvae on certain experimental plots were so high that *Lotus* was virtually eliminated, and many larvae vacated these plots, presumably in search of food.

6. The only hymenopterous parasite of Zygaena larvae at Dungeness was Apanteles zygaenarum, and this caused a low percentage of mortality, due usually to the mycoses resulting from infected emergence wounds. Telenomus parasitised 95% of Zygaena eggs in 1967, thus producing a population "crash". No bacterial disease was found, the only primary microbial disease was caused by Beauveria bassiana in hibernating larvae.

7. In the microbiological studies, a reliable, non-lethal method for the surface sterilisation of Zygaena larvae was devised, then samples of haemolymph and gut contents were obtained after aseptic dissection, and the flora analysed. At the same time, the epiphytic bacteria of the food plant were also identified.

8. The haemolymph was found normally to be sterile, and the fore-gut to contain a flora almost identical with that of the food plant. In the mid-gut, however, a restricted and remarkably constant flora was found, indicating the selective inhibition of certain incoming bacteria. It was also found that streptococci, known to be present on their food plant, were apparently eliminated from the mid-gut of Zygaena larvae from Thursley Common, whilst these were part of the normal mid-gut flora in larvae from Dungeness. The significance and possible pathogenicity of the mid-gut flora is discussed.

9. The possible mechanisms of this selective inhibition of bacteria were studied. Plate inhibition test failed to demonstrate anti-bacterial substances in the food plant, or in the mid-gut contents of the larvae, or any bacterial antagonism, and so attention was

turned to the physical conditions of the mid-gut lumen. The mid-gut pH was measured and found to be 9.6. Alkaline conditions alone did not produce the inhibition observed, and so it was thought possible that the redox. potential might play an important role, particularly as strongly-reducing bacteria were known to exist in the mid-gut. Time did not permit this potential to be measured, but tests made at pH 9.6 and in the absence of gaseous oxygen produced the selective inhibition noted in the larvae. It seemed, therefore, that physical conditions were a likely cause of the selective inhibition.

10. Streptococci were the predominant bacteria in larvae from Dungeness, although absent from Thursley Common larvae. Aseptic rearing was attempted in order to elucidate their role in the physiology of the host, and an apparently successful technique for the aseptic culture of Zygaena larvae was devised, but all sterile larvae were killed as a result of an equipment fault. A method is also described for the tracing of streptococci, or other cellular antigens, in insect tissues by means of fluorescent labelled antibodies.

11. A report is made of a possible nuclear polyhedrosis of Zygaena filipendulae. If confirmed this would be the first record of a polyhedrosis in the family Zygaenidae.

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