Studies of

the Reproductive Biology and of the Structure, Composition, and Physiology of the Egg of

Graphognathus leucoloma Boheman

(Coleoptera: Curculionidae)

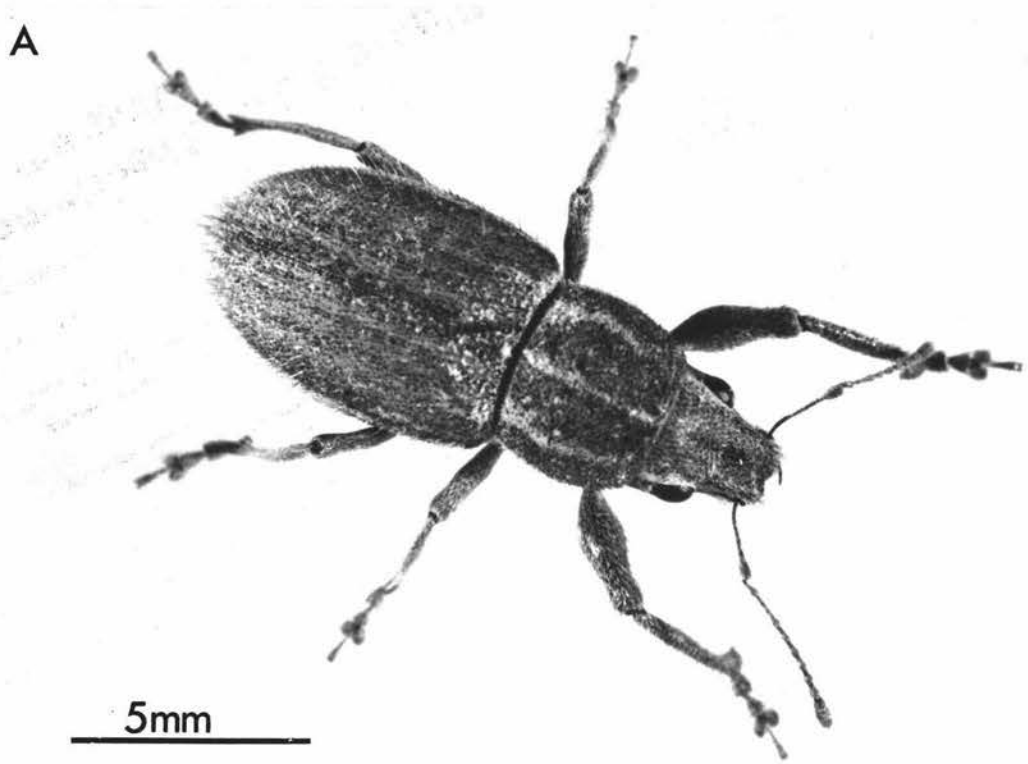
A thesis presented in partial fulfilment
of the requirements for the degree of
Master of Science in Zoology
at Massey University

David George Holdom

1977

A. Graphognathus leucoloma Boheman, adult.

B. Graphognathus leucoloma Boheman, eggs.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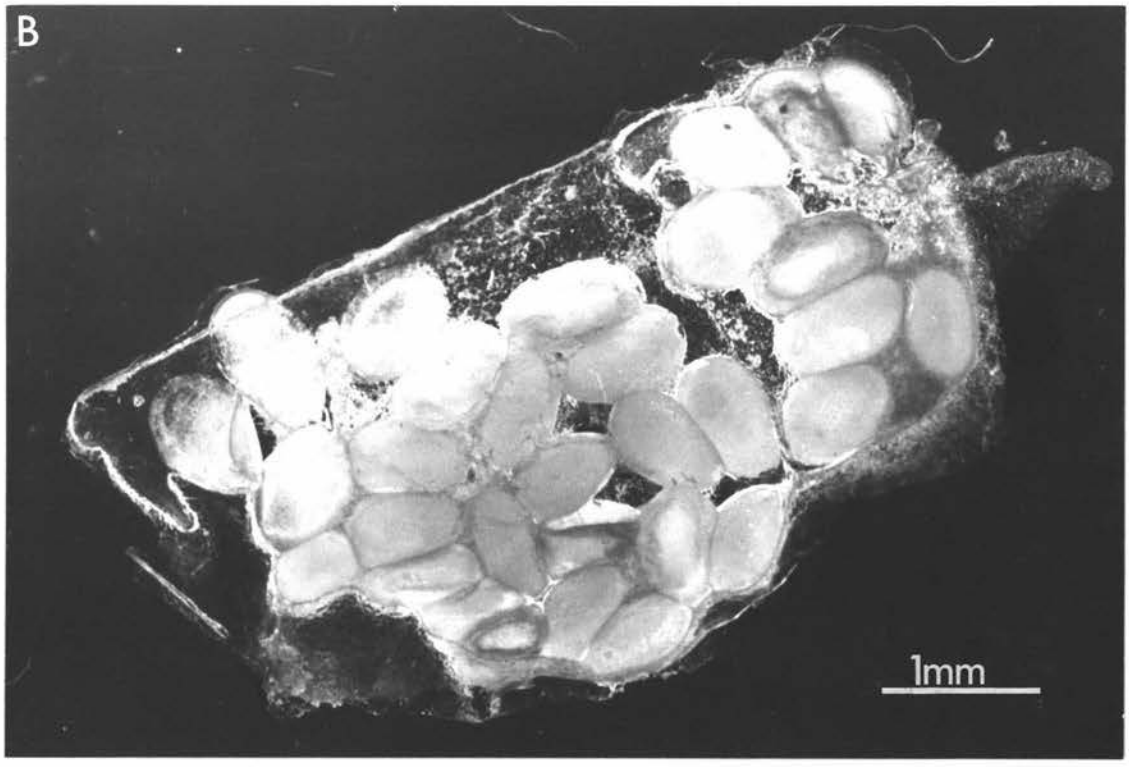
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To the late Stan and Blanche Bason,
whose enthusiasm and love of nature
were a major influence
on my interest in biology

ABSTRACT

The published information regarding the biology and pest status of Graphognathus leucoloma Boheman is summarized.

A study was made of egg development at temperatures ranging from 4 to 37.8°C and relative humidities ranging from 40 to 100%. Egg hatch was frequently very low and showed considerable variability. Median duration of development ranged from about 14 days at 31.5°C to 95-97 days at 15°C, with the developmental-hatching threshold between 12.1 and 13.5°C. Sub-threshold temperatures were lethal. Virtually no hatching occurred below 100% RH but the eggs could withstand considerable dessication and would hatch when moistened. Possible effects of humidity on the duration of development and of parental age on egg viability are also discussed. Some reasons for the very low egg viability are suggested, along with modifications to the experimental design to eliminate them.

Studies were made of adult and pupal size, and of longevity, pre-oviposition period and reproductive output. Some reasons for unexpectedly low fecundity and long pre-oviposition period are discussed.

The structure of the egg envelopes and the cement in which the eggs are laid was studied, using scanning and transmission electron microscopy, and Nomarski differential interference microscopy. A histochemical study of these structures, supplemented by some simple chemical tests, was also made, to elucidate in part their composition. The chorion is 4-9µm thick and composed entirely of protein; some 250,000 aeropyles are scattered over the surface, and its structure is such that it could probably act as a plastron when the egg is submerged. There is no micropyle. Disulphide linkages are probably important in the structure of the chorion, as in many other species, but unlike other beetles so far studied, no crystalline proteins were found. The vitelline membrane was found to be a three-layered structure 0.3-0.5µm thick composed of protein and acid mucopolysaccharides, and to be highly resistant to chemical attack. Waterproofing is probably provided by a layer of lipid on the outer surface of the vitelline membrane. The cement was found to be a complex, variable and heterogeneous mixture of protein and up to five acid mucopolysaccharides, which is unlike that of any other species reported. Some possible functions of the cement and the significance of its composition are suggested.

A note on the citation of publications, and the referral to species and their systematic status.

Any publication by three joint authors is cited in full the first time it is referred to and thereafter is abbreviated to the form: senior author *et al*. Publications with more than three joint authors are cited in the form: senior author *et al*. each time they are cited, including the first. The specific name of any organism is given in full only on the first time it is referred to, and the names of insect species and genera and their taxonomic status are listed in Appendix Five.

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Credit for the frontispiece and Figs. 3.1, 3.2 and 3.3 must go to the staff of the Central Photographic Unit, who also processed the light micrographs. Miss A. Turner drew Figs. 2.1 to 2.6.

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ABBREVIATIONS USED IN THE TEXT

°C	degrees celcius
P	Probability
RH	relative humidity
≏	approximately equal to
<	less than

ABBREVIATIONS USED IN THE FIGURES

aer	aeropyle
B	bacteria
C	chorion
cem	cement
G	glycogen granules
ic	inner layer of chorion
ilin	inner lining of chorion
ivm	inner layer of vitelline membrane
lae	lining of aeropyle
mvm	middle layer of vitelline membrane
oc	outer region of chorion
ovm	outer layer of vitelline membrane
trl	trabecular layer
vtm	vitelline membrane

CHAPTER ONE

THE STUDY ANIMAL

1.1 ORIGINS AND OCCURRENCE

The white-fringed weevil, Graphognathus leucoloma Boheman (Coleoptera, Curculionidae, Brachyderinae), is a native of South America, where it occurs in Argentina, Brazil, Chile, Peru and Uruguay (Berry, 1947; Young et al., 1950). Its known range now includes many south-eastern states of the U.S.A. as far north as Maryland (Anonymous, 1969), all five mainland Australian states (Chadwick, 1970), South Africa (Joubert, 1951) and New Zealand. In New Zealand, G. leucoloma was found in Northland in 1944, where it was believed to have been introduced with American war equipment about 1940 (Cottier, 1962), but it now occurs throughout the North Island and has been found in Nelson (Perrott, 1964) and Ashburton (May, 1975) in the South Island.

Buchanan (1947) recognised three species of Graphognathus: G. leucoloma, G. peregrinus and G. minor, and six races of G. leucoloma, of which five occur in the U.S.A. Only G. leucoloma is recorded outside North and South America, but no details regarding strains appear to have been published.

1.2 PEST STATUS, LIFE CYCLE, AND BIOLOGY

G. leucoloma is a serious pest in many areas (Berry, 1947; Young *et al.*, 1950; Joubert, 1951; Wright, 1962; Todd, 1964), and chemical control has proved difficult, with no acceptable economical method existing at present. It attacks a wide variety of plants and Young *et al.* (1950) reported that it was known to feed on 365 plant species in the U.S.A. In New Zealand, legumes, brassicas, maize (*Zea mays* L.), some cereals, potatoes (*Solanum vulgare* L.), water melons (*Citrullus lanatus* (Thunb.) Mansf.), pumpkins (*Cucurbita maxima* Duch.) and pine seedlings (*Pinus* spp.) are amongst the recorded hosts (Todd, 1964; May, 1966, 1975; Nelson, 1971; Given, 1973).

Todd (1964) reported that the white-fringed weevil has a two year life cycle but that because of overlapping generations, larvae at various stages of development could be found throughout the year. May (1975) noted that a single generation spans a minimum of 10 months in the north of New Zealand, but can take up to two years under less favourable conditions, and almost certainly requires two years in the Manawatu (M.J. Esson, personal communication). Berry (1947) found that in South America the life cycle occupies one year in the warmer regions, but two in the cooler regions, a situation apparently similar to that in New Zealand.

The eggs (frontispiece b) are oval, white and soft, and measure on average 0.62 by 0.56mm. They are laid in batches amongst ground litter and on plants where they are pushed between adjoining surfaces and cemented together (May, 1966). The cement is a translucent, white, viscous liquid when the eggs are laid but dries to a tough, white mass. The time taken by the eggs to hatch varies greatly, depending on conditions (Young *et al.*, 1950; May, 1966). Under dry conditions the eggs develop but do not hatch until sufficient moisture is present (May, 1966). Young et al. (1950) reported that eggs of "*G. leucoloma fecundus*" hatch in about 17 days in summer but take more than 100 days in winter, while Bass and Barnes (1969) found that eggs of "*Graphognathus* spp" took an average of 21 days to hatch on moist filter paper at 26.7°C. The eggs can also survive in a quiescent state for at least 8 months under suitable conditions (Gross *et al.*, 1972b).

The larvae feed on the roots of the host plants and are responsible for most of the damage caused by this insect (Todd, 1964). In general, plants with fibrous roots are damaged less severely than those with tap

roots, in which the larvae may sever the main root (Anonymous, 1956). Relatively low infestations are required for economic damage in root crops such as potatoes and carrots (Daucus carota L.) because the marketable part of the crop is damaged. Damage may vary from minor in one part of a field to destruction of plants in another part (Anonymous, 1956, 1969; Todd, 1964). Severely affected plants wilt, turn yellow and eventually die. Norton (1974) found that mortality of lucerne (Medicago sativa L.) plants depended on larval population density and on soil moisture, with plants able to withstand higher infestations if the soil was moist. Gross et al. (1972a) found that the time of introduction of larvae into the soil directly influenced the rate of larval development and the ability of the larvae to damage stands of rye (Secale cereale L.) and soya beans (Glycine max Ner.), with those introduced early doing more damage. Barnes and Bass (1972) found that a suitable period of cooling of full-grown larvae increased survival and shortened the larval period.

Mature larvae move to the surface from July on and become prepupae. The prepupa forms a cell by rotating its body and exuding drops of mucus from its anus to form a strong, smooth lining and after several weeks it moults to form a pupa (May, 1975). The pupal stage has been reported as lasting about 10 days (May, 1975) and as approximately 13 days in summer but longer in cooler weather (Young et al., 1950).

The adults (frontispiece a) are parthenogenic, and no males have been found. They are also flightless. Young et al. (1950) noted that adults took from 5 to 25 days to begin laying eggs and lived for up to 150 days, although most died within 90 days of eclosion. Longevity, fecundity and egg viability have all been shown to be affected by adult diet (Anonymous, 1956; Barnes and Bass, 1973; East, 1976). Fecundities averaging from four to 1,600 eggs per weevil, depending on diet, with one individual laying over 3,000 eggs on the best diet (peanut (Arachis hypogaea L.)), have been reported (Anonymous, 1956). East (1976) also found that populations of G. leucoloma increased sharply under legumes, but not under pure stands of the grass Paspalum dilatatum Poir.

1.3 THE PRESENT STUDY

Since the white-fringed weevil is frequently found in large numbers in areas which become very dry in summer, when the eggs are laid, it could be expected that the eggs would show considerable resistance to dessication.

Work carried out during the first season was intended to provide information on the responses of the eggs to temperature and humidity. However, although this provided an indication of the responses of the eggs to these factors it did not provide accurate data, since the eggs showed an unexpectedly large variability in hatch, which was not allowed for in the experimental design. Therefore, during the second season the experimental design was accordingly modified to accommodate this, and the scope of the work was narrowed to an investigation of the effects of temperature only. At the same time variability in the adults was studied and supplementary work was undertaken to compare the relevance of laboratory studies to those made under conditions approaching the field situation.

Since it can be expected that the structure and composition of the egg envelopes should be related at least in part to the resistance of the egg to dessication, a study of them, particularly in relation to waterproofing, was initiated during the first season. This was then expanded into an investigation, extending into the second season, involving scanning and transmission electron microscopy, supplemented by Nomarski differential interference microscopy. In addition, a histochemical study was made, supplemented by some simple chemical tests.

The aims and relevant literature of each aspect of this study are discussed more fully in the appropriate chapters, whilst a number of miscellaneous observations, which were made during the study, are outlined in Appendix One.

CHAPTER TWO

STUDIES ON THE DEVELOPMENT AND HATCHING OF EGGS

2.1 INTRODUCTION

Little work has been done on the development and hatching of white-fringed weevil eggs in response to different temperatures and humidities, although the eggs of many other species of insects have been studied with regard to temperature only (e.g. Messenger and Flitters, 1958; Butler and Wardecker, 1971, 1973; Mussen and Chiang, 1974), temperature and humidity (e.g. Johnson, 1940; Sharma, Sahni, and Sinha, 1973; Sarin and Saxena, 1973; Wong and Davis, 1975) or temperature and soil moisture (e.g. Wightman, 1973). Eggs of weevils studied include those of Sitona lineata L.(Andersen, 1950), Sitophilus oryzae L.(Birch, 1944a,b; Khan, 1949; Howe, 1952), S. granarius L.(Khan, 1949), Anthonomus grandis Boheman (Bacheler and Bradley, 1975). Anthonomus signatus Say (Clarke and Howitt, 1975), Hypera brunneipennis Boheman (Madubunyi and Koehler, 1974), H. postica Gyllenhal (Roberts, De Witt, and Armbrust, 1970; Guppy and Mukerji, 1974; Morrison and Pass, 1974), Listronotus oregonensis Le Conte (Martel, Svec, and Harris, 1976), Lissorhoptrus oryzophilus Kuschel (Raksarat and Tugwell, 1975), Otiorhynchus sulcatus Fabr (Shanks and Finnigan, 1973) and Hylobius pales Herbst (Speers and Cody, 1975).

Gross et al. (1972b) studied the effects of a number of temperatures and humidities on eggs of "Graphognathus spp". They used temperatures of 7.2, 12.7, 18.3, and 23.8°C and relative humidities of 75, 80, 85, 90 and 95% and held the eggs at 26.6°C and 95% RH for 1-7, 8-14, 15-21 or 22-28 days before exposure to the experimental conditions for up to 8 months. The highest survival was obtained from those eggs stored at 95% RH at the three lower temperatures and survival was reduced at 23.8°C. The size of larvae at hatching diminishing noticeably as length of storage of eggs at this temperature increased. Maturity of eggs was important; more mature eggs showed greater percent eclosion at 85% RH and above, but the trend was reversed at lower humidities. In addition, the tolerance of the eggs to lower temperatures increased with increasing maturity. Larvae from different treatments also showed different survival rates; those from eggs exposed to 18.3°C and 95% RH for eight months survived better than larvae from eggs exposed to 7.2 or 12.7 and 95% RH for the same length of time.

The only other published information concerning the effects of

specified conditions on the duration and survival of the egg stage of Graphognathus is that by Bass and Barnes (1969) who found that eggs of "Graphognathus spp" placed on moist filter paper at 26.7°C began hatching after 12 days with an average time to eclosion of 21 days and a 90% hatch. In addition, Young et al. (1950) stated that the eggs take 11-30 days to hatch in summer, with a mean of 17 days, while in winter they may take more than 100 days. Wright (1961) gave the incubation time as 10-14 days under "suitable conditions", while May (1966) stated that the time ranges from 6 to 70 days "depending on conditions".

The ability of eggs to overwinter should also be related to their tolerance to low temperatures. Some eggs of G. leucoloma apparently overwinter and hatch in spring although winter is normally passed in the larval stage (Anonymous, 1969).

Several authors have reported that G. leucoloma eggs can withstand considerable periods of dessication (Wright, 1961; Todd, 1964; May, 1966, 1975), but the only data published is that of Gross et al. (1972b) mentioned above. Young et al. (1950) also stated that development is completed under dry conditions, but that eggs would not hatch until moisture was present.

The present investigation was undertaken to provide a fuller understanding of the responses of eggs to temperature and humidity, since this is pertinent to the ability of eggs to overwinter and to survive dry conditions during the summer.

The first season's work was designed as an investigation of the ability of newly laid eggs to withstand various combinations of temperature and humidity and the effect of these conditions on incubation times. However, the results only gave an indication of the responses of eggs to low temperatures and to humidity and the effects near the upper temperature limits were not examined. Also, the results were confused by considerable variability in overall hatch and were not amenable to statistical analysis.

During the second season I therefore decided to concentrate on the effects of those temperatures which had been shown to be above the lower threshold for development and hatching. In addition, the number of temperatures used was increased, the temperature range was extended upward and the experimental design was improved to permit statistical analysis of the results. No further studies of the effects of humidity were made. Adult size, pre-oviposition period, longevity, fecundity and

overall egg viability were also examined, in order to further illustrate the problems of variability encountered during the first season and to add to knowledge of adult biology.

In addition, a number of adults were maintained in a sheltered outdoor situation to investigate whether the studies made under constant conditions in the laboratory were relevant to field conditions. The same data as for laboratory-held adults were recorded. This approach was also designed to test the ability of eggs to overwinter in the field. Results pertaining to eggs are presented in this chapter and those pertaining to adults in Chapter Three.

2.2 MATERIALS AND METHODS

2.2.1 First Season

2.2.1.1 Collection and maintenance of adults

Adult weevils were collected by sweep netting at night from lucerne crops in the Manawatu (mainly in the Longburn and Rangiotu districts), in January, February and March of 1975. Up to approximately 2500 weevils were maintained at any one time and periodic collections were made to replace those that died.

The insects were maintained by a method similar to that described by Gross and Bartlett (1972). Ten cylinders of 22 guage galvanised metal, approximately 25cm high and 38cm in diameter, with aluminium foil bases were used as cages (Fig. 2.1), and a layer of petroleum jelly was smeared around the top 2-3cm inside the cages to prevent any weevils from escaping. Pieces of aluminium foil 1-2cm square scattered in the cages provided oviposition sites from which eggs could be easily removed and counted. Fresh white clover clippings were provided daily as food. These were initially inserted through holes in the lids of 500cm^3 polystyrene containers, half filled with water, to keep the cuttings fresh and to prevent the weevils from drowning. However, this method was later abandoned and the clippings were subsequently placed loosely in the cages. Clover was either grown in the glasshouse or collected from an area of waste ground on the Massey University campus. The weevils were kept in a room in which the temperature, humidity and photoperiod were not controlled but approximated conditions outside.

2.2.1.2 Maintenance of constant temperatures

The equipment used to maintain constant temperatures is listed in Table 2-I.

2.2.1.3 Maintenance of constant relative humidities

Relative humidities of 40 $\pm$ 1.5, 50 $\pm$ 1.5, 60 $\pm$ 1.5, 80 $\pm$ 1.0 and 95 $\pm$ 0.5 percent were maintained in 600cm^3 "Agee" preserving jars fitted with seals and screw tops using dilute solutions of sulphuric acid, for which accurate temperature-relative humidity relationships are available (Wilson, 1921; Solomon, 1951), and a humidity of 100% was provided by distilled water. Approximately 150cm^3 of distilled water or the appropriate solution was placed in each of these humidity chambers and a platform of 2cm grid plastic netting supported on a piece of 4cm PVC pipe was placed in each jar to support containers of eggs (Fig. 2.2). Dilute

Table 2-I Facilities for maintenance of constant temperatures

Temp °C	Facility and Comments
4 ± 1.5	Cool room. Air circulated by a fan in the cooling unit.
10 ± 2	Old refrigerator, modified. Air circulated by a fan.
15 ± 1.5	New refrigerator, modified. Air circulated by a fan.
20 ± 1	Fibreglass insulated wooden cabinet. Fan in series with a thermostat provided heat. Intermittent air circulation by the fan.
25.5 ± 1.5	a) Old refrigerator cabinet. Heat provided by two 40 watt blackened light bulbs connected in parallel with each other and in series with a thermostat. Air circulation by convection only.
	b) For assessment of survival. Controlled temperature room with air circulated by two fans.

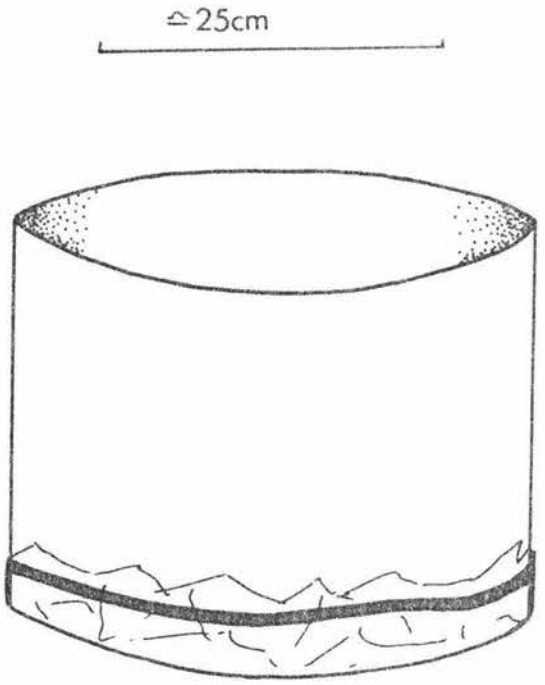

Fig. 2.1 Metal oviposition cage with aluminium foil floor used for housing adults during the first season.

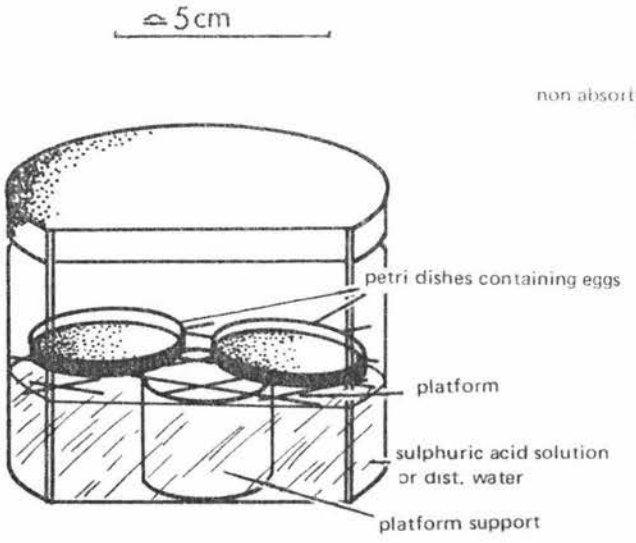

Fig. 2.2 Jar used for holding eggs at constant humidity during the first season.

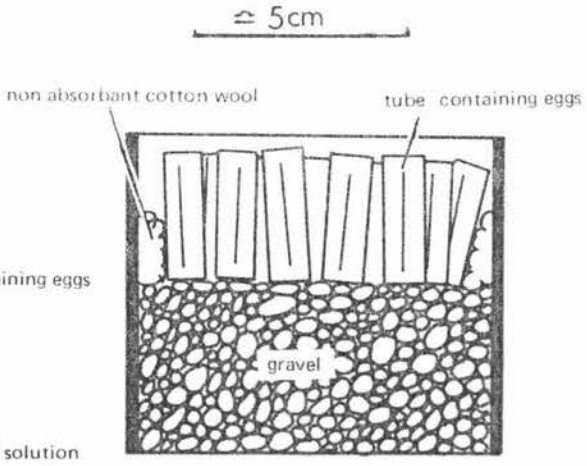

Fig. 2.3 Jar used to maintain eggs in laboratory during the second season.

solutions of sulphuric acid were prepared according to the method of Solomon (1951) and concentrations of sulphuric acid were determined with British standard series S50N hydrometers, together with tables and appropriate corrections (British Standards Institution, 1959, 1960).

2.2.1.4 Assessment of temperature and humidity effects

Eggs were collected within 10 hours of being laid and those for each temperature-humidity combination were divided into two samples, one of which was counted at approximately 100 eggs and the other estimated at approximately 1000 eggs by weighing. Each sample was then placed on filter paper (Whatman No. 1) in an uncovered 4.25cm plastic petri dish in the appropriate humidity chamber which was then placed in the dark at the designated temperature.

The smaller samples from each treatment were checked once or twice daily, and the larvae which had hatched were counted and removed. Subsamples of approximately 50 or 100 eggs were taken from the larger samples at 1, 2, 3, 4, 6 and 8 weeks and thence every 4 weeks from the time of laying, in order to assess survival. For this a modification of the method of Gross et al. (1972b) was used. Eggs were flooded for six hours with distilled water, blotted dry and held as described above at 100% RH and at 25.5 ± 1.5°C in the dark. The method used by Gross et al. (1972b) was to hold the eggs at 95% RH with weekly floodings, but this was found to be time consuming and gave unreliable results, with eggs hatching only within 24 hours of flooding and some still hatching after several floodings resulting in a greatly extended hatch period. Samples were checked once or twice daily and the larvae were counted and removed until none had hatched for one week - provided that three weeks had elapsed from the time of flooding.

A temperature of 25.5°C was considered to be suitable for assessing survival, as Gross et al. (1972b) used 26.6°C for the preliminary incubation and for assessment of survival, and Bass and Barnes (1969) obtained a 90% hatch at 26.7°C. This indicates that although the eggs do not store well at higher temperatures (Gross et al., 1972b), development and hatching is not adversely affected.

Some quantification of the variability evident in the results was desired in addition to a more accurate assessment of percent hatch at various temperatures. Therefore, in a separate experiment, seven samples of about 100 and five of about 50 eggs were placed at 25.5 ± 1.5°C, eight of about 100 eggs were similarly placed at 20 ± 1°C and one extra sample

of about 100 eggs was placed at 15 ± 1.5°C. All samples were maintained at 100% RH. While samples kept at 20 and 25.5°C were checked twice daily from 14 and 10 days after laying respectively, and hatching was considered to be complete when no further larvae had emerged for one week, samples held at 15°C were checked weekly until the hatching of subsamples taken for survival assessment indicated that development was nearly complete. From this time, samples at 15°C were checked every one or two days until no further eggs hatched for three weeks. However, the samples at 20 and 25.5°C were checked at irregular intervals for up to three months and those at 15°C for up to seven months after laying. Any larvae present were counted and removed.

2.2.1.5 Effects of Tyrophagus putrescentiae Schrank (Acari, Acaridae)

Some subsamples of eggs were found to be infested with this mite, but no clear effect on survival was apparent, so a small experiment was set up to further examine this. Ten samples of about 50 eggs were collected, of which five were infested with five adult T. putrescentiae each and five were left as controls. All were incubated at 25.5°C and 100% RH.

2.2.2 Second Season

2.2.2.1 Maintenance of constant temperatures

The temperatures used are listed in Table 2-V. Cooled incubators were used to maintain temperatures of 15.0 and 17.5°C while a constant temperature room provided a temperature of 20°C. All the other temperatures were obtained by using waterbaths.

2.2.2.2 Maintenance of adults and assessment of temperature effects and egg survival

Three hundred adults of known age were housed individually and fed on an artificial diet as described in Chapter Three. These adults were randomly allocated into ten groups, one of which was maintained in the shade house at approximately ambient conditions, and of the remainder, eight groups were paired and "pre-allocated" to two experimental temperatures each as set out in Table 2-II. This was done to facilitate management of the weevils and the collection of the eggs and their placement at the experimental temperatures, which were originally intended to be at 2.5° increments starting at 17.5°C.

Table 2-II Allocation of eggs to test temperatures through allocation of adults

Eggs from lots	to temperatures	T_1(°C)	and	T_2(°C)
1 and 2		17.5		20.0
3 and 4		22.7		25.4
5 and 6		25.4		27.3
7 and 8		31.5		37.4

The three temperatures used but not listed in Table 2-II (15.0°C, 33.3°C, 35.2°C) were decided upon on the basis of the results of the treatments listed in the table, or, in the case of 15°C, to make the series more complete at the lower end of the range.

At this stage it is appropriate to define two terms: a "clump" is defined as the eggs laid in a single mass, while a "batch" consists of all the eggs laid by a single weevil in one day, and consists of one or more clumps each containing one or more eggs.

Single batches of 10 or more eggs were placed in labelled tubes and allocated as follows: a suitable batch laid by a particular weevil was placed at "survival assessment" conditions (25.4°C as described below), the next allocated to T_1, the next to T_2 and the next to survival assessment. A total of 30-40 batches were placed at each temperature. A temperature of 25.4°C was used as a standard with which to compare the effects of the temperature treatments. For this reason two paired lots were allocated to 25.4°C. The placement of batches on either side of those allocated as in Table 2-II at "survival assessment conditions" provided for a "double comparison" of results with standard conditions.

Insufficient eggs were obtained for 17.5 and 20°C because of low fecundity of the adults in lots one and two, and waterbath failures resulted in the loss of two sets of samples at 37.4 and 31.5°C. The extra eggs required for 17.5 and 20°C, the replacements for 37.4°C and those placed at all other temperatures were collected as they were laid from all weevils maintained in the laboratory. Most eggs from weevils in lot 9, batches of less than 10 eggs and batches not otherwise used were placed at 25.4°C for the assessment of survival.

The eggs were placed in specimen tubes and labelled with the date of

egg collection, adult identification and the number of eggs including the number (if any) destroyed during collection and counting. Twelve to 40 tubes per jar, depending on tube size, were placed in "Agee" 600cm³ preserving jars and supported in an upright position by a small quantity of non-absorbant cotton wool placed around the sides of each jar (Fig. 2.3). Each jar contained enough washed gravel to cause it to submerge and the gravel itself was just covered with distilled water. When closed, the jars were placed in the appropriate temperature cabinets, the controlled temperature room, or waterbaths.

Samples other than those used to assess survival only were checked daily (every second day at 15°C) and any larvae present were recorded and removed. Tubes were checked for three weeks after the first hatch, except at 15°C, where they were checked for four weeks and then placed at 20°C for one week to check for further hatching.

Survival was assessed at a temperature of 25.4°C. At first the samples were checked daily but as the number of samples increased this became very difficult for one person to do, necessitating a change in technique. Tubes were then covered with "parafilm", which was perforated finely to allow humidity equilibrium while preventing larvae from escaping*. These eggs were subsequently checked four weeks after laying, then allowed to dry out at 20°C and any larvae which had hatched were recorded and removed after one week. The eggs were then flooded with distilled water and the process was repeated at weekly intervals for five weeks. All eggs from the temperature treatments were alternatively dried and flooded in the same manner on completion of the period of daily checking.

2.2.2.3 Shade house experiment

The weevils maintained in the shadehouse were checked daily for eggs, and the first, second, and third batches laid by a given weevil were placed at 25.4°C (for assessment of survival), at 100% RH in the shadehouse, and exposed to ambient conditions on the soil surface in the shadehouse respectively. The cycle was repeated for further batches.

Eggs maintained at 100% RH in the shadehouse were held in specimen tubes closed off with nylon bolting cloth (0.1mm mesh) held by rubber

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* It was found that larvae could escape from tubes by climbing in the film of moisture on the tube walls. During both seasons escapes were uncommon provided larvae were not left for more than one day.

bands. These tubes were inverted and placed over distilled water in a 600cm^3 jar, as described for maintenance of humidities for the first season's work (Section 2.2.1.3). A layer of plastic netting (2mm mesh) prevented the tubes from slipping through the supporting grid (Fig. 2.4). Eggs maintained under ambient conditions were held in 12mm diameter glass tubes, 3-5cm long with a double layer of bolting cloth glued across the bottom, and with the top covered by a single layer, held in place by a rubber band (Fig. 2.5). These tubes were placed upright in a group at the base of a clump of grass (Fig. 2.6) in a small area adjacent to where the adults were housed in which the vegetation was kept at a height of between 15 and 30cm (Fig. 3.3). These eggs were checked at weekly intervals, and any larvae which had emerged were counted and removed.

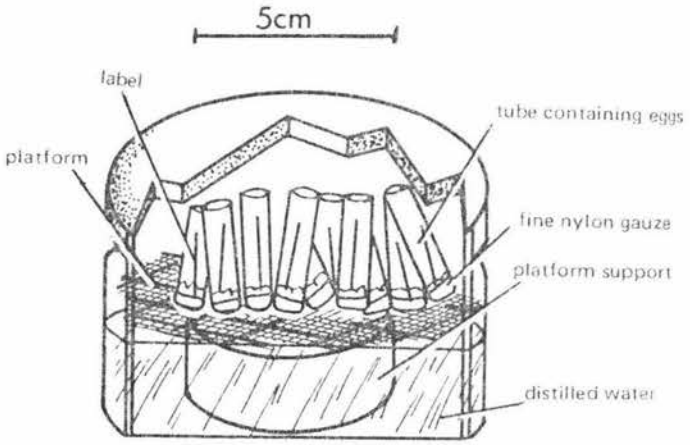


Fig. 2.4 Jar used to maintain eggs at high humidity in the shadehouse.

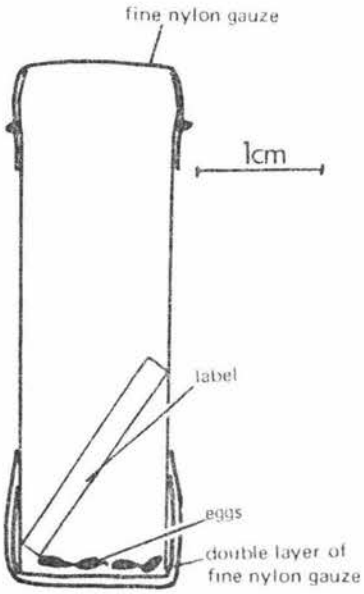


Fig. 2.5 Tube used to contain eggs exposed to ambient conditions in the shadehouse.

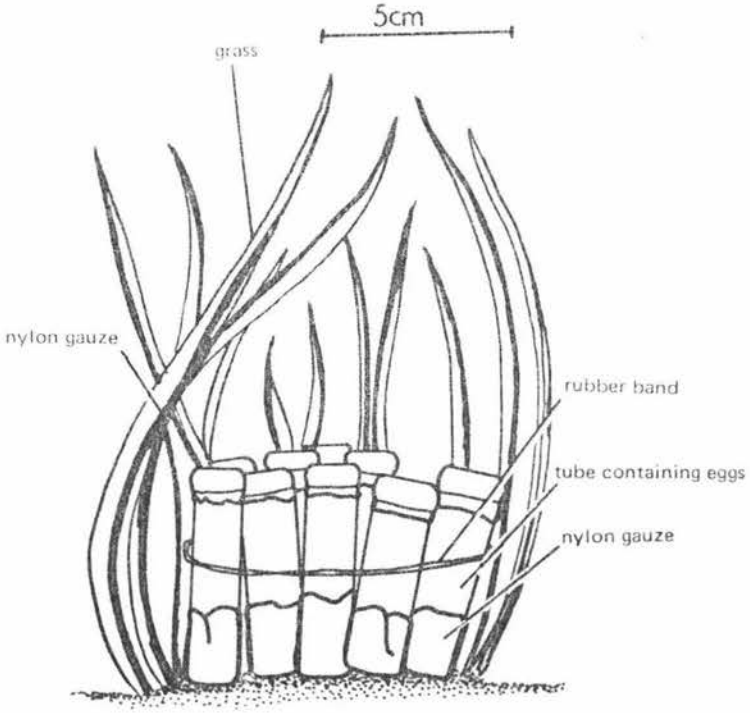


Fig. 2.6 Maintainance of eggs at ambient conditions in the shadehouse.

2.3 RESULTS AND DISCUSSION

2.3.1 Effects of Temperature and Humidity on Survival

The survival in relation to time, at the combinations of temperature and humidity to which eggs were exposed during the first season's work is given in Table 2-III. No eggs hatched after more than three weeks at 4 or 10°C, and the effects of humidity were not well defined at these temperatures.

At the other temperatures however, survival was greater at higher humidities and the detrimental effects of dry conditions increased with increasing temperature. Eggs hatched freely at 100% RH but apart from a single egg at 25.5°C and 95% RH none hatched below saturation. Eggs survived particularly well at 95% RH, with some hatching after 32 and 36 weeks (the last time of sampling) at 15 and 20°C respectively. Some eggs hatched after 16 weeks at 25.5°C and 95% RH but none hatched after 28 or 32 weeks, although no samples were taken at 20 or 24 weeks. Survival progressively decreased below 95% RH. One egg survived from a sample of 55 kept at 25.5°C and 40% RH for four weeks and held at 95% RH after flooding, but apart from this one survivor, no eggs hatched after more than two weeks at 40% RH and 25.5°C, or three weeks at 20°C and 15°C. This single hatching does, however, indicate that under field conditions a small proportion may be able to survive prolonged dessication.

These results show similarities to those obtained by Gross et al. (1972b), who found that eggs would survive for at least eight months at 18.3°C and 95% RH, but that they survived for this length of time at 7.2 or 12.7°C only if mature or nearly so when placed at the experimental conditions. They also found that survival decreased as humidity decreased, but they recorded a generally greater hatch than was observed in the present study.

Eggs of many insects show reduced survival at low humidities, although some are highly resistant to dessication (Bursell, 1974b). The increased effect of low humidities at higher temperatures is at least partly accounted for by the observation that water loss depends more on saturation deficit than on relative humidity, and the former is greater for a given relative humidity at higher temperatures (Bursell, 1974b).

Continuous exposure to 40 or 50% RH for several days is highly unlikely to occur in the field in New Zealand, and it is possible that some recovery from the effects of detrimental conditions could occur

Table 2-III Survival of G. leucoloma eggs after exposure to different temperatures and humidities.

Temp. °C	Time exposed (weeks)	Percent eclosion at various relative humidities					
		40% RH	50% RH	65% RH	80% RH	95% RH	100% RH
4	1	24	25	55	22	2	26
	2	7	7	2	11	6	4
	3	0	0	2	0	0	0
	3	2		-	-	4	1
	4	0		0	0	0	0
10	1	3	26	36	56	30	46
	2	0	0	1	4	4	4
	3			0	7	0	0
	4				0		
15	1	14	29	53	58	22	23
	2	20	13	35	33	38	21
	3	2	14	23	54	54	49
	4	0	7	13	22	44	33
	6		3	9	6	28	14
	8		0	3	28	54	21
	12			0	3	15	5*
	16				4	32	23*
	20				0	19	7**
	24				1	3	hatching
	28				0	4	complete
	32					7	
20	1	44	29	6	28	48	63
	2	15	30	33	57	55	59
	3	1	62	5	10	64	63
	4	0	8	0	44	47	85
	6		1	1	16	39	hatching
	8		1	1	5	59	complete
	12		0	1	3	35	
	16			0	13	22	
	20				1	48	
	24				0	-	
	28					8	
	32					6	
	36					4	
25	1	29	29	77	63	66	61
	2	5	12	41	47	59	78
	3	0	2	11	28	43	hatching
	3		38	52	79	3	complete
	4		0	59	25	52	
	6			13	26	38	
	8			15	15	27	
	12			0	18	36	
	16				7	14	
	20				0	-	
	24					-	
	28					0	
	32						

* Hatching of sample completed prior to flooding

** Hatching of sample almost completed prior to flooding

between intermittent exposures. It must also be noted that within crops humidities may be much higher and temperatures lower than in the air above the plants (Cloudsley-Thompson, 1962; Pinter, Hadley, and Lindsay, 1975). From the present study it is concluded that high temperatures and low humidities such as probably occur in crops during summer in New Zealand are unlikely to have any significant detrimental effects on eggs of G. leucoloma. However, careful monitoring of crop microclimates is required before laboratory data can be related reliably to the field. Further, the single or at the most two samples which were used in the present study did not allow for statistical analysis, the need for which is illustrated by the variability evident from Tables 2-III and 2-IV.

2.3.2 Duration of Development

2.3.2.1 Measurement and representation of the duration of the egg stage

The developmental period may be expressed using four statistics; the modal, mean and median hatch times and the time taken for the first eggs to hatch. The mode is satisfactory only if hatching follows a sigmoidal pattern with a distinct, transient peak rate. If this does not occur this statistic will be difficult if not impossible to determine. The remaining three are discussed by Howe (1967). The time to first hatch, which has been used, for example, by Turnipseed and Rabb (1965) is influenced by sample size, and will decrease as sample size increases. In addition, accidental inclusion of a partially developed egg will produce a biased result. Howe (1967) argues that there is little to choose between the remaining two (mean and median). Many workers have used the mean (for example Peairs, 927; Singh, 962; Butler and Wardecker, 1971, 1973; Sarin and Saxema, 1973; Raksarat and Tugwell, 1975; Speers and Cody, 1975; Martel et al., 1976), but it is susceptible to extreme values and a few eggs taking a very long time to hatch can markedly influence the mean while having a relatively small effect on the median. The hatch-time curve for insects often has a strong positive skew (Howe, 1967) and for this type of sample the median may be a better expression of the central tendency of the population (Sokal and Rohlf, 1969; Zar, 1974). On this basis Messenger and Flitters (1958) argue strongly for the use of the median and Howe (1952) and Wightman (1973) also use the median. In addition, Howe (1952) gives the time to first hatch and Wightman gives the mean and range of hatching times. Huffaker (1944) used the mean of the time required for the first half of samples of mosquito eggs to hatch, claiming that the second half are more likely

to be subject to limited food supplies.

The disadvantages inherent in the use of the mean to express the duration of the egg stage are demonstrated by the results of the work carried out during the first season with samples of eggs kept at 15, 20 and 25.5°C and 100% RH (Table 2-IV). Although the criterion of no eggs hatching for a set period as already described was used to define arbitrarily the completion of hatching, checking of samples at less regular intervals after this time revealed some further eggs hatching, here termed "tail enders". To differentiate between totals including and excluding these tail enders, the terms "total" and "arbitrary total" respectively have been used. At 15, 20 and 25.5°C the tail enders amounted to approximately 7.5%, 0.2% and 3.5% of the total hatch respectively. These produced increases of 5.4, 0.4 and 7.3% in the means but only 1.4, 0.0 and 0.6% in the respective medians. Thus it can be seen that these tail enders have a much greater influence on the mean than on the median. For this reason, although the mean, median and time to first hatch are all included in Table 2-IV the median has been used in plotting graphs for both the first and second season's work.

The values and their standard errors given in Table 2-IV are the means of the values for individual samples. As there were only two samples at 15°C this approach was not possible, but the data for the two were combined and treated as a single sample. Mean hatching times for individual samples were calculated from the times taken by individual eggs to hatch. The individual medians were estimated from a plot of cumulative percent hatch against time for each sample. The cumulative curves of the totals of eggs kept at each temperature are given in Fig. 2.7.

Messenger and Flitters (1958) used a probit method to estimate the median, plotting percent hatch as probits against the logarithm of time. This method resulted in the hatch curve being close to a straight line in the medial region but it had some sigmoidal deviation at each end. An estimate of the median was made by ignoring the ends of the graph. When the present results were plotted in this manner they yielded a similar result except that the graph did not deviate as much from a straight line at the lower end as that of Messenger and Flitters (1958) but it deviated to a greater extent at the upper end, particularly for results from the second season. However, the median estimated by this method proved to be no more accurate than that estimated from a linear plot, and therefore the probit method was not used.

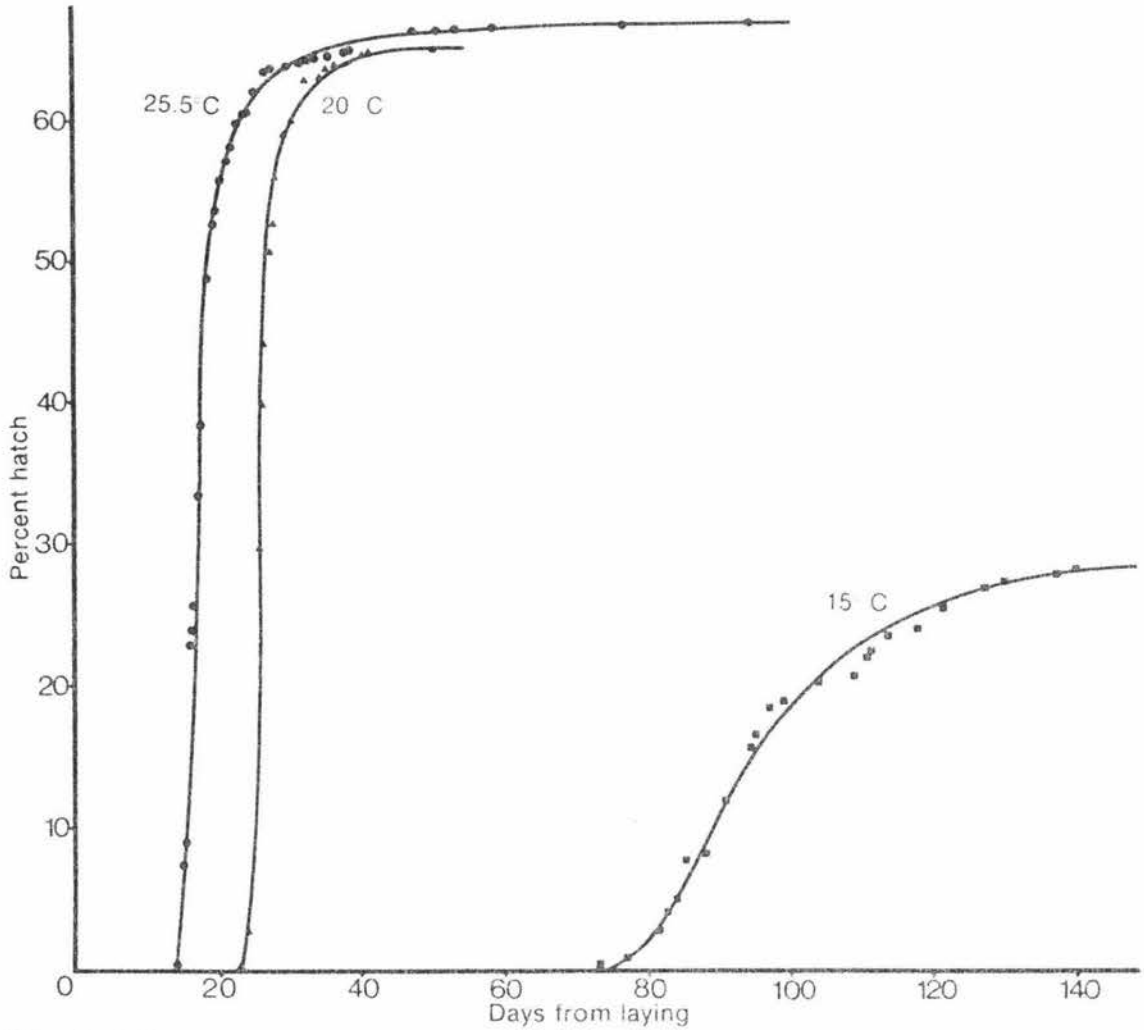


Fig. 2.7 Cummulative hatch curves of samples held at 100% RH and specified temperature (first season).

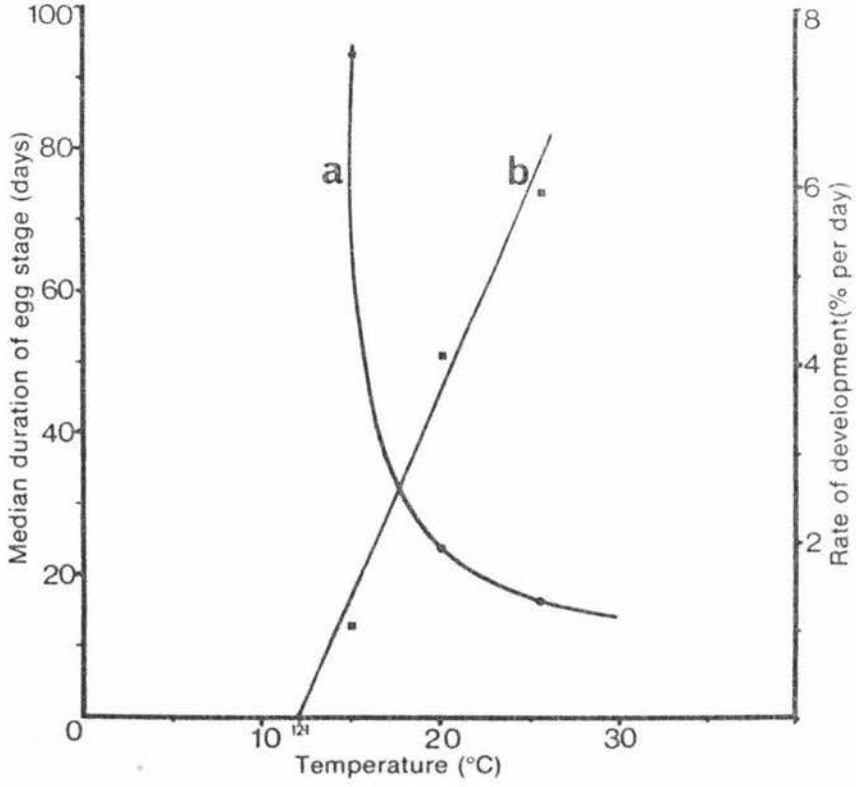


Fig. 2.8 (a) Median duration and (b) rate of egg development in relation to temperature (first season).

2.3.2.2 Some possible effects of humidity on the duration of the egg stage

At 20 and 25.5°C development was virtually complete after four and two weeks respectively, with 2% of the sample taken from 20°C and 100% RH after four weeks incubation having hatched before this time. The cumulative hatch curves for eggs kept at various humidities at 20°C for three and four weeks and then placed under conditions suitable for hatching were plotted, along with the corresponding curves for 25.5°C for one and two weeks. After two weeks at 25.5°C differences were apparent in the time taken from flooding until hatching between the various humidities with the time increasing as humidity decreases. No clear trend was evident after one week however; nor was any trend evident in samples taken at three or four weeks from 20°C. Observations of the appearance of head capsules made under the dissecting microscope indicated that development at lower humidities may take slightly longer. However, this assessment is based on a small number of observations and is somewhat subjective.

The duration of embryonic development has been found to increase as relative humidity decreases in a number of insects (Bursell, 1974b). These include S. lineata (Anderson, 1930), Ptinus tectus Boield (Coleoptem, Ptinidae) (Howe and Burges, 1953), and Galleria mellonella L (Lepidoptera, Pyralidae) (Barbier and Chauvin, 1974), and appears to be the most common situation. There are some insects, however, which have a distinct optimum at less than 100% RH and this has been found to occur, for example, in Locusta (Bursell, 1974b) and in the homopteran Adelges piceae Ratzeburg (Amman, 1968). There are also species which are unaffected by humidity, as for example the bedbug Cimex lectularius L. (Hemiptera, Cimicidae) (Johnson, 1940), Thermobia domestica Packard (Thysanura, Lepismatidae) (Sweetman, 1938) and Bruchus obtectus Say (Coleoptera, Bruchidae) (Menusan, 1934).

Any increase in the time taken by eggs to hatch at lower humidities could be due to any of the following:

a) Direct retardation of development by low humidities,
b) weakening of larvae resulting in a reduced ability to break through the chorion or requiring some time for recovery before hatching can occur, or both
c) a hardening of the chorion making escape from the egg difficult.
d) a random effect stemming from the low hatches occurring at low humidities.

No firm conclusions can be drawn from the present study, although

there may be a small increase in the duration of development of G. leucoloma eggs at low humidities. More detailed investigation is therefore required to clarify this point.

From an ecological point of view the inhibition of hatching which results from a lack of moisture is far more significant. Apart from one egg at 25.5°C and 95% RH, none hatched below 100% RH. As the eggs developed, the mandibles and then the entire head capsules became visible through the chorion, and the larvae could frequently be seen moving within the eggs and it was found that they completed development regardless of humidity provided of course that they were not killed by dessication first. Even at 40% RH a few apparently fully developed larvae were observed, although they were not seen to move within the eggs. The advantages of this are that the eggs will only hatch when conditions are suitable for the survival and entry into the soil of newly hatched larvae, and that they will be ready to hatch as soon as such conditions occur. In contrast, the eggs of O. sulcatus will hatch at 60% RH but at this humidity the larvae quickly die (Shanks and Finnigan, 1973).

2.3.2.3 Some effects of temperature on the duration of the egg stage

In insects generally, the development time falls rapidly as temperature increases above a lower threshold. The rate of this decrease also falls steadily as the temperature rises until, just below the upper threshold, the duration of development may increase again (Bursell, 1974a). Since this curve approximates a hyperbole its reciprocal should approximate a straight line, but in practice it is a shallow sigmoid (Messenger and Flitters, 1958; Howe, 1967; Bursell, 1974a). A number of workers have used the temperature-rate curve (using the reciprocal of the duration of development as an expression of developmental rate) to obtain an estimate of the developmental-hatching threshold (for example Singh, 1962; Roberts et al., 1970; Wightman, 1973; Madubuny and Koehler, 1974; Mussen and Chiang, 1974). There has been, however, some confusion in the literature with regard to the definition of thresholds. Bursell (1974a) outlines four threshold effects with regard to insect eggs:

a) the "developmental threshold", below which no development will occur,
b) the "developmental-hatching threshold" below which some development may occur but will not be completed.
c) the "hatching threshold" below which hatching will not occur, though development may or may not be completed. (Johnson (1940) found that the developmental-hatching threshold of C. lectularius was 13°C while the

hatching threshold was only 8°C).

d) the "hatching-survival threshold" as defined by Hodson and Al Rawy (1956). Below this, hatching may occur but the larvae will not survive to maturity.

The threshold estimated by the estension of the straight line portion of the rate curve is the developmental-hatching threshold using this terminology, although other terms have been used in the literature including "developmental threshold" (Wightman, 1973), "alpha temperature" (Madubuny; and Koehler, 1974), "highest low-temperature death point" (Huffaker, 1944), "minimum development temperature", "base temperature" (Roberts et al., 1970) and "threshold of development" (Singh, 1962). Terms such as "developmental threshold", "developmental zero", "minimum development temperature" and "threshold of development" are best reserved for the developmental threshold as defined above, which will generally be below the developmental-hatching threshold. In the present account the term "threshold" is used to refer to the developmental-hatching threshold unless otherwise stated.

Because the temperature-development curve is a shallow sigmoid rather than a straight line its extrapolation to estimate the threshold must be treated with some caution. Messenger and Flitters (1958) found that the relationship was very close to a straight line at medial temperatures for three species of fruit flies and extrapolation from this region gave a good approximation of that determined experimentally. The inclusion of the region of the curve below this gave an estimate which was too low. Kelvin (1934), however, found that with muscoid flies the deviation from a straight line to be too great for extrapolation. Howe (1967) considered that developmental limits were impossible to determine experimentally, but Messenger and Flitters (1958), using samples of about 1000 eggs of fruit flies obtained 0.1% and zero hatch temperatures 0.28°C apart and concluded that the threshold lay between the two. Nevertheless as the temperature approaches the threshold the hatch falls rapidly to zero and varies erratically from sample to sample (Howe, 1967). For this reason, and the often very long duration of development at these low temperatures careful extrapolation may be the most suitable method of estimation of the threshold.

It must also be noted that temperature may interact with other factors such as humidity, light, and adult diet and vigour (Howe, 1967). Messenger (1959) noted that the threshold is not a completely stable characteristic

but is modified by acclimation, variations in temperature during exposure and other factors, and Wightman (1973) found that the threshold in one population of Costelytra zealandica White (Coleoptera, scarabaeidae) was 7.9°C but that in another it was 5.7°C.

A straight line drawn through the three points of the developmental rate-temperature plot determined from the present study yielded an intercept of 12.1°C (Fig. 2.8). In view of the above discussion this estimate of the threshold can at best be regarded as approximate, but as no eggs hatched and all quickly died at 10°C, the threshold should lie between 10 and 15°C which is in agreement with this figure.

Howe (1967) suggests that much of the careful analysis to which data is subjected is invalidated by inaccuracies in determining the developmental period. He suggests that the hatch period should be covered by at least 10 observations, a criterion satisfied in the present study. A more serious problem however is temperature maintenance. Since the cabinets used in the present study at both 20 and 25.5°C relied on convection or intermittent fanning some doubts can be raised as to their reliability. However, standard errors of the means of the parameters used to measure duration of development were small, suggesting that temperature variation from place to place within a single cabinet was small. On the other hand monitoring of temperatures was less rigorous than was desirable and the thermometer was accurate to 1°C only. Results must therefore be treated with some caution.

The lethal effects of 4 and 10°C, both sub-threshold temperatures, show a similar pattern to the results of Gross et al. (1972b), who found a reduced tolerance to 7.2 and 12.7% in less mature eggs. They used the mean developmental time for eggs laid in summer of 17 days reported by Young et al. (1950) as a basis for the assumption that some of the eggs in the 15-21 day old group and most in the 22-28 day old group were mature. However, the results from the present study indicate that at 26.7°C most eggs in the 15-21 day old group would have been mature. This assumption is supported by a sharp increase in survival from the 8-14 day old to the 15-21 day old group under most conditions at 7.2 and 12.7°C in the work of Gross et al. (1972b). The higher of these two temperatures is slightly above the threshold estimated in this study. It is known, however, that acclimation can alter the threshold and that thresholds can vary between populations and this indicates that 12.7°C could well be below the threshold for the population studied by Gross et al. (1972b) who, in addition, do not state which species or strain of Graphognathus was used.

A similar trend of higher susceptibility to cold in less developed eggs has been found in other species, for example Diatraea grandiosella Dyar (Lepidoptera, Pyralidae) (Stewart and Walton, 1965); Conoderus vespertinus Fabr. (Coleoptera, Elateridae) Turnipseed and Rabb, 1965); Trichoplusia ni Hübner (Lepidoptera, Noctuidae) (Kishaba and Henneberry, 1966); Tribolium confusum Duval (Coleoptera, Tenebrionidae) (Watters, 1966); Plodia interpunctella Hübner (Lepidoptera, Pyralidae) (Cline, 1970); and H. postica (Morrison and Pass, 1974). In the species investigated by Kishaba and Henneberry (1966) and Morrison and Pass (1974), a sudden increase in sensitivity to cold occurred at the time of head capsule formation. The experiments reported here did not examine the effect of egg maturity on susceptibility to cold, and although it appears from the work of Gross et al. (1972b) that this decreases with increasing maturity, further work is required before firm conclusions can be drawn.

2.3.3 Second Season

2.3.3.1 Assessment of survival

It became apparent at an early stage that a considerably smaller proportion of eggs were hatching and that they were taking longer to hatch than was observed during the first season. Moisture levels in tubes used during the second season were higher than those in the petri dishes used during the first season, and this was suspected as contributing to these results. For example, of one sample of 98 eggs, only 14% hatched but the tube was flooded by condensation. When this was drained off and the eggs allowed to dry a further 30% hatched. For this reason the tubes were alternatively allowed to dry out and then flooded as described above, resulting in a substantial increase in the proportion which hatched in some instances. However, additional factors were probably also involved in the reduction and delay of hatching.

As already described eggs were at first checked daily but later on this became very difficult because of the large numbers of samples, so the tubes were covered and checked four weeks later. There was also a transitional series left for a portion of the four week period only, and as the time of enclosure increased a sharp decline in the proportion of eggs hatching occurred and an unpleasant odour developed in many of the jars, suggestive of anaerobic microbial activity. Any larvae that had hatched were dead and very few eggs hatched after the jars were opened. Rather than introduce any further changes, it was decided to continue with this method since by the time this trend had become obvious a large number of

samples had started this treatment. This experiment was rendered almost worthless due to virtually complete mortality of the eggs. Some compensation was provided however by a set of 26 batches placed at 25.4°C and checked daily as for eggs allocated to the experimental temperatures but some weeks later. Furthermore two jars were set up as for those used to assess survival but with 0.5 and 1% copper sulphate solution respectively instead of distilled water. In the former jar few eggs hatched but the smell was barely discernible, whilst in the latter hatching wass markedly increased and there was no unpleasant smell. From the 13 batches of greater than 10 eggs in the jar with 1% $CuSO_4$ a hatch of 5.4% of 411 eggs was obtained. For the total of 18 batches (429 eggs) the overall hatch was 5.1%. This compares with 1.8% from 26 batches totalling 836 eggs laid approximately six weeks previously and checked daily as already described.

It is concluded that microbial growth resulted in anoxia coupled with a build-up of toxic gases such as carbon dioxide and hydrogen sulphide. To prevent this the gravel could have been sterilized before use and 1% or stronger $CuSO_4$ solution or some other bacteriostatic solution which would not reduce the humidity significantly should have been used.

2.3.3.2 Some effects of temperature on survival

As with the first season's work a small proportion of eggs hatched after the arbitrary time limit had been exceeded. However, their effect on the overall results was negligible and they have not been included.

The results of work carried out during the second season are summarized in Table 2-IV. Plots of cumulative percent hatch against time for each temperature are given in Fig. 2.9 and of survival in relation to temperature in Fig. 2.10. The variation in percent hatch within any temperature treatment was higher in relation to total egg hatch than that observed during the first season (the standard error of the means being 19% of the mean or more compared to less than 8% of the mean in the first season).

In addition, differences in the viability of the different groups of weevils from which eggs were allocated were found (see Chapter Three). Fortunately, eggs allocated to survival assessment as part of the programme listed in Table 2-II were laid early enough to avoid the difficulties associated with much of that section of the work which have been described above. No significant difference was found in a comparison by one way analysis of variance of the test samples with each other or with the

Table 2-IV Hatching of G. leucoloma eggs during first season

Temp. °C	No. of Samples	Total No. of eggs	Time to first hatch (days)	Inclusion of tail enders	Mean duration of egg stage (days)	Median duration of egg stage (days)	Rate of development (% per day) = 100/median	Percent hatch	Range of time taken to hatch (days)
15	2	216	72.7	no	98.9 ± 2.0	93.2	1.07	28.7	72.7 - 139.5
				yes	104.1 ± 3.0	94.5	1.06	31.1	72.7 - 197.6
20	8	833	21.8	no	26.5 ± 0.1	24.4 ± 0.2	4.10 ± .03	64.3 ± 4.9	21.8 - 39.8
				yes	26.6 ± 0.1	24.4 ± 0.2	4.10 ± .03	64.4 ± 5.0	21.8 - 48.9
25.5	13	1056	14.1	no	17.8 ± 0.1	16.9 ± 0.3	5.92 ± .11	66.0 ± 4.3	14.1 - 32.9
				yes	19.1 ± 0.3	17.0 ± 0.4	5.88 ± .14	67.9 ± 3.9	14.1 - 93.9

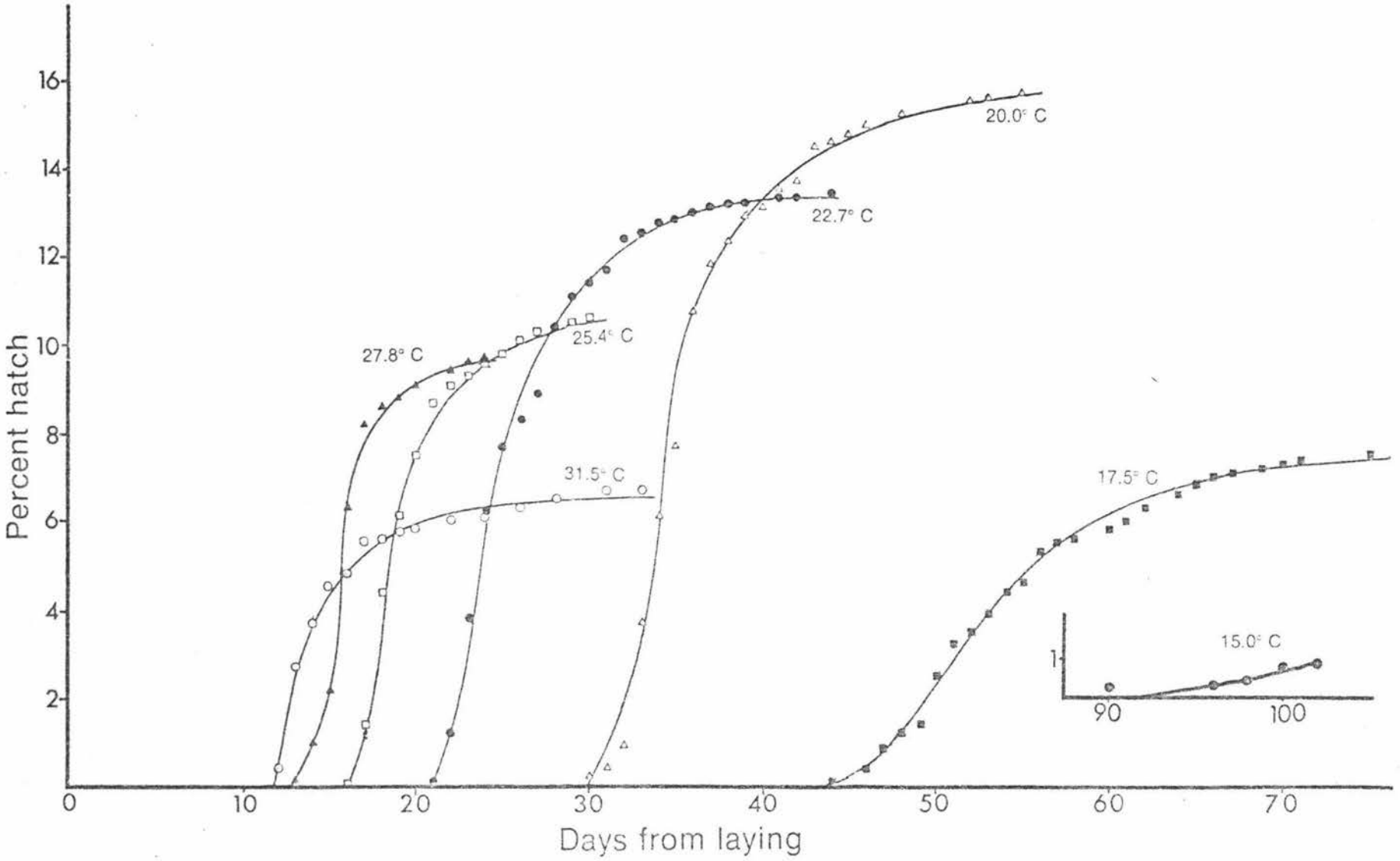


Fig. 2.9 Cumulative hatch curves at specified temperatures (second season).

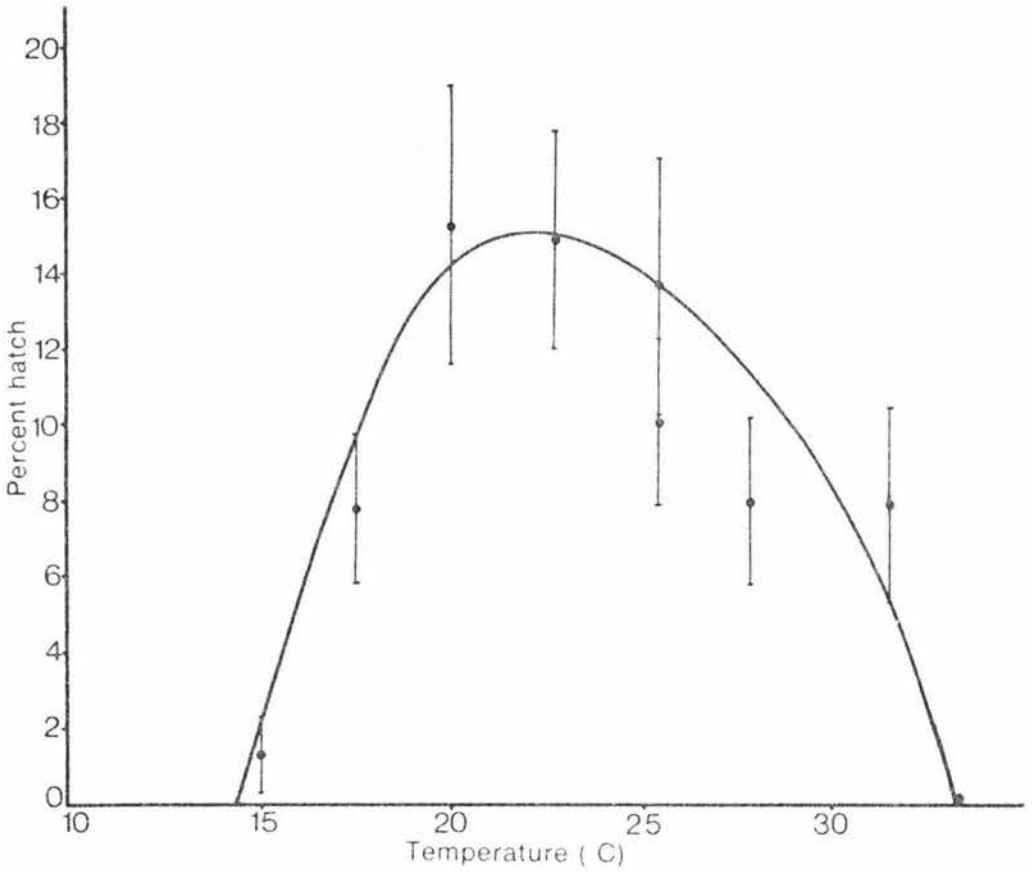


Fig. 2.10 Survival of eggs in relation to temperature (second season).

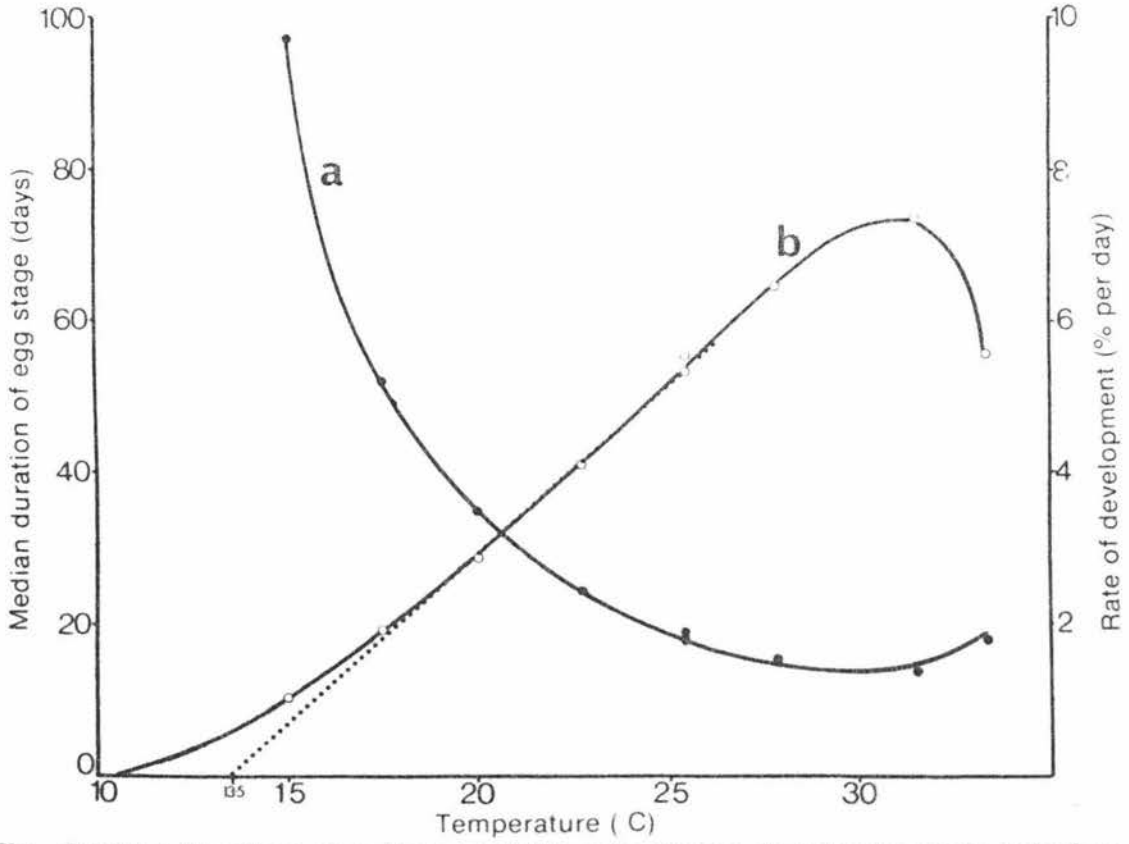


Fig. 2.11 (a) Median duration and (b) rate of egg development in relation to temperature (second season).

appropriate survival assessment eggs (Section 2.2.2.2) but there was a highly significant difference between eggs collected from adults in lots one and two and those collected from the other lots and placed at 17.5°C and 20.0°C ($P < .01$ in both cases). At 15.0°C and 33.3°C too few eggs hatched for analysis to be valid. No eggs hatched at 35.2°C or 37.4°C.

Few conclusions regarding the effects of temperature on survival can therefore be drawn, except that eggs will hatch at least from 15°C to 33.3°C. Because of the very low overall hatch (a maximum of 15.8% at 20.0°C) the upper thermal limit cannot be reliably estimated, although it is probably close to 35°C. It is possible that samples placed at 15.0°C and 33.3°C were adversely affected by parental age (see Section 2.3.3.4), but in view of the results obtained during the first season the reduction in hatch at 15°C is probably at least partly due to temperature.

2.3.3.3 Effects of temperature on the duration of the egg stage

The developmental period was much less variable within a treatment than was survival of eggs but was slightly more variable than during the first season (Table 2-V). The medians were estimated from the cumulative batch curves presented in Fig. 2.9. Plots of median developmental time and median developmental velocity (100/median time) against temperature (Fig. 2.11) yield the typical curves described by Bursell (1974a) in spite of the duration of development being somewhat greater at both 20°C and 25.4°C than it was during the first season. The durations of development at 15°C, 20°C and 25.5°C during the first season are consistent with what could be predicted from Fig. 2.11 for 15.2°C, 22.7°C and 26.5°C respectively during the second season.

If the velocity curve (Fig. 2.11b) is extended downward it cuts the temperature axis at 10.6°C. It is, however, more usual to extend the straight line portion of this curve and this gives an estimate of the threshold of 13.5°C, which is somewhat higher than that obtained during the first season. The reasons for these differences are not clear. Errors in measuring temperatures, particularly during the first season, may be partly responsible but it is unlikely that this was entirely responsible. Another possible explanation is the higher level of moisture in the tubes (Section 2.3.3.1). During the first season very few eggs hatched during flooding, although this may have been due to the limited time of flooding (six hours). However, the result of allowing the tubes to dry out duringthe second season as already described, is a further indication that excessive moisture may inhibit hatching. The use of

Table 2-V

Effects of temperature on survival and duration of development of G. leucoloma eggs - 2nd season

Temp. °C	No. of samples	Total No. of eggs	Percent of total eggs hatching*	Mean percent hatch (mean of individual samples) ± sem	Time to first hatch (days)	Mean time taken to hatch (days) ± sem	Median time taken to hatch (days)	Rate of development (percent per day) = 100 / median
15.0 ± 1.5	30	910	0.8	1.3 ± 1.0	90	96.6 ± 1.8	97.0 ± 0.5**	1.03
17.5 ± 1.5	36	1148	7.3	7.8 ± 2.0	44	55.0 ± 0.8	52.0 ± 0.5	1.92
20.0 ± 1.5	34	917	15.7	15.3 ± 3.7	30	36.6 ± 0.4	34.8 ± 0.5	2.87
22.7 ± 0.05	44	1611	13.4	14.9 ± 2.9	21	26.3 ± 0.3	24.4 ± 0.5	4.10
25.4 ± 0.1	39	1496	9.6	10.1 ± 2.2	17	20.1 ± 0.2	18.8 ± 0.5	5.32
25.4 ± 0.1	36	1210	11.7	13.7 ± 3.4	16	19.8 ± 0.3	18.0 ± 0.5	5.56
27.8 ± 0.05	30	973	9.8	8.0 ± 2.2	13	16.7 ± 0.3	15.5 ± 0.5	6.45
31.5 ± 0.5	37	1320	6.7	7.9 ± 2.6	12	16.0 ± 0.5	13.6 ± 0.5	7.35
33.3 ± 0.1	28	1011	0.2	0.2	17	18	18.0 ± 0.5	5.56
35.2 ± 0.1	32	1188	0	0	-	-	-	-
37.3 ± 0.1	31	971	0	0	-	-	-	-

* = Total no. hatching / Total no. of eggs x 100

** Error of reading from graph

tubes instead of petri dishes meant that a relatively greater area of side wall was available to act as a "catchment" for condensation. In any event it is unlikely that anoxia was responsible for the differences particularly at lower temperatures as the jars were opened daily. One further possibility is that the increase in developmental times was intrinsic in the eggs, and this may also be an explanation of the low rates of survival. This may have resulted from some factor or factors affecting the adult weevils since the adults laid fewer eggs than has been reported in the literature for those fed on artificial diet (Barnes and Bass, 1973).

It is possible to make few comparisons with the literature in regard to egg viability and even less with regard to developmental time. The only developmental time reported for a stated temperature was by Bass and Barnes (1969) who found that at 26.7°C 90% of eggs hatched, beginning at 12 days with an average of 21 days before eclosion. Thus their stated time of first hatch is the same as that reported here for 31.5°C during the second season and predicted for 26°C during the first. The mean, however, is equivalent to that obtained or predicted for temperatures below 25°C in both seasons even if the tail enders are included. It must also be pointed out that there are three species of Graphognathus in the U.S.A. and several recognised strains of G. leucoloma, and the above authors do not state which species or strain they used, so it is quite possible that these differences are due to their having used different species or strains from that occurring in New Zealand. Elsewhere, percentage hatches similar to or higher than those obtained here during the first season and substantially higher than those obtained during the second have been published. Young et al. (1950) give egg viability as over 90% in summer while Barnes and Bass (1973) report 93% and 60% eclosion on an artificial diet and lucerne respectively. The viabilities reported by the latter authors also demonstrate that adult diet may affect the eggs.

Finally, it must be pointed out that the results obtained during the present study do not indicate the ability of the larvae to develop into fertile adults. Gross et al. (1972b) found differences in the survival of larvae of "Graphognathus spp" from eggs kept under different conditions and Hodson and Alrawy (1956) found that nymphs of Oncopeltus fasciatus Dallas (Hemiptera, Lygaeidae) which hatched near the thermal limits failed to develop into adults even though a high proportion of eggs had hatched in some cases. Therefore it can be expected that the range of suitable temperatures for full development of G. leucoloma will be narrower than that for egg hatching.

2.3.3.4 Effect of parental age on egg viability

Eggs laid in early May by adults kept during the first season showed a lower hatch than eggs laid in February or March (approximately 25% and 64% respectively at 25.5°C) whereas eggs laid in both February and March showed a similar hatch.

The results obtained during the second season are given in Table 2-VI

Table 2-VI Viability of samples collected at different times and incubated at 25.4°C

Date of collection of eggs	No. of eggs	Percent hatch at 25.4°C	Comments
23/2/76 - 19/3/76	1496	10.1 ± 2.2	Checked daily
24/2/76 - 20/3/76	1210	13.7 ± 3.4	Checked daily
13/4/76 - 28/4/76	836	1.8 ± 0.8	Checked daily
7/6/76 - 14/6/76	411	5.4 ± 0.5	Tubes enclosed over 1% $CuSO_4$ for one month before checking

Although the results indicate the possibility of a downward trend in egg viability with increasing parental age, firm conclusions cannot be drawn and more rigorous experiments designed to test this point are required.

2.3.4 The Shadehouse Experiment

A total of 821 eggs in 48 batches were maintained in the laboratory, 425 eggs in 14 batches at 100% RH in the shadehouse and 153 eggs in 8 batches exposed to ambient conditions in the shadehouse. Of these 5.8%, 0.9% and 0.7% respectively, hatched, with a further 1.4% of the eggs from 100% RH in the shadehouse hatching after they were moved to 20°C in the laboratory. In view of the described problems with the eggs maintained and the very low hatches obtained with all the eggs from weevils kept in the shadehouse conclusions cannot be drawn with regard to comparisons between eggs from laboratory and shadehouse maintained adults and between laboratory and shadehouse maintained eggs from the latter.

2.3.5 Effect of T. putrescentiae on G. leucoloma eggs

The mean percentage hatches of eggs infested with mites and those not infested were 29 ± 7.2 and 22 ± 8.9 respectively. This difference was not significant and there was no consistent reduction in hatching of samples in

which mites were found during the studies of the effects of temperature and humidity during the first season. The conclusion reached was that the mites had no effect on egg viability.

2.4 GENERAL CONCLUSIONS

The following conclusions were drawn regarding the eggs of G. leucoloma. Firstly, eggs hatch at least over the range 15°C to 33.3°C, the developmental-hatching threshold is probably between 11°C and 14°C, and the upper lethal limit is probably close to 35°C. The eggs can withstand levels of dessication and high temperatures in excess of those likely to be encountered in the field in New Zealand but will not hatch unless moist conditions prevail. Dry conditions may retard development very slightly and there is some evidence that excessive moisture will inhibit hatching. This may ensure that eggs only hatch when the soil is moist (e.g. after rain) but not when it is so wet as to drown larvae or inhibit their entry into the soil. Anonymous (1969) reports that a heavy, week long rain can kill many small larvae. Cold, wet conditions may reduce survival to some extent, particularly amongst eggs laid late in the season, and sub-threshold temperatures are lethal to newly laid eggs but tolerance may increase as development proceeds. The viability of eggs may decline slightly as the age of the adults increases.

The following suggestions may be made concerning any future investigations in the light of the experience gained in these studies. Firstly, the eggs themselves should be randomly allocated to different temperature treatments instead of the adults being randomly allocated into groups and then eggs being allocated from specific groups to specific temperatures. The original method broke down because some factor or factors intervened to make originally identical lots of adults behave differently (see Chapter Three), whereas if the eggs had been allocated randomly this would not have affected the temperature treatments.

Relatively larger samples should be placed below 17.5°C and above 31°C to delineate more accurately the lower and upper temperature limits for development. A more sensible approach to maintaining the eggs would perhaps be to reduce the total number of samples to a level which allows their daily checking and samples could be taken periodically (say every four weeks) in order to assess the effects of parental age on egg viability. Nevertheless, even if these changes had been incorporated into the present study, the very low proportion of eggs which hatched during the second season would still have cast some doubts on the validity of the results obtained.

It would also seem desirable, before carrying out any further work on the effects of temperature, to explore various methods of maintaining

eggs so as to define optimum conditions for hatching. The fact that eggs may develop fully and remain quiescent until suitable conditions for hatching prevail demonstrates the independence of these two processes. The method used during the first season produced results but required much space and time and is not suitable for use with waterbaths (which are more accurate than temperature cabinets) unless a method can be devised for keeping the jars submerged and stable. However, a direct comparison of the two methods should be made. Since there was virtually no hatch at 95% RH but an apparently unhibited hatch at 100% RH the threshold for full hatching must lie at or close to saturation. As there is evidence of free moisture inhibiting hatching some investigation of humidities between 95% and 100% may be fruitful in reducing problems with condensation without inhibiting hatching. One suggestion is the use of saturated potassium dichromate solution, which maintains a relative humidity of 98% at 25°C (Solomon, 1951), since it would effectively reduce microbial growth in the water (or gravel if the latter is used). Other possibilities are very dilute sulphuric acid or sodium hydroxide solutions. Once satisfactory results are obtained the work reported in this Chapter could be repeated with the changes suggested or others having the same effect being implemented.

Another important consideration is post-hatching survival, which can be approached from two points of view. Firstly, the survival of larvae from eggs kept at different temperatures is particularly important in view of the findings of Gross et al. (1972b) described above regarding survival of larvae from different temperature and humidity treatments. Alternatively, the survival of larvae which hatch at different times during the hatch period may be important. It was noted, particularly during the first season, that the tail enders were apparently much weaker than those hatching close to the median time. Therefore larvae could be taken at frequent intervals as hatching proceeds and kept separately under identical conditions to assess survival. In ecological terms it is the survival from one generation to the next which matters, and in this respect the "tail enders" may not be significant.

CHAPTER THREE

STUDIES OF VARIABILITY AND REPRODUCTIVE CAPACITY IN ADULTS AND PUPAE

3.1 INTRODUCTION

It is a matter of common observation that variability is a normal property of living organisms. This variability can be spectacular amongst the insects as in the New Zealand brentid Lasiorhynchus barbicornis Fabr. in which size may vary almost five-fold in males and three-fold in females (Meads, 1976). Other species may have a number of morphs, often with intermediate forms (e.g. Ittamies, 1971), while features normally to show a continuous variation about a mean (or more than one mean if a number of strains exist).

Considerable variability in the viability of G. leucoloma eggs has already been described in Chapter Two. The approach taken during the second season was an attempt to assess the effects of temperature on the eggs more accurately (Chapter Two) and to investigate variability in a number of features in addition to egg viability.

If a relationship between reproductive capacity and adult size exists in G. leucoloma such a knowledge may be useful in assessing the reproductive potential of field populations. However, as diet has been shown to affect fecundity (Young et al., 1950; Barnes and Bass, 1973; East, 1976) and egg viability (Barnes and Bass, 1973) to a considerable extent any relationship between size and reproductive potential must be considered together with diet, climate, and any other factors which may reduce the actual reproductive output below the potential maximum.

An attempt was also made to compare weevils kept under semi-field conditions with those maintained in the laboratory. A readily accessible situation with conditions at least approximating those which could be expected to occur in crops but which provided protection from weather extremes, wandering farm and domestic animals and vandalism was required. The shadehouse was suitable for this purpose.

3.2 MATERIALS AND METHODS

3.2.1 Collection and Maintenance of Weevils

Pupae and apparently mature larvae were collected by digging from the four sites listed below:

Site one: A lucerne crop on Number One Dairy Unit, Massey University.

Site two: Pasture separated from site one by a metalled farm road.

Site three: A lucerne crop near the Manawatu River at Longburn. The insects were collected only from large patches of weeds, which consisted almost entirely of yarrow (Achillea millefolium) with some grasses, clover and other weeds.

Site four: Pasture adjacent to site three.

The pupae were placed in individually identifiable cells in plastic ice cube trays (90 cells per tray) and were weighed as soon as possible after collection, but some moulted to the adult stage before this could be done. The trays were wrapped in plastic film and stacked on moist sand in a plastic aquarium which was covered with a sheet of glass and kept in a controlled temperature cabinet at 20 $\pm$ 1.5°C with a 15 : 9 hr day : night light regime. Trays were checked twice daily for any adults that had hatched. The adults had almost completely darkened after four days, when they were removed, weighed, randomly allocated into 10 lots of approximately 30 and placed in the individually identifiable containers described below. Any which showed signs of abnormalities were rejected.

Adults were maintained singly in 200cm³ polystyrene "ice cream sundae" containers with waxed cardboard lids, and which were modified by the insertion of a No. 3 stainless steel entomological pin through the centre of the base, supported by a small piece of cork underneath (Fig. 3.1). A small piece of an agar based artificial diet (Barnes and Bass, 1973) was placed on the pin to provide food for the adults and was replaced daily. Preliminary work with the diet during the first season showed that the adults readily laid eggs under pieces of it if these were placed on the floor of the cage. Eggs laid in this situation were frequently difficult to recover and count, and placing it on the pin held it to the floor of the container, and so avoided the problem. The composition and preparation of the diet are described in Appendix Two.

A drop of water was placed in the container and replenished whenever necessary to maintain a high humidity so that the diet would not dry out

Fig. 3.1 Container used to house adults singly during the second season (natural size)

ad	artificial diet
op	aluminium foil ovipository


ad
op

as fast and to provide drinking water for the weevil if it was required. The humidity inside the containers was not measured. A small piece of aluminium foil, as was used during the first season's work was placed in each container as an oviposition site.

One lot of 32 weevils was maintained in the shadehouse as described below and the remainder were initially kept in two constant temperature cabinets at 20 ± 1.5°C with a 16 : 9 hr day : night photoperiod. They were transferred to the constant temperature room when it became operational, and were there maintained under the same conditions of temperature and photoperiod as in the cabinets.

All containers were examined daily for signs of feeding and for the presence of eggs. Any eggs which were present were counted, removed and used as described in Chapter Two. An attempt was made to ascertain the time from eclosion to commencement of feeding by noting signs of feeding on the diet together with the production of faeces. The first indicator proved to be very unreliable in practice, because the diet often dried to some extent, was not cut cleanly, or because it was damaged while being placed on the pin. Therefore large quantities of the diet had to be consumed before a reliable determination of feeding could be made, and this did not happen often. The second indicator also proved to be unreliable in some cases because small fragments of diet, which frequently broke away, often resembled faeces when dry, so the record of the commencement of feeding was not complete.

Any dead weevils were removed, the date of death noted and the pronotum length and width and head width were measured. Pronotum length was measured dorsally and medially, pronotum width at the widest point and head width across the rear margins of the eyes.

All of the above procedures allowed the following data to be recorded for individual weevils: pupal weight of most, adult weight four days after eclosion, head width, pronotum width, pronotum length, pre-oviposition period, longevity, age when each batch of eggs was laid, the number of eggs in each batch, and the proportion of many of those clumps which hatched.

3.2.2 Shadehouse Experiment

The shadehouse consisted of an enclosure set into a hillside facing north-east at the top of a valley so that the top of the shadehouse was level with the flat hill top. Three sides were formed by the hill and the top and front were constructed of wire netting on a wooden frame. In addition the top was covered with black plastic netting with a 2mm mesh

("shade cloth") (Fig. 3.2). The front of the enclosure was shielded by trees approximately 12m high and some 12m from it. A row of trees some 2.5m high was sited 2m from the north-west side.

A shelter was constructed inside the shadehouse to protect the containers and weevils from rainfall and from heating by direct sunlight. It consisted of a sheet of hardboard approximately 1m square supported on wooden pegs about 30cm high at the rear and 20cm high at the front. The rear was further protected by a sheet of softboard leaning against the hardboard top (Fig. 3.3).

Temperature was recorded by a Tomo thermohygrograph which was checked daily against a thermometer measuring to 0.5°C. Both were housed under the shelter along with the weevils. Humidity was not recorded since the containers were closed and a drop of water was placed inside as already described, and because difficulties were encountered in calibrating the humidity element of the thermohygrograph. However, conditions in the shadehouse were fairly damp for much of the time, particularly later in the season.

Fig. 3.2 The shadehouse

The shelter beneath which the weevils were kept (arrow) can be seen through the left-hand door.

Fig. 3.3 The shelter beneath which the adults were kept, with the cover removed, as seen from the left-hand door of the shadehouse

cw container used to house weevils individually.

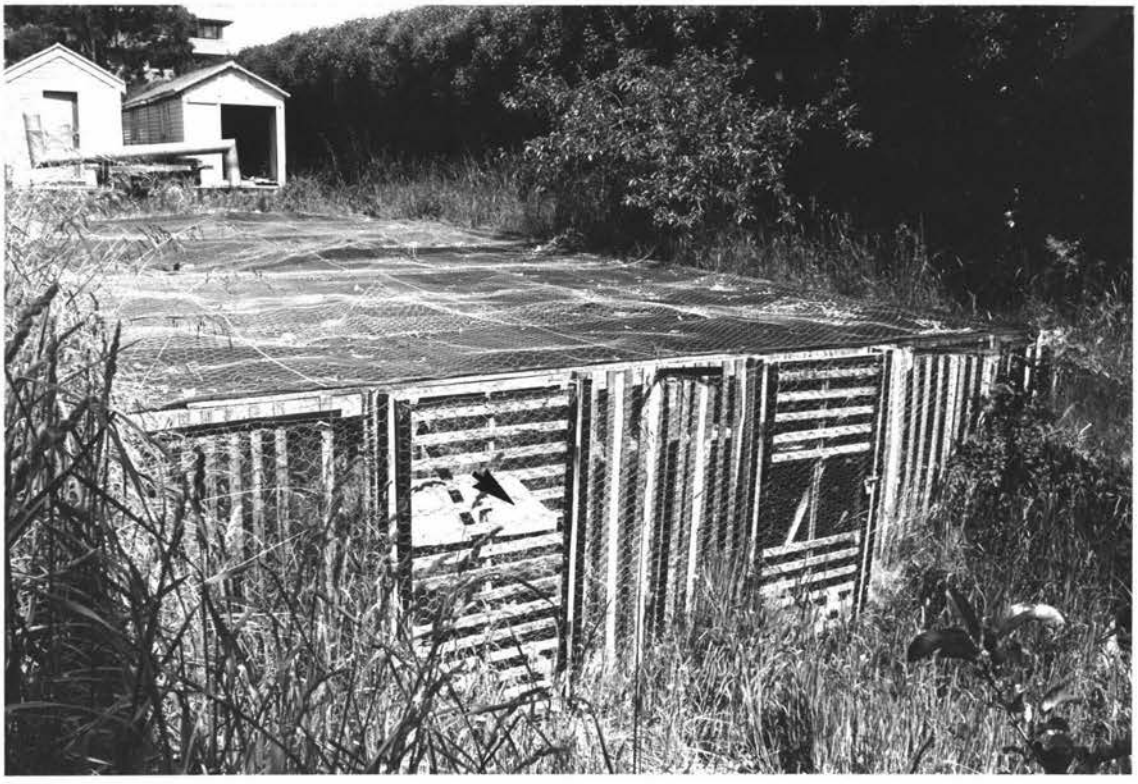
ea area behind shelter where eggs exposed to ambient conditions were placed.

es jar containing eggs kept at saturated humidity.

hc hardboard cover.

sb softboard shield.

tg Thermohygrograph.




hc
sb
tg
ea
es
cw

3.3 RESULTS

3.3.1 Size Characteristics of Adults and Pupae

The weights of adults and pupae were analysed separately for each collection site and the results are given in Table 3-I. Size did not differ significantly between sites one and two (the Massey sites) or between sites three and four (the Longburn sites) but those from the Massey sites were highly significantly heavier than those from the Longburn sites ($P < .01$). For this reason the data for the two Massey sites was combined, as was that for the two Longburn sites, in histograms of pupal and adult weight distribution (Figs. 3.4 and 3.5 respectively). Adult weight was highly significantly correlated ($P < .001$) with the linear dimensions measured and with pupal weight (Table 3-II).

3.3.2 Longevity, Pre-oviposition Period and Reproductive Capacity

Most weevils commenced feeding between 12 and 20 days from eclosion, with an average of about 15 days and an observed range of 8-28 days. The mean pre-oviposition period, longevity, egg batch size and fecundity for each of lots one to nine, for the total of lots one to nine and three to nine and for lot 10 (the weevils kept in the shadehouse) are given in Table 3-III. Fecundity is given both including and excluding those weevils which did not lay any eggs.

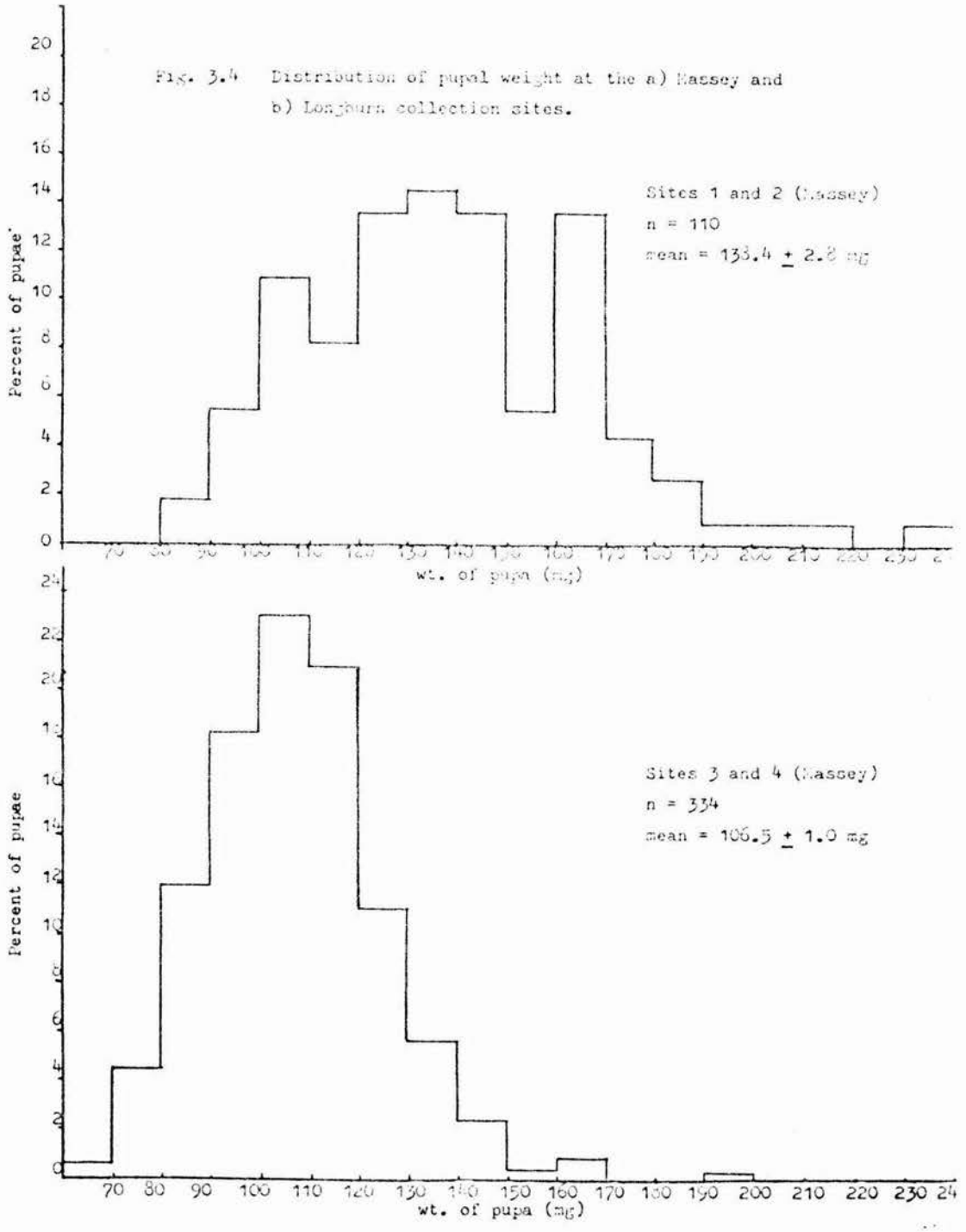
Considerable differences were observed between lots for some data, so lots one to nine were compared by one-way analysis of variance. There were no significant differences in pre-oviposition period or egg-batch size, whereas differences in longevity and number of eggs per female were significant ($P < .05$). However, if lot one was omitted the differences were not significant ($P \simeq 0.27$ in both cases) and if lot two was also omitted the value of P rose above 0.4. For this reason only data from lots three to nine were used in calculation of coefficients of linear correlation and in plotting histograms as these lots could definitely be regarded as belonging to the same population.

Longevity ranged from 12-273 days with a mean of 93 $\pm$ 3 days in lots one to nine and from 25-273 with a mean of 97 $\pm$ 3 days in lots three to nine (Fig. 3.6) and the pre-oviposition period ranged from 29-75 days and averaged 48.5 $\pm$ 0.7 days (Fig. 3.7). Egg production was extremely variable, ranging from 0-1032 eggs with a mean of 170 $\pm$ 14 for lots three to nine (Fig. 3.8) and 152 $\pm$ 11 for lots one to nine and 22% of the weevils failed to lay any eggs (21% of lots three to nine). Weevils frequently

Table 3-I Weight Characteristics of Pupae and Adults from Different Collection Sites

Collection site	Pupae				Adults			
	n	Mean ± sem (mg)	s.d.	Range (mg)	n	Mean ± sem (mg)	s.d.	Range (mg)
1. (Massey Lucerne)	50	141.2 ± 3.4	24.1	96 - 203	32	116.4 ± 3.2	18.0	83 - 151
2. (Massey Pasture)	60	136.1 ± 4.2	32.3	81 - 236	52	110.5 ± 3.2	22.9	72 - 178
1 + 2	110	138.4 ± 2.8	28.9	81 - 236	84	112.9 ± 2.3	21.3	72 - 178
3. (Longburn Lucerne)	277	106.8 ± 1.0	17.3	69 - 165	219	88.1 ± 0.9	13.8	50 - 134
4. (Longburn Pasture)	57	104.8 ± 2.7	20.6	70 - 192	56	84.7 ± 2.3	16.9	59 - 146
3 + 4	334	106.5 ± 1.0	17.8	69 - 192	275	87.4 ± 0.9	14.5	50 - 146
Total	444	114.4 ± 1.2	25.2	69 - 236	359	93.4 ± 1.0	19.5	50 - 178

Fig. 3.4 Distribution of pupal weight at the a) Massey and b) Longburn collection sites.



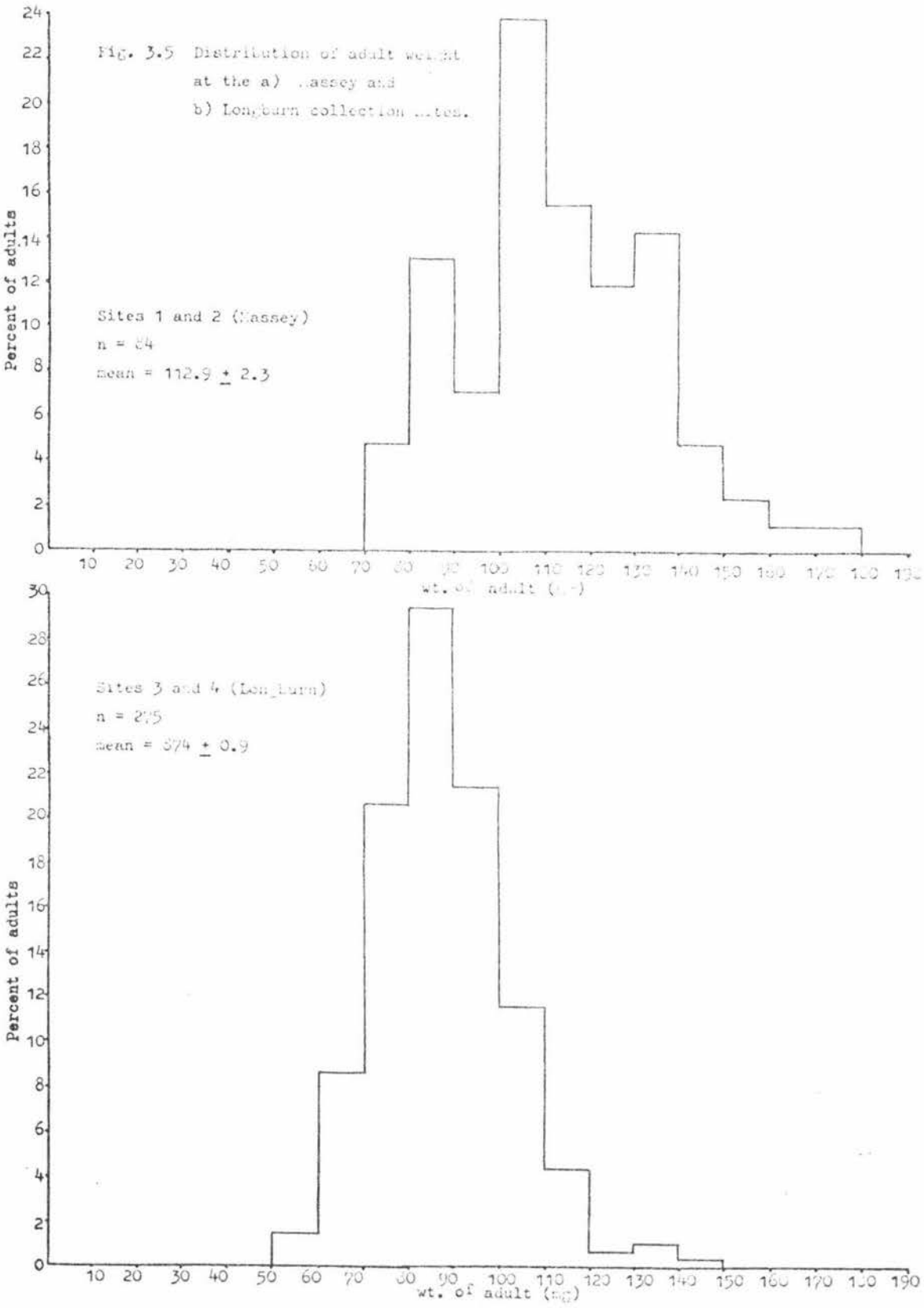


Fig. 3.5 Distribution of adult weight at the a) Massey and b) Longburn collection sites.

Table 3-II Size characteristics of adults used in the experiments and of the pupae from which they emerged.

Variate	No. in sample (n)	Mean ± sem	s.d.	Range	Correlation with adult wt.
Adult wt. (mg)	297	92.2 ± 1.1	18.5	50 - 176	-
Pupal wt. (mg)	264*	112.8 ± 1.4	23.4	70 - 217	0.97
Pronotum length (mm)	297	2.633 ± 0.01	0.16	2.33 - 3.13	0.88
Protonum width (mm)	297	3.37 ± 0.01	0.20	2.94 - 4.16	0.84
Head width (mm)	297	2.31 ± 0.01	0.14	2.08 - 2.82	0.93

* Some pupae moulted before they could be weighed

Table 3-III Longevity, Pre-oviposition Period and Reproductive Capacity of *G. leucoloma*

Lot No.	Pre-oviposition period (days)				Longevity (days)				Mean egg-batch size				Total egg production				Total egg production per weevil excluding those that did not lay			
	No. in sample	Mean ± sem	s.d.	range	No. in sample	Mean ± sem	s.d.	range	No. in sample	Mean ± sem	s.d.	range	No. in sample	Mean ± sem	s.d.	range	No. in sample	Mean ± sem	s.d.	range
1	21	54 ± 2.3	10.6	36-69	30	70 ± 3.9	21.3	12-113	21	30 ± 3.2	14.8	9-20	30	65 ± 12	66	0- 237	21	93 ± 13	61	9- 237
2	22	47 ± 2.0	9.2	34-64	29	83 ± 4.9	26.2	34-130	22	32 ± 2.5	11.5	11-55	29	111 ± 22	117	0- 467	22	147 ± 24	113	11- 467
3	25	51 ± 1.8	8.9	35-72	29	92 ± 5.5	29.8	34-143	25	28 ± 1.8	9.2	2-51	29	167 ± 26	141	0- 554	25	193 ± 27	134	2- 554
4	22	48 ± 2.5	11.9	31-68	28	96 ± 9.2	48.7	28-234	22	26 ± 2.5	11.7	7-43	28	144 ± 31	166	0- 758	22	183 ± 36	167	21- 758
5	25	48 ± 2.1	10.4	31-75	30	102 ± 7.7	42.1	31-205	25	27 ± 2.4	11.9	4-58	30	150 ± 30	162	0- 682	25	180 ± 32	162	4- 682
6	20	51 ± 2.2	9.8	35-73	30	110 ± 10.9	59.5	27-244	20	34 ± 2.8	12.6	10-67	30	175 ± 34	185	0- 609	20	262 ± 37	167	20- 609
7	22	47 ± 2.5	11.7	29-65	29	97 ± 11.0	59.2	36-273	22	26 ± 2.2	10.3	6-46	29	182 ± 55	295	0-1032	22	240 ± 68	319	6-1032
8	27	46 ± 1.9	9.9	30-68	30	110 ± 7.5	40.9	38-192	27	32 ± 1.8	9.2	13-48	30	241 ± 34	188	0- 668	27	268 ± 34	179	40- 668
9	21	46 ± 1.7	8.0	36-63	29	83 ± 9.0	48.5	25-233	21	27 ± 3.0	13.9	2-56	29	129 ± 40	215	0- 897	21	179 ± 51	235	4- 897
total 1 - 9	205	49 ± 0.7	10.2	29-75	264	93 ± 2.7	44.6	12-273	205	29 ± 0.6	11.8	2-80	264	152 ± 11	184	0-1032	205	195 ± 13	187	2-1032
total 3 - 9	162	48 ± 0.8	10.2	29-75	205	97 ± 3.3	47.9	29-273	162	29 ± 0.9	11.4	2-67	205	170 ± 13.9	199	0-1032	162	215 ± 16	201	2-1032
10	19	62 ± 3.3	14.3	40-93	32	87 ± 6.0	33.9	10-133	19	31 ± 4.0	17.2	3-47	32	48 ± 10	56	0- 198	19	81 ± 12	51	3- 198

Fig. 3.6 Longevity of weevils in lots 3 - 9

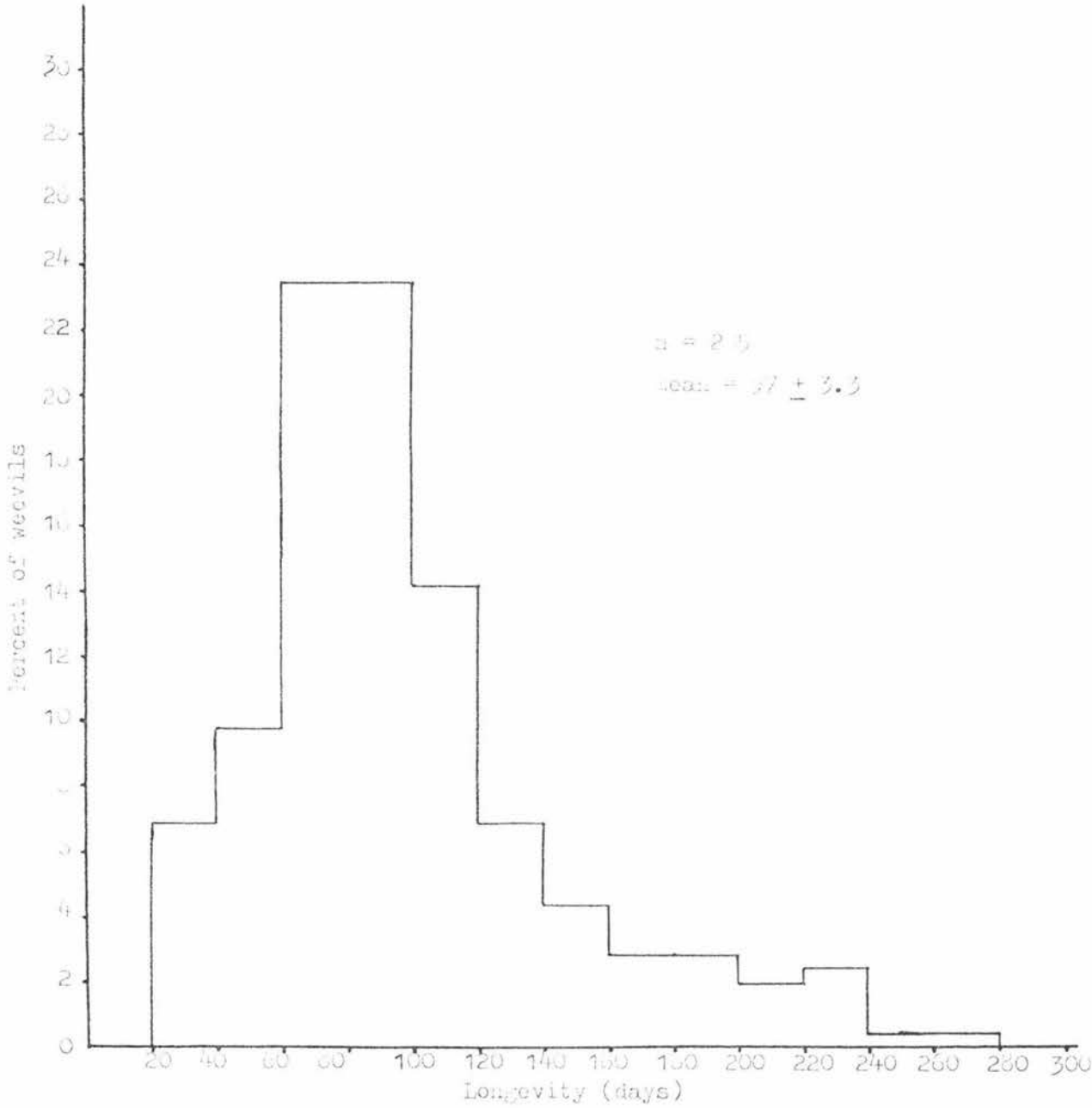
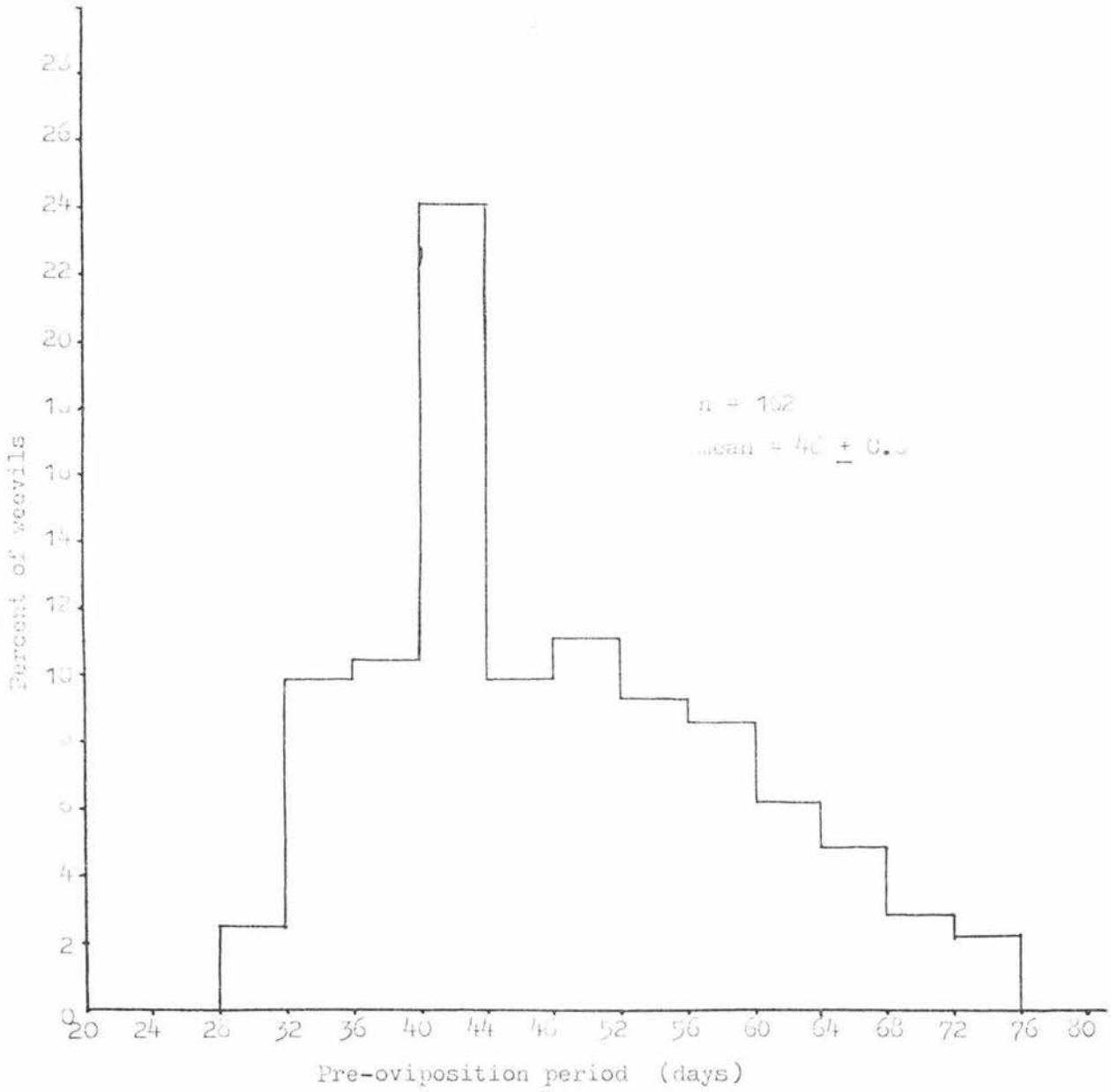


Fig. 3.7 Pre-oviposition period of weevils in lots 3 - 9



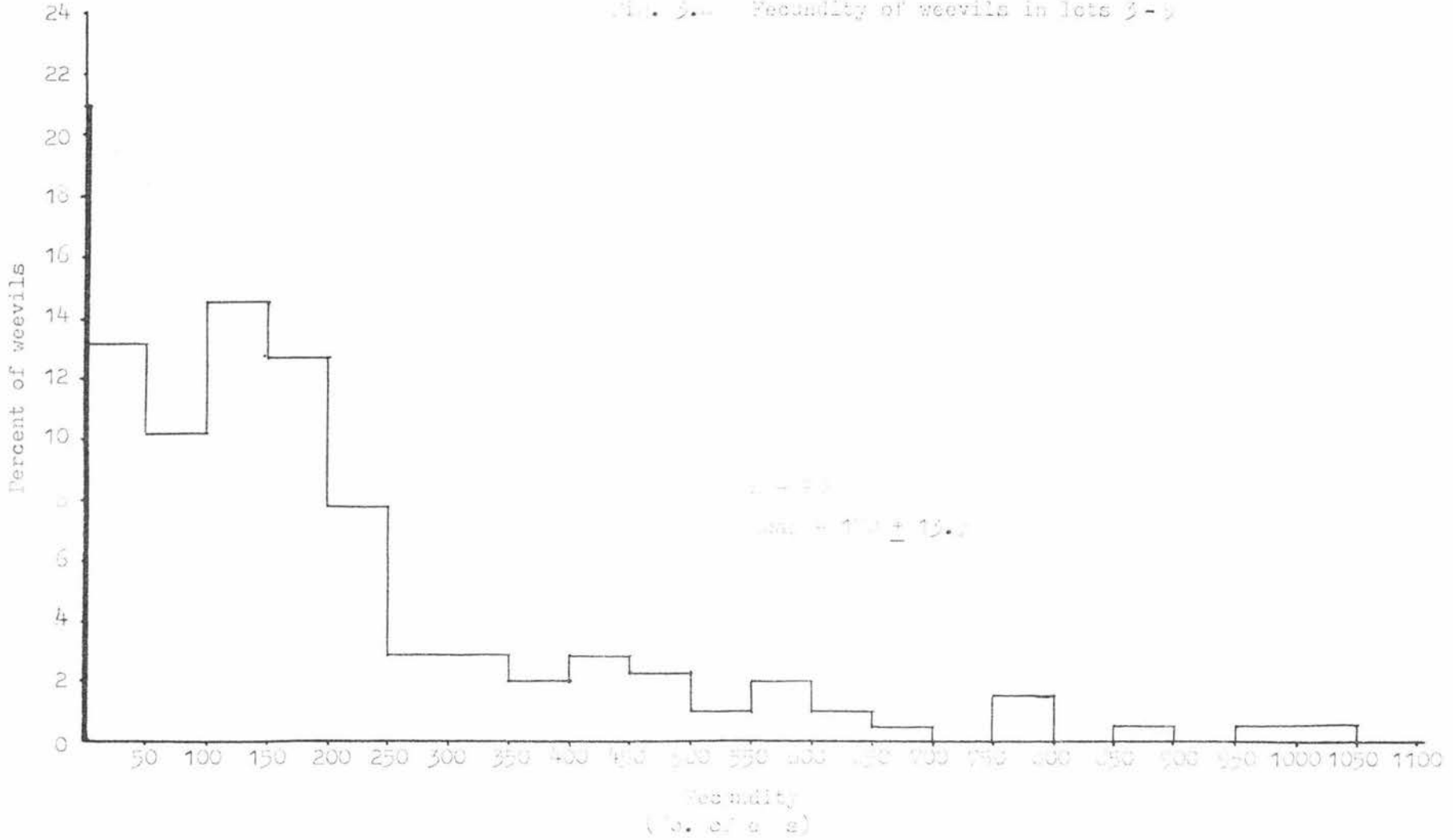
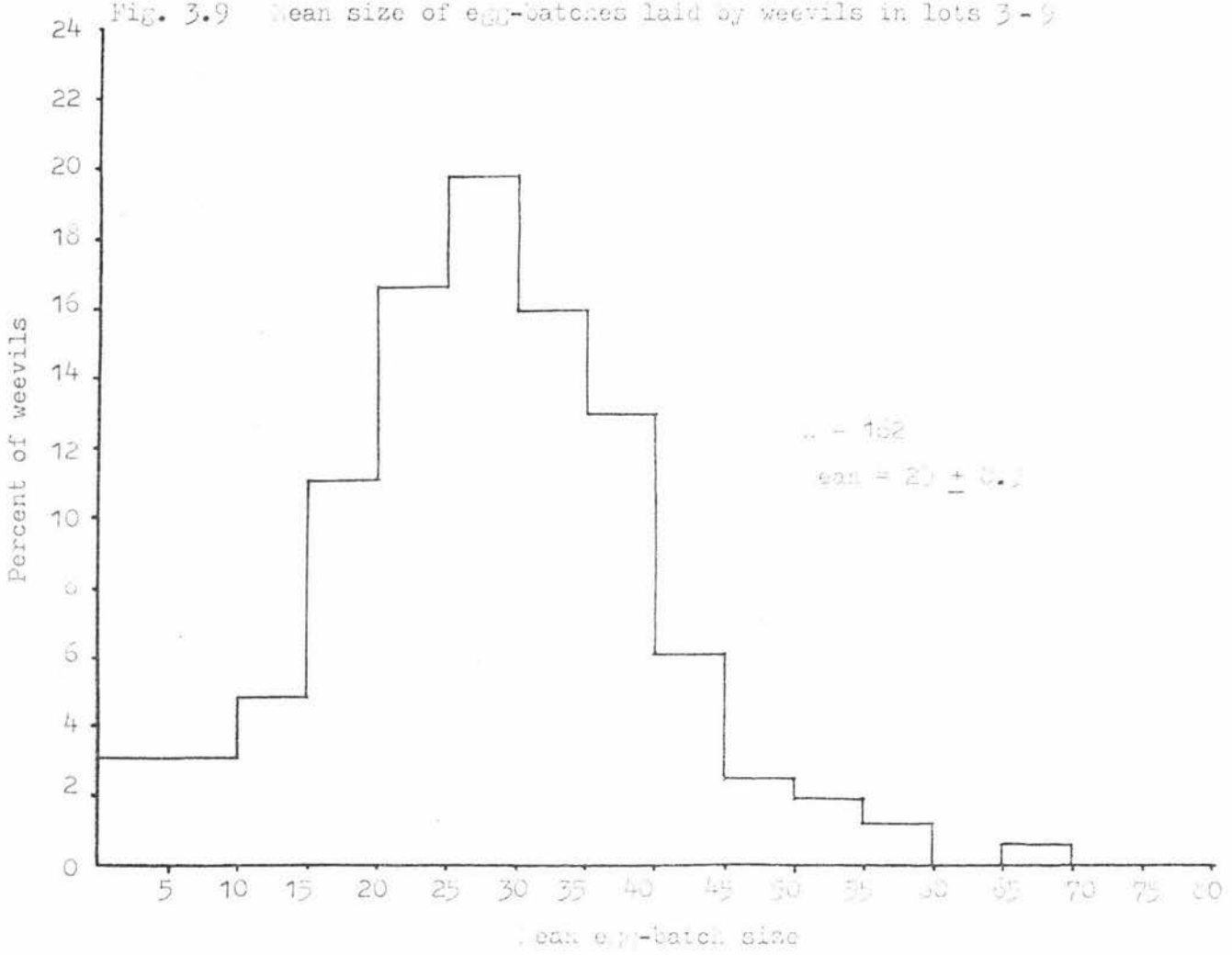

Percent of weevils
24
22
20
18
16
14
12
10
8
6
4
2
0
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000 1050 1100

Fig. 3.9 Mean size of egg-batches laid by weevils in lots 3 - 9



laid eggs in several dumps and both clump and batch size varied widely from a single insect. Any disturbance during oviposition resulted in the immediate cessation of egg laying, but weevils evidently moved of their own accord quite frequently while laying. Egg batch (and clump) size varied from 1-105 eggs and the mean batch size for individual weevils ranged from 2-80 eggs (2-67 in lots three to nine) with an overall mean of 29 ± 1 (Fig. 3.9) but mean clump size was undoubtedly somewhat lower than this.

No relationships were found between adult size and longevity, pre-oviposition period, total egg production, mean batch size or egg viability, and there was no difference between weevils from different collection sites. A comparison of batch size with egg viability yielded contradictory results with a small negative coefficient of linear correlation which was significant in some samples but not others.

3.3.3 Shadehouse Experiment

The results of this experiment are summarised in Table 3-III. Mean batch size and longevity were within the range of these of weevils maintained in the laboratory, with means at 31 ± 4 and 67 ± 6 respectively. Longevity ranged from 10-133 days, individual batch sizes from 2-67 eggs and means of batch size ranged from 3-47 eggs. Pre-oviposition period ranged from 40-93 days with a mean of 62 ± 3 days and was significantly greater than that of laboratory maintained weevils ($P < .01$). Total egg production ranged from 0-193 eggs with a mean of 40 ± 10 and was significantly lower than that of all of lots two to nine considered singly and significantly lower than that of lots one to nine collectively ($P < .01$ in all cases) but was not significantly different from fecundity in lot one. Thirteen of the 32 weevils in lot 10 failed to lay eggs.

3.4 DISCUSSION

Differences in sizes of individuals from different collection sites can be related in part to population density which was substantially higher at the Longburn sites than the Massey sites. However, in both cases density was much greater in the lucerne crops than in adjacent pasture and was very patchy in all four sites. Other factors must therefore be involved: different nutritional states of the plants at the various sites may be involved but other factors such as soil type, compaction and microclimate may be very important, and no conclusions can be drawn from a simple study such as this.

The close relationship between linear dimensions and weight was to be expected and demonstrates that any of the measurements made in this study can be used as a reliable indicator of size of individuals.

The lack of a relationship between the size of individuals and egg production contrasts with the positive relationships between size and potential or actual fecundity which have been found in some other species. For example, Schmidt and Blume (1975) found that significant correlations existed between pupal weight and head width and between these measurements and ovariole number in the horn-fly Haematobia irritans L (Diptera, Muscidae) and Farrell (1973) found that the initial body weight in C. zealandica was significantly correlated with fecundity and that longer lived females were slightly heavier than short lived females. Geier (1963) demonstrated a relationship between weight of virgin females and potential fecundity in Laspeyresia pomonella L. (Lepidoptera, Tortricidae), but Wearing and Fergusson (1971) found that fecundity was highly variable and was different on different apple cultivars and in different seasons and they could not find a relationship between weight and actual fecundity, even on the most suitable apple cultivar. Presumably, therefore, factors which limit egg laying in the field are important enough to override factors intrinsic in the moths. Spiller (1964) found that Anobium punctatum Degeer (Coleoptera, Anobiidae) females laid from zero to 123 eggs with a mean of about 55 and that in approximately 60% of adults fecundity was highly significantly correlated with weight, while the remainder laid fewer eggs than the weight-fecundity relationship of the first 60% would predict. He did not arrive at any explanation for this observation.

However, in view of the low fecundity and egg viability reported here, these results should be treated with caution as a relationship may become apparent under ideal conditions. Whether a relationship exists between

egg clump or batch size and egg viability is also doubtful and although a negative relationship has been suggested in the present study no conclusions can be drawn.

The differences in longevity and egg production amongst lots were a complicating factor with regard to the analysis of data in both this and the previous chapter. No explanation can be offered as all lots were treated identically. It appears that lot one and to a lesser extent lot two were acted upon by some factor or factors after the weevils were allocated and the result was a reduction in viability.

However, the weevils as a whole demonstrated an unexpectedly low egg production and long pre-oviposition period in addition to the low egg viability described in Chapter Two. Egg production was substantially below that reported by Barnes and Bass (1973) for weevils fed on an artificial diet and lucerne (means of 783 and 500 eggs respectively, and by East (1976) for weevils fed on samfoin (631 eggs) and clover, lotuses and lucerne (313 eggs) but was similar to that of weevils fed on Desmodium spp (165 eggs) and substantially higher than that of those fed on grasses (10 eggs). However, East used young field collected adults which may have laid some eggs before being collected, so their true fecundity is not known.

The pre-oviposition period was also much longer than had been expected. Adults are present in the field for 3-4 months (mid December to early April) in the Manawatu (M.J. Esson, personal communication) although May (1975) reports adults as being present from mid-September to early June with peak numbers from mid-November to late January, but her observations may relate to the north of New Zealand only. Thus the seven week pre-oviposition period reported here would amount to virtually half of the season and this would be a highly unlikely occurrence in the field.

Longevity was slightly below the mean of 105 days reported by Barnes and Bass (1973) for weevils fed on the artificial diet used in the present study but was greater than the mean longevity of 84 days of weevils fed on lucerne by the same authors. It was slightly to substantially higher than that reported by East (1976) of weevils fed on a range of diets, although it must be noted that as with the observations on fecundity described above the true longevity of his weevils cannot be known.

It is probable that the high proportion of adults which did not lay eggs was related to the low average fecundity, low egg viability and long pre-oviposition period. Dissection of a number of non-layers did not

reveal any abnormalities apart from a single specimen in which the tip of the spermatheca was forked. Some, however, had not fed and while little development of the reproductive system had occurred in some, considerable numbers of apparently normal eggs were present in others. These observations appear to indicate that the insects used in this study were exposed to some influence(s) which reduced viability substantially. There are a number of possible contributing factors which could account for this and those which possibly affected egg viability by acting directly on the eggs have already been discussed in Chapter Two. Diet has been shown to have a marked effect on longevity and fecundity (Young et al., 1950; Barnes and Bass, 1973; East, 1976), and it is possible that the diet was deficient, possibly because of poor quality of one or more of the ingredients or because of deterioration after preparation or something in the method of preparation. The diet was made up in batches which were stored in petri dishes in the refrigerator for up to 2-3 weeks until needed. Considerable care was exercised in preparation and no post-preparation deterioration was evident. In some instances the diet dried out completely after being fed to the weevils and frequently dried slightly, so some deterioration in food value was likely at this stage, but it was usually reasonably moist when it was replaced. Even when the diet appeared to be completely normal when it was replaced it was very rarely largely consumed, so there appeared to be always an adequate supply of food. It is however quite possible that the diet was unpalatable and that this resulted in reduced consumption. This relates particularly to the quality of the ingredients, which could not be assessed, but it is possible that as the diet was developed in the U.S.A. the ingredients such as lucerne leaf meal could be different and more work is required to evaluate this diet under New Zealand conditions.

A further possibility is that the polystyrene containers were toxic to the weevils. Hutt and White (1972) demonstrated that polystyrene was toxic to codling moths and it seems likely that it could have contributed in this case. Further experimentation is needed to clarify this point. These suggestions do not, however, explain the differences which occurred between lots, and, although they may be related to the overall poor viability of the weevils, these differences remain unexplained. All of the variates considered here require further study and conditions for maintenance of adults should also be further investigated.

The increased pre-oviposition period and reduced egg production observed in the shadehouse are easily explained on the basis of the lower

mean temperatures recorded there. This experiment has not been successful in testing the applicability of laboratory results to the field, largely because of the very low egg production and viability found in both situations in the present study, which casts doubt on the validity of any results obtained in relation to any other situation, and it appears likely that the same detrimental factors which operated in the laboratory also operated in the shadehouse.

CHAPTER FOUR

STUDIES ON THE STRUCTURE AND COMPOSITION OF THE EGG SHELL AND CEMENT OF G. leucoloma

4.1 INTRODUCTION

The egg shell of a terrestrial insect must protect it from physical injury, dessication, and attack by microorganisms and predators. It must therefore contain elements which provide strength, waterproofing and resistance to chemical attack while still allowing gaseous exchange to occur. Since the oxygen molecule is larger than the water molecule, the requirements for waterproofing and gaseous exchange are therefore in some ways contradictory.

Beament (1946a) described the chorion as "that part of the egg lying outside the oocyte cell membrane, which is secreted by the follicle", and considered that the vitelline membrane was not formed by the follicles but from the oocyte cell membrane; "the egg cell membrane, ... becomes the vitelline membrane, and is the base or substrate for the deposition of the chorion" (in the hemipteran Rhodnius prolixus Stahl). King and Koch (1963), working with Drosophila melanogaster Meigen (Diptera, Drosophilidae), showed that the vitelline membrane was in fact formed by the follicle cells using the egg cell membrane as a substrate. This is also true of a number of other species (de Wilde and de Loof, 1974).

The gross morphology and size of insect eggs varies widely between species. They may be sausage shaped, oval, round or conical (Chapman, 1971) and may possess an operculum or cap which facilitates hatching, as in R. prolixus (Beament, 1946a), while others possess a pair of hatching lines between which the structure of the chorion may differ markedly from that of the rest of the egg (e.g. Hinton, 1967, 1969; Jones, 1972). Some eggs possess horns or other processes which extend from the surface and which frequently have a respiratory function (e.g. Hinton, 1961; Klug, Campbell and Cummings, 1974). Also, many possess more or less complex surface sculpturing or ridges (e.g. Stringer, 1968; Hinton, 1969, 1970; Rowley and Peters, 1972; Audo, 1973; Salkeld, 1973), and hence egg morphology can be of taxonomic value (e.g. Horsfall, Voorhees and Cupp, 1970). Most insect eggs possess specialised openings through the chorion, the micropyles, which allow for the entry of sperm but the number and form at the micropyles varies greatly from species to species, and in some species, in which the eggs are fertilized in the ovary, micropyles

may be absent (Chapman, 1971).

The structure of the chorion is frequently very complex and shows considerable variation between species, but some basic features are common to many species: most chorions consist of several regions and possess a system which allows gas exchange while conserving water (Hinton, 1970). There is frequently a layer consisting of struts which separate a thin inner layer from the bulk of the chorion and enclosing air spaces (the trabecular or inner meshwork layer). The inner layer is usually solid, as in Tetrix spp (Orthoptera, Tetrigidae) (Hartley, 1962), Calliphora erythrocephala Meigen (Diptera, Calliphoridae) (Anderson, 1960), R. prolixus (Hinton, 1969) and Atrachya menetriesi Faldermann (Coleoptera, Chrysomelidae) (Ando, 1973), but in the Muscidae (Diptera) it forms an open meshwork (Hinton, 1962, 1967, 1969; Hinton and Cole, 1965), and in Hyalophora cecropia L. (Lepidoptera, Saturniidae) it consists of a porous layer (Smith, Telfer and Neville, 1971). The extent of the inner meshwork layer varies widely, occupying 20% or more of the chorion in some Diptera (e.g. Hinton, 1967) but less than 1% in H. cecropia (Smith et al., 1971). The outer layers of the chorion are even more variable, although there is generally a more or less solid region which may be penetrated by aeropyles. Outside this there may be a further meshwork layer such as occurs in muscoid flies (Hinton, 1967, 1969), and in some species there is a thin, often perforated layer outside this (Hinton, 1962, 1967). In many species, however, the outer meshwork layer is absent and the aeropyles open directly onto the egg surface as in Galleria mellonella L. (Lepidoptera, Pyralidae) (Barbier and Chauvin, 1974) and A. menetriesi (Ando, 1973). On the other hand, a middle region, comprising some 20% of the chorion and containing a continuous network of fine passages which connect with the aeropyles, occur in H. cecropia (Smith et al., 1971). In Cerura vinula L. (Lepidoptera, Notodontidae) the chorion consists of more than 40 distinct gas filled layers (Hinton, 1969) while the chorion of the egg of Lytta viridana Le Conte (Coleoptera, Meloidae) is only 0.2 - 0.3μm thick and consists of a single layer with no respiratory system (Sweeny et al., 1968). The chorion of L. viridana appeared to be chemically homogeneous, but Beament (1946a) found seven chemically distinct layers in the egg of R. prolixus and King and Koch (1963) found that the chorion of D. melanogaster consisted of two layers; the innermost of which contained a protein, a lipid and a neutral polysaccharide, and the outermost large amounts of acidic mucopolysaccharides. However, protein has been found to be the major component of insect egg shells

and Kawasaki, Sato and Suzuki (1971a,b, 1972, 1974) have found a number of protein fractions, although these authors also found varying but generally low levels of glucosamine and galactosamine in egg shells of species from several orders. A number of amino acid analyses have been made of insect egg shells which showed some common features but considerable variation occurred between species (Wilson, 1960; Furneaux and Mackay, 1972; Kawasaki et al., 1971a,b, 1972, 1974).

The vitelline membrane is generally thinner and simpler in structure than the chorion. In the phasmid Bacillus libanicus Uvarov, for example, it is some 3µm thick while the chorion is 30 - 50µm thick (Koscona, 1950) and in R. prolixus it is 0.2µm thick compared with a chorion of about 20µm (Beament, 1946a). In Amanthes c-nigrum L. (Lepidoptera, Noctuidae) it is only 0.2 - 0.3µm thick, but varies within a single egg (Salkeld, 1973), and in L. viridana it is only slightly thinner than the already thin chorion (Sweeny et al., 1966), while in dragon flies the vitelline membrane appears to be thicker than the chorion (Kawasaki et al., 1974).

No studies are known to me concerning the structure and composition of the egg-shell of a weevil, in spite of the fact that the Curculionidae comprise the largest family of any insect order. Any information in this respect would therefore be of value in adding to the overall knowledge of this aspect of insect biology, as well as for its intrinsic interest in gaining an understanding of how the egg of G. leucoloma meets the requirements of gas exchange and water conversation coupled with physical protection outlined above. Further interest is added by the fact that G. leucoloma is parthenogenic, since no parthenogenic species of higher insects appear to have been studied, and what has been done appears to be limited to the Phasmida, in which all species appear to possess a micropylar apparatus. The present study may also be of assistance in choosing ovicidal chemicals, since these must pass through the chorion or inhibit its function in some way to be effective.

This study consisted of an examination of the physical structure of the vitelline membrane, chorion, and cement of the egg of G. leucoloma by means of both scanning and transmission electron microscopy supplemented by Nomarski differential interference microscopy. In addition, a histochemical study was undertaken in order to elucidate in part the chemical nature of these structures, and was supplemented by some simple chemical tests.

4.2 MATERIALS AND METHODS

Details of the formulae and methods used are given in Appendix Three, and a synonym and source list of chemicals used is included in Appendix Four.

4.2.1 Transmission Electron Microscopy (TEM)

The eggs proved to be extremely difficult to fix and embed due to the impervious nature of the shell, so each egg was pierced with a fine pin when in the fixative. Clumps of eggs or portions of them were fixed in a buffered mixture of 3% glutaraldehyde and 2% formaldehyde (pH 7.2), post fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series followed by propylene oxide and embedded in Fluka epoxy resin. Pale gold ($\simeq$ 80nm) sections were cut with a diamond knife on an LKB ultramicrotome and the sections were subsequently stained with lead citrate and uranyl acetate, and examined using a Philips EM 200 transmission electron microscope. The shell of the eggs became very hard and brittle during embedding and this made section cutting difficult, resulting in many of the sections being of less than the desired quality.

4.2.2 Scanning Electron Microscopy (SEM)

Clumps of unpierced eggs were fixed as for TEM in glutaraldehyde/formaldehyde followed by osmium tetroxide. They were then frozen rapidly in freon 12 which was itself cooled with liquid nitrogen, placed in liquid nitrogen and fractured, and finally freeze-dried. The specimens were then mounted on aluminium stubs with conductive cement, sputter-coated with 10 - 20nm of gold in a Film-vac Minicoater and examined and photographed in a Cwikskan 100 field emission scanning electron microscope.

4.2.3 Light Microscopy

4.2.3.1 Histological methods

Similar difficulties occurred with respect to fixation and embedding the eggs as those encountered during preparation for TEM and the eggs were therefore also pierced with a fine pin during fixation. Most of the eggs were fixed in Carney's fixative or in Lillie's alcoholic lead nitrate fixative for mucosubstances (Pearse, 1968) but a few were fixed in 10% neutral buffered formalin or in the cetyl pyridinium chloride-formalin fixative for mucosubstances described by Pearse (1968). Studies on carbohydrates were carried out mainly on alcoholic lead nitrate fixed

eggs, while those on protein were carried out mainly on Carnoy fixed eggs. The eggs were dehydrated in ethanol and double embedded in celloidin and wax (paraffin + ceresin, congealing point 60°C) using the celloidin in methyl benzoate method (Gurr, 1962), which was the simplest method producing adequate sections. Sections were cut at 5µm or occasionally 7µm on a Reichert rotary microtome.

Some difficulty was experienced in attaching the section to slides; and the best results were achieved by floating ribbons on 0.25% gelatine solution on the slides, blotting them firmly with filter paper (Whatman No. 50) and placing the slides at 40 - 50°C in formaldehyde vapour overnight (Gray, 1954; Humason, 1962). Since formaldehyde blocks the reactions of many protein groups (Pearse, 1968), this method was unsuitable for mounting sections to be used for protein histochemistry and therefore the sections were instead floated on albuminised slides with 0.5% gum arabic, blotted firmly and dried (Gray, 1954).

Stained sections were examined using a Reichert Diapan microscope fitted with a Nikkon Microflex mode MFM.D double camera attachment and photographed using Ilford FP4 black and white film or Kodak EHB 135 colour film. Unstained sections were examined with the same microscope using both normal and Nomarski differential interference optics and photographed as above.

4.2.3.2 Histochemistry

4.2.3.2.1 Carbohydrates

The following techniques were used for the characterisation of carbohydrates:

a) 1% Alcian Blue 8GX at pH 1.0 or 2.5 for 30 minutes (Pearse, 1968).

b) Critical electrolyte method: 0.1% Alcian Blue 8GX at pH 2.5 and 5.7 in the presence of 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0M $MgCl_2$ for 18 hours (Scott and Dorling, 1965; Ashhurst and Costin, 1971a).

c) 0.02% Azure A at pH 1.5, 3.5 and 4.0 for 30 minutes and examined as aqueous mounts.

d) 0.5% aqueous Toluidine Blue for 30 seconds and examined as aqueous mounts (Ashhurst and Costin, 1971a).

e) Periodic acid Schiff (PAS) (Pearse, 1968).

f) Modified PAS reaction for uronic acid-containing glycosaminoglycans (Scott and Dorling, 1969; Ashhurst and Costin, 1971a,b).

g) Periodic acid-diamine (PAD) reaction (Spicer and Jarrels, 1961; Pearse, 1968).

h) Methylation sections were stained with 1% Alcian Blue 8GX at pH 1.0 or 2.5 after treatment with methanolic thionyl chloride for 30 minutes or 4 hours (Pearse, 1968).

i) Both the tetrazonium method (Pearse, 1960) and the diaminobenzidine method (Bussolati, 1971).

j) The carbodiimide reaction (Geyer, 1971).

k) Enzyme digestions:

i) Hyaluronidase. Sections were incubated in a solution of ovine testicular hyaluronidase and stained with Alcian Blue at pH 1.0 or 2.5.

ii) Neuraminidase. Sections were incubated in a solution of neuraminidase followed by staining with Alcian Blue at pH 2.5.

4.2.3.2.2 Proteins

Only a limited number of amino acids can be distinguished histochemically (Pearse, 1968): these include tyrosine, tryptophan, arginine, cysteine, cystine and amino acids with sidechain carboxyl groups (glutamic and aspartic acids). The methods used in the present work are described by Pearse (1960) or are modifications of them. Tyrosine was demonstrated by the diazotization-coupling method; tryptophan by the dimethylaminobenzaldehyde (D.A.B)-nitrite method or the "tryptophan method for formalin fixed tissues" (glyceric aldehyde condensation method); arginine by the Sakaguchi-dichloronaphthol method; cysteine by the dihydroxydinaphthyl disulphide (DDD) reaction; cystine by the DDD reaction using N-ethyl maleimide to block existing sulphydryl groups and thioglycollate to reduce disulphides to sulphydryls which react with the DDD reagent. The alcohol-ether washing schedule of the DDD reaction (Pearse, 1968) was increased as some reagent tended to remain in the tissues, giving a falsely positive staining reaction (Appendix Three). Side chain carboxyl groups were demonstrated with the mixed anhydride method. As a general protein stain the chloramine-T Schiff reaction was used, this demonstrates protein bound amino groups (Pearse, 1968) and a strong reaction would indicate a high level of amino acids with sidechain amino groups or proteins which consist of short polypeptide chains. A weak reaction would indicate the reverse or that the amino groups were bound in some way.

4.2.4 Chemical Tests Performed on Fresh Whole or Dechorionated Eggs

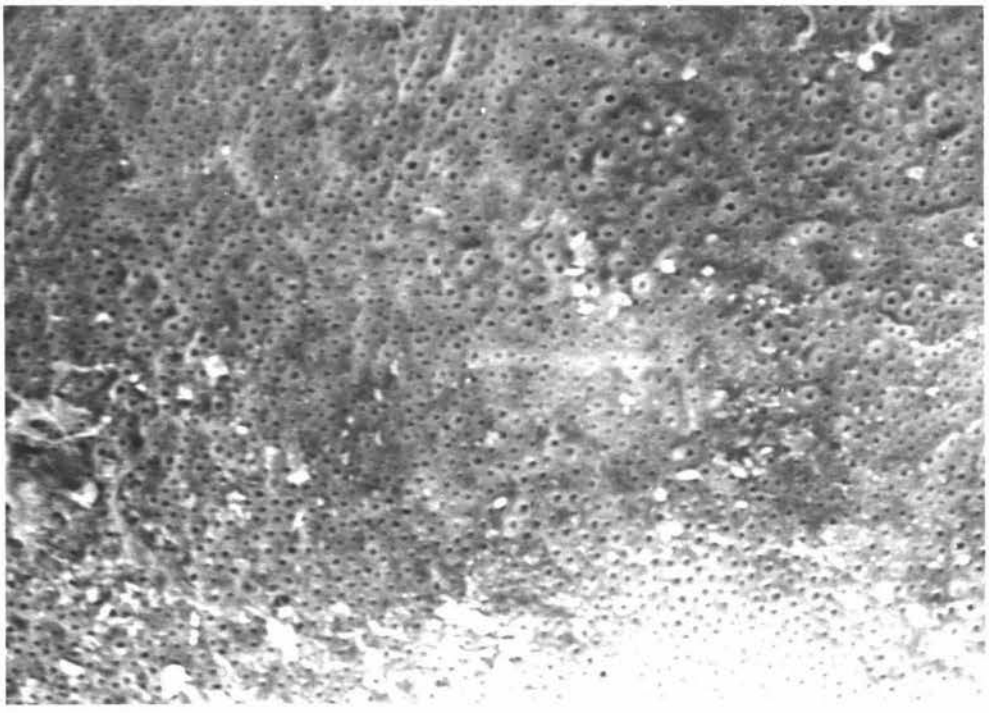
Clumps of eggs were treated with 1% NaOCl, 0.1M and 10% NaOH and 0.1M and 10% HCl, all in aqueous solution. They were also heated in 10% NaOH and HCl. Eggs were dechorionated by treatment for one to four hours with 1% NaOCl or by dissection after the chorions and cement were softened by treatment overnight with 2% aqueous chloramine-T. The latter softened, but did not dissolve the chorions. Dechorionated eggs were placed in octane for varying periods of time after which some were placed in distilled water while the others were exposed to the atmosphere for 30 minutes, together with untreated eggs as controls, before being placed in distilled water.

4.3 RESULTS AND DISCUSSION

The egg of G. leucoloma is white and measures on average 0.62 by 0.56mm (May, 1966). It is broadly oval but the chorion is flexible and the egg can be deformed to some extent, as can the chorion of some other insects (e.g. Ando, 1973). It lacks sculpturing or any protuberances and there are no micropyles, but aeropyles are distributed over the entire surface (Figs. 4.1 - 4.3). The lack of micropyles can perhaps be expected, as this is in line with the fact that this insect is parthenogenetic. The egg shell is composed of two readily distinguished parts; an inner, thin membrane, the rupture of which releases the egg contents, and a thicker outer layer. The terms vitelline membrane and chorion are used for these respective layers, and this terminology appears to be consistent with that in the literature on other species. The eggs are surrounded and held together in clumps by a variable layer of cement, which is liquid when the egg is laid but dries to form a tough mass. The cement is not an egg membrane.

4.3.1 Structure and Composition of the Chorion

The chorion of the egg of G. leucoloma varies from 4 - 9µm in thickness, and is composed of four regions (Figs. 4.4 to 4.6). The bulk of the chorion is solid except for a large number of aeropyles which run radially or slightly obliquely through it (Figs. 4.4 - 4.7). These occur over the entire surface of the egg (Figs. 4.1 - 4.3) and are approximately 1µm in diameter at the surface but soon constrict to a diameter of from 0.25 - 0.5µm, with the narrowest regions close to their inner and outer ends. The aeropyles communicate directly with a region approximately 0.2 - 0.5µm wide (the trabecular layer) which consists of interconnecting air spaces bridged by struts of varying thickness. Directly below this is a solid, homogeneous inner layer of approximately the same thickness. There is no difference between the appearance of the material comprising these three layers under the electron or light microscopes (see below). The inner surface of the chorion is, however, lined by a layer 20 - 25nm thick which is more electron dense than the bulk of the chorion but this is not visible under the light microscope. The aeropyles have a lining which appears to be distinct from the cement and which is about 10nm thick (Figs. 4.4, 4.5, 4.8a). Their surface is also irregular, with ridges running around the canal at right angles to its long axis. The lower regions of the pores are sometimes, but not always, blocked by a very thin plug of material which is similar in



25µm

2·5µm

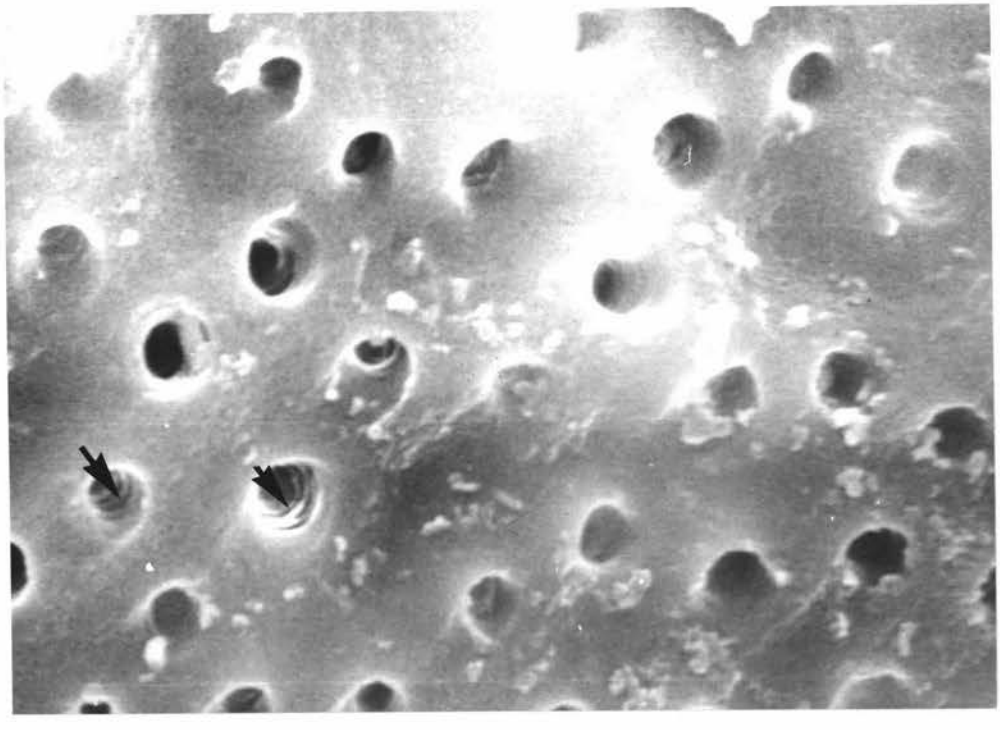
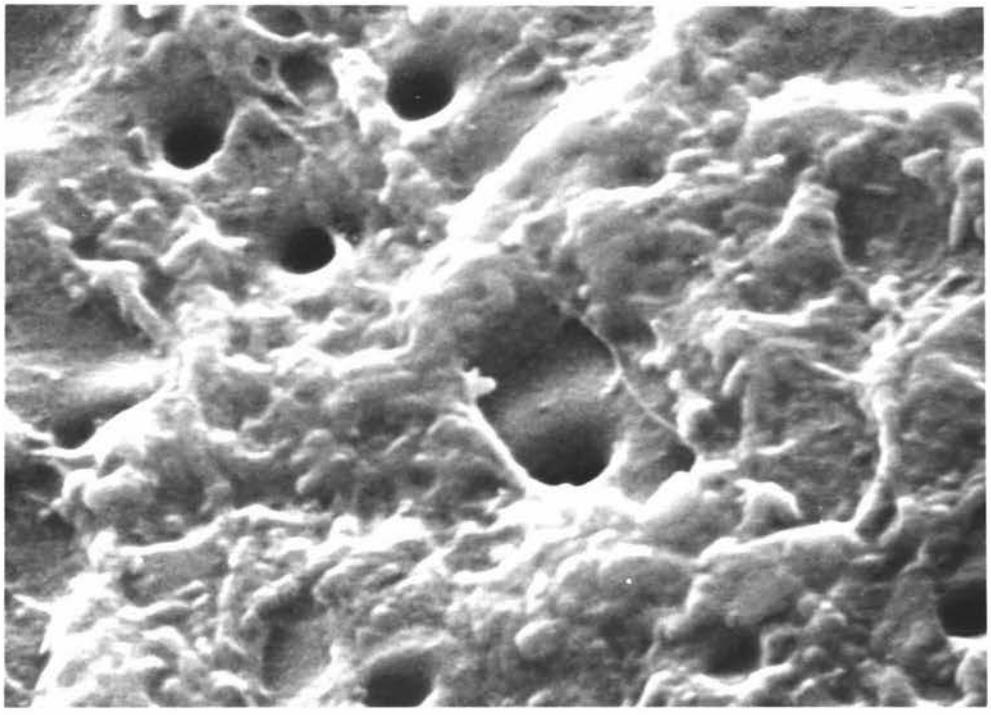


Fig. 4.3 Scanning electron micrograph showing pores exposed through gaps in the cement



2·5 μm

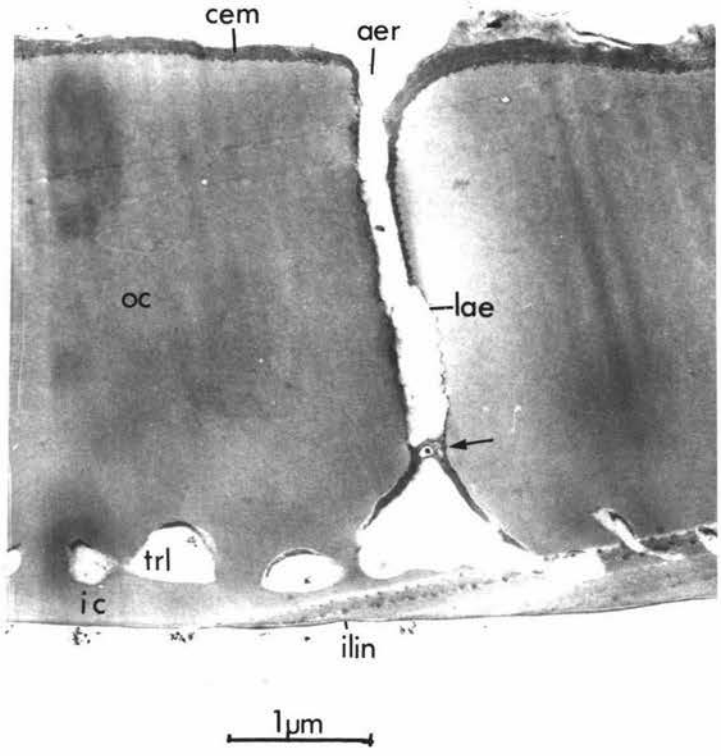
Fig. 4.4 Transmission electron micrograph of the chorion and cement

The cement extends into the aeropyle but does not cover it, although there is a "plug" which may be cement near the end of the aeropyle (arrow). "Plugs" of this type were frequently but not always seen.

Fig. 4.5 Transmission electron micrograph of the chorion and cement.

In this case the cement covers the aeropyle but does not extend into it.

aer	aeropyle
cem	cement
ic	inner layer of chorion
ilin	inner lining of chorion
lae	lining of aeropyle
oc	outer (main) region of chorion
trl	trabecular (inner meshwork) layer


cem
aer
oc
lae
trl
ic
ilin
1µm

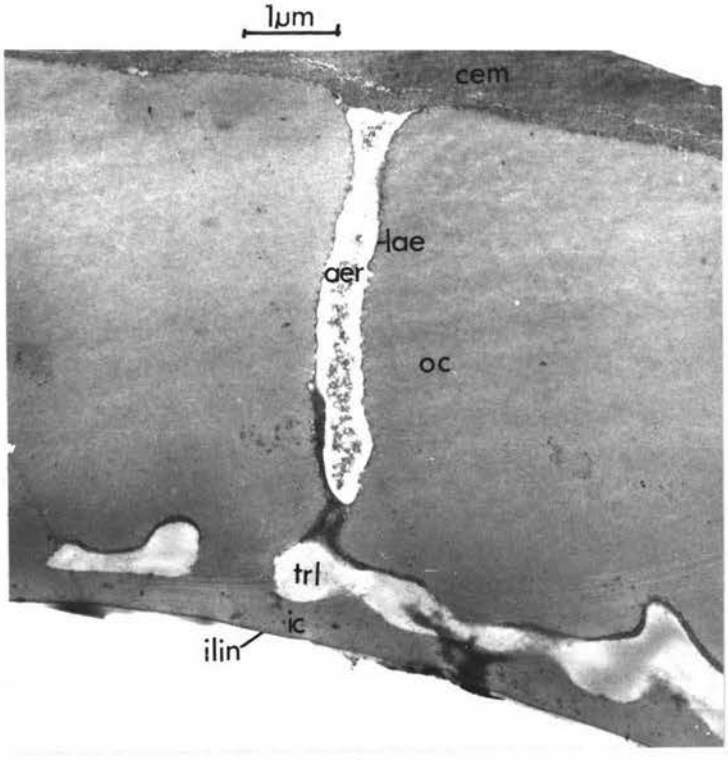

1µm
cem
lae
aer
oc
trl
ic
ilin

Fig. 4.6 Scanning electron micrograph of a freeze fractured egg showing the broken edge of the chorion

In this case the egg is covered by a fairly thick layer of cement. The thin layer on the surface of the vitelline membrane (arrows) may be a layer of wax.

Fig. 4.7 Unstained section photographed using Nomarski optics.

The chorions of two eggs can be seen, separated by cement.

aer aeropyle

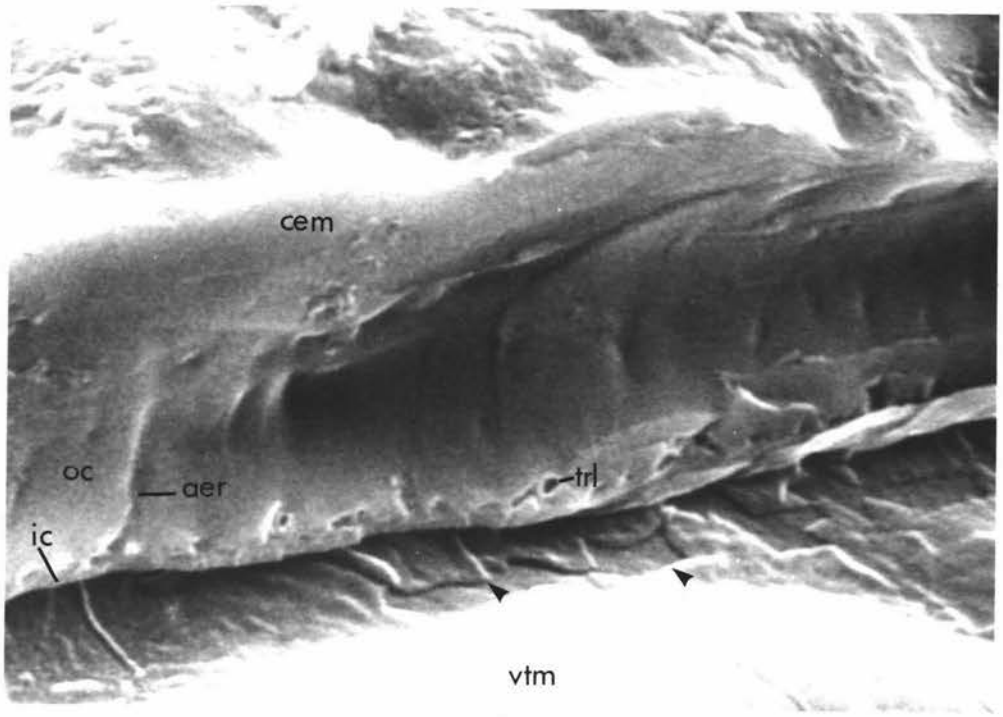
cem cement

ic inner layer of chorion

oc outer region of chorion

trl trabecular layer

vtm vitelline membrane



5 µm

20 µm

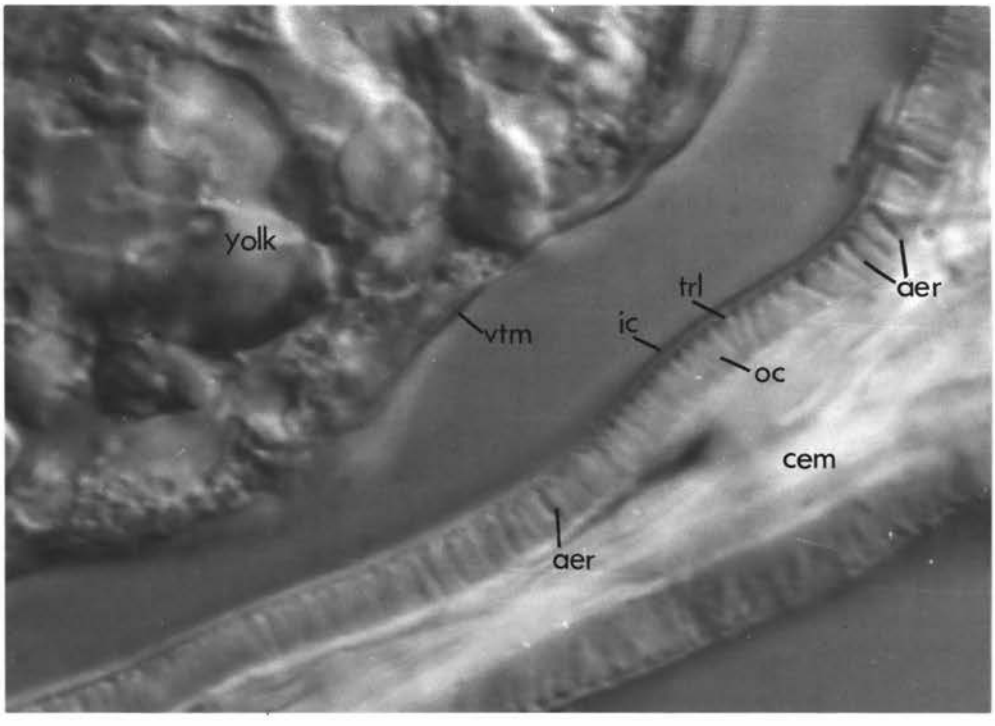


Fig. 4.8 Transmission electron micrographs of the vitelline membrane and inner region of the chorion

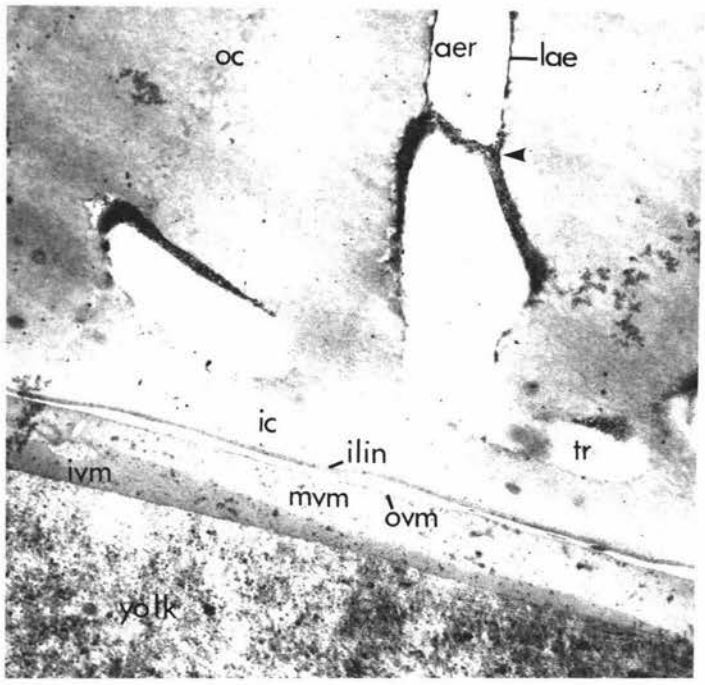
The vitelline membrane can be seen to consist of three layers.

In (A) a very thin "plug" can be seen in the aeropyle (arrow).

In (B) the inner layer of the vitelline membrane extends right across to the thin outer layer near the centre of the region of the membrane shown.

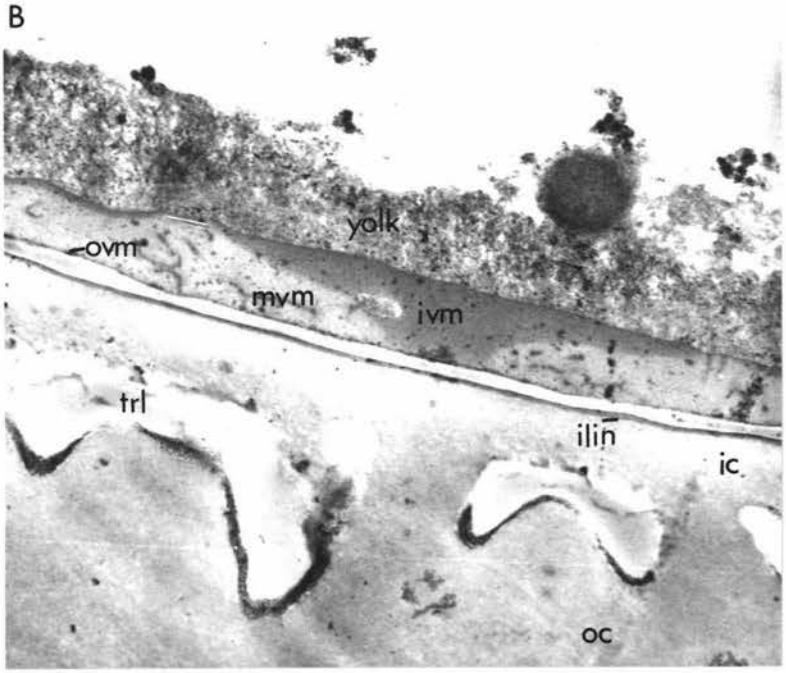
aer	aeropyle
ic	inner layer of chorion
ilin	inner lining of chorion
ivm	inner layer of vitelline membrane
lae	lining of aeropyle
mvm	middle layer of vitelline membrane
oc	outer region of chorion
ovm	outer layer of vitelline membrane
trl	trabecular layer

A


oc
aer
lae
ic
ilin
tr
ivm
mvm
ovm
yolk

1µm

B


yolk
ovm
mvm
ivm
trl
ilin
ic
oc

appearance to the cement (Fig. 4.4, 4.8a). The cement may cover the aeropyles completely (Figs. 4.3, 4.6) particularly when two eggs lie adjacent to one another as in Fig. 4.7, but in some regions they are visible through gaps in it (Fig. 4.3). The cement may extend into the aeropyles (Fig. 4.4) but does not necessarily do so, even when it covers their openings (Fig. 4.5).

The eggs of a large number of terrestrial insects are structured so that the chorion can act as a plastron, or physical gill, enabling the egg to respire if they become submerged provided that the water is well aerated (Hinton, 1969, 1970). In order to act in this way, the outer regions of the respiratory system must be hydrophobic and also extensive so as to provide a large water-air interface (Hinton, 1969). Taking the dimensions of the egg of G. leucoloma as 0.82mm by 0.56mm (May, 1966) then it has a surface area of approximately 1.5mm^2 and from scanning electron micrographs it was calculated that there are about 250,000 aeropyles. Considering only the outer diameter of each as 1µm, then the total area of these aeropyles is 0.2mm^2 or 13% of the egg surface, while if only the inner diameter of 0.4µm is considered (the average diameter about 0.5 - 1µm from the surface) then the total area occupied by them is 0.03mm^2 or 2% of the surface area of the egg. Hinton (1969) has stated that the plastron of most insect eggs has an air-water interface of 10^5 - $10^6$$\mu m^2$ per mg of egg, and gives 15,000μm^2 per mg as the minimum area of an effective plastron. It follows that for an egg the size of that of G. leucoloma, the aeropyles would have to occupy 0.17% or 11% of the surface area of the chorion to provide interfaces at 15,000μm^2/mg or $10^6$$\mu m^2$/mg respectively (Hinton, 1969) so it appears that on this basis the respiratory system of G. leucoloma eggs could act as a plastron. Even if much of the chorion is covered by cement the minimum requirement of 15,000μm^2/mg will still be met provided that between 1.3% and 8.5% of the surface is exposed (based on the maximum and minimum pore diameters of 1.0µm and 0.4µm given above). It also seems likely that some diffusion could occur through the cement, particularly where it is very thin (as in Figs. 4.4 and 4.5) and in this event even less of the chorion would need to be exposed. The large number of aeropyles scattered uniformly over the surface of the egg may be a device to ensure that, no matter what the orientation of the egg in the cement, an adequate number of aeropyles will be exposed for respiration to occur. It still remains, however, to be shown whether the outer part of the respiratory system is hydrophobic in this egg in order to demonstrate its ability to act as a

plastron. Confirmation of this function could perhaps be best obtained by studies on the respiration rates of both dry eggs and those submerged in aerated water.

The chorionic structure of G. leucoloma is unlike that of any other Coleoptera so far reported. In Ocypus olens Müller (Coleoptera, Staphylinidae) the chorion is 40 - 50µm thick with some 4,000 aeropyles concentrated mainly in an equatorial band around the egg, and trabecular layer is deeper and more complex than that of G. leucoloma (Lincoln, 1961), while the chorion in A. menetriesi is 7µm thick and consists of three layers; a solid inner layer 1.4µm thick, a porous layer of similar thickness and a 4µm thick outer layer which is penetrated by "aeropylar pits". The surface is sculptured into a hexagonal pattern which is moulded by the follicle cells and the whole egg is covered by a thin "extrachorion" which is possibly produced in the calyx of the ovary (Ando, 1973) and which probably corresponds to the cement in G. leucoloma. The surface of the eggs of four species of Diabrotica (Chrysomelidae) is sculptured in a similar manner (Rowley and Peters, 1972), while amongst the Meloidae the chorion of L. viridana is a simple envelope 0.2 - 0.3µm thick which lacks a respiratory system; presumably it is thin enough for gas exchange to occur by diffusion through it (Sweeny et al., 1966). The chorion of the latter insect has an ordered ultrastructure (Sweeny et al., 1966) and this suggests that it is composed of crystalline protein (Furneaux and Mackay, 1972). The latter authors also found crystalline proteins in the chorions of 14 species of beetle from 13 families in addition to the Meloidae, and the only exceptions were two Dermestidae, whose eggs lacked a chorion, but no weevil was studied. Representatives of a number of other insect orders were also found to possess chorions composed at least partly of crystalline protein but no crystalline structures were found in G. leucoloma and it is therefore the beetle in which to my knowledge this has been found to be so. In addition, the chorion did not have a lamellar ultrastructure, a feature apparently restricted to the Lepidoptera. It appears that the gross structure of the chorion in G. leucoloma is most like that of Galleria mellonella but the trabecular layer of the former is only half as wide as that of the latter and the layer beneath it is thicker. The ultrastructure and composition of the chorion in G. mellonella is, however, more complex, and consists of several distinct types of material (Barbier and Chauvin, 1974) while only one major type has been found in G. leucoloma.

Only protein was detected in the chorion, which appeared to be of

uniform composition apart from the various linings observed under the electron microscope. However, in view of the findings of Kawasaki et al. (1971a,b, 1972, 1974), it is probable that several different proteins are present. The mixed anhydride reaction for glutamic and/or aspartic acids (Fig. 4.10) and the DDD reaction for cystine (Fig. 4.11) were strongly and uniformly positive. The DDD reaction demonstrates the presence of sulphydryl (SH) groups, either free (cysteine) or, after oxidation by an appropriate reagent (in this case thioglycollic acid), those engendered from disulphide groups (cystine) as well. Blocking of existing SH by reagents such as N-ethyl maleimide prior to oxidation renders the technique specific for cystine (Pearse, 1968). The DDD reaction gives a red colouration with low levels of SH, a blue colour with high levels and intermediate shades with intermediate levels (Pearse, 1968). The DDD reaction for cystine gave an intermediate shade in the chorion of G. leucoloma, which indicated a low to moderate level of substrate. Cysteine was also present, but at markedly lower levels (Fig. 4.12) whereas tyrosine gave a moderate reaction (Fig. 4.13), while arginine gave a very weak reaction (Fig. 4.14), and tryptophan was not detected (Fig. 4.15). The chorion was rapidly dissolved by 1% NaOCl and hot 10% NaOH, but was not dissolved by overnight exposure to HCl or cold NaOH and this indicated a moderate level of chemical stability.

It must be noted that histochemical tests are qualitative or at best semi-quantitative (as with the DDD reaction) in their indication of the amount of a particular substance present and that they will not detect substances below a minimum level which will depend on the sensitivity of the particular test. For these reasons they cannot be used to compare the absolute amounts present and the terms "low", "moderate", "high" and so on refer to the intensity of reaction rather than the actual amount which would be determined in a biochemical analysis. One substance may therefore give a more intense reaction than another even though it is present at a lower level. However, the levels of cystine and cysteine as detected by the DDD reaction can be compared to some extent and cystine is definitely present at considerably higher levels than is cysteine. This is significant as disulphide linkages are considered to be important in contributing to the strength of the chorion of a number of species. Kawasaki et al. (1971a, 1972, 1974) found structural proteins in which disulphide bonds were important in the shells of the eggs of Bombyx mori L., B. mandarina Moore (Lepidoptera, Bombycidae) and Antheraea mylitta Drury (Lepidoptera, Saturniidae), and in Sympetrum infuscatum Selys and

Fig. 4.9 Micrograph of an unstained section

Note that both the vitelline membrane and the inner surface of the chorion show as dark lines (arrows).

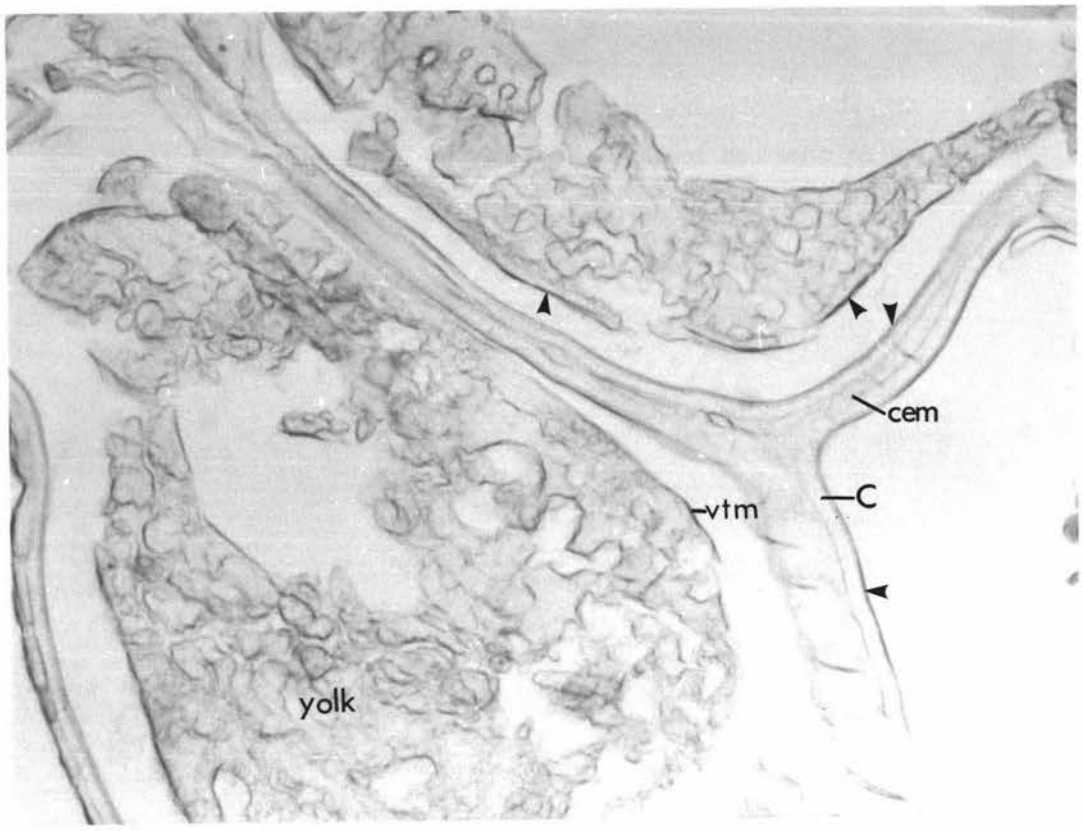
Fig. 4.10 Mixed anhydride reaction for side-chain carboxyl groups (glutamic and aspartic acids)

The chorion is the most strongly staining (red) structure.

C chorion

cem cement

vtm vitelline membrane



100µm

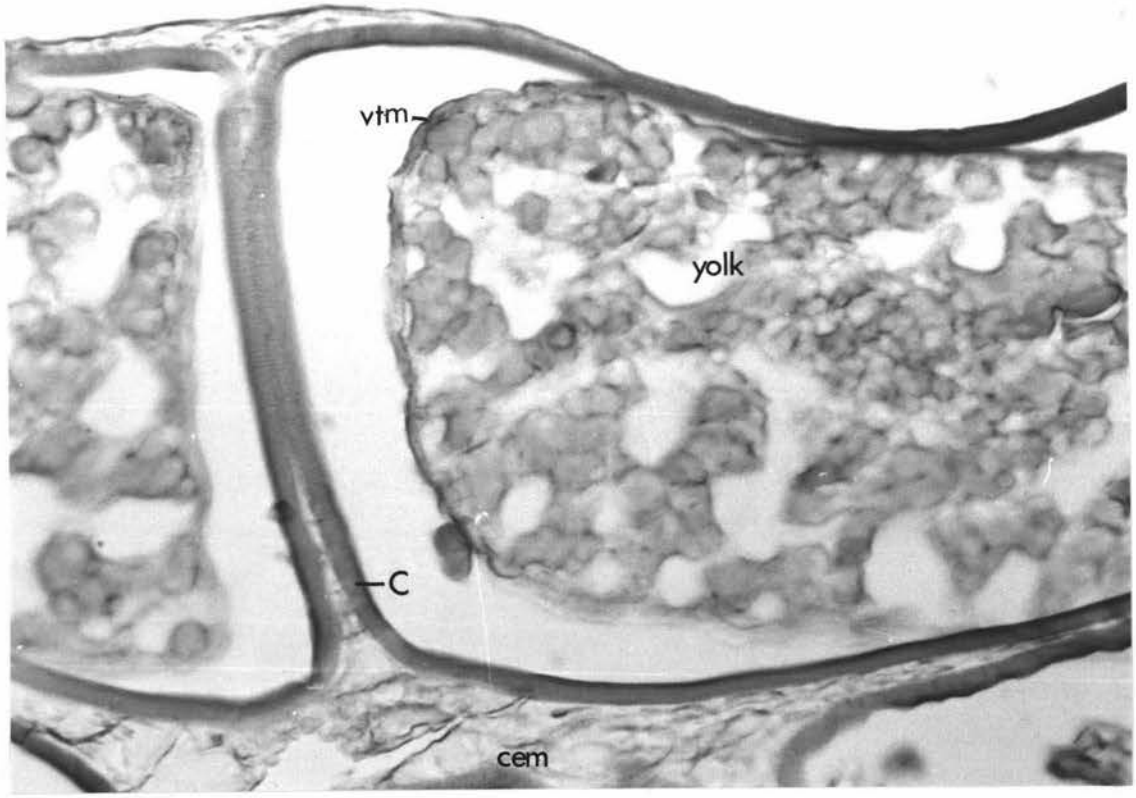


Fig. 4.11 The DDD reaction for disulphide groups (cystine)

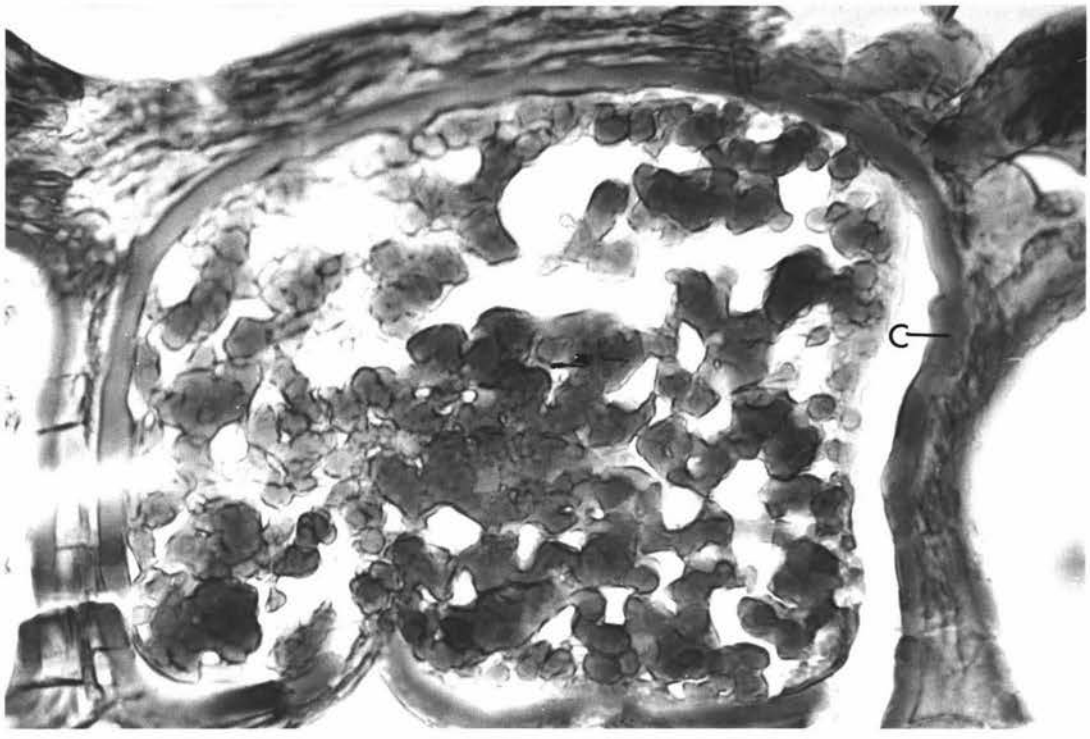
The cement stained red, though unevenly, while the chorion gave a pinkish-lilac colour.

Fig. 4.12 The DDD reaction for sulphydryl groups (cysteine)

Staining of all structures was weaker than was staining for cystine.

C chorion

See Figs. 4.9 and 4.10 for a fuller explanation of structures.



100µm

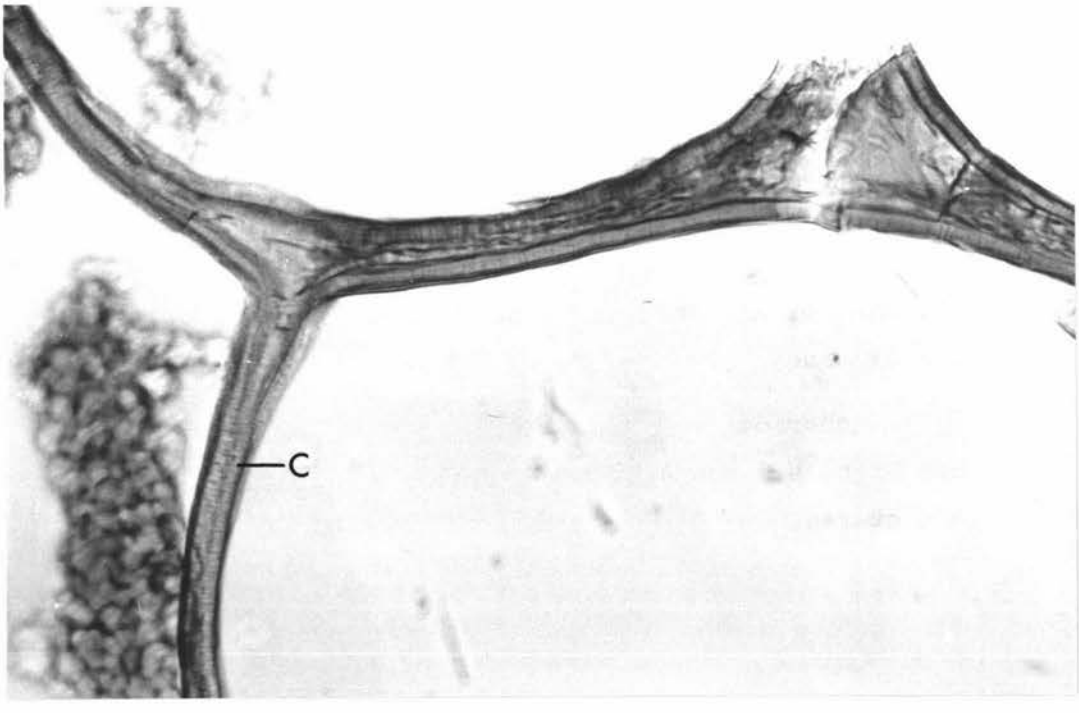


Fig. 4.13 The diazotization-coupling method for tyrosine

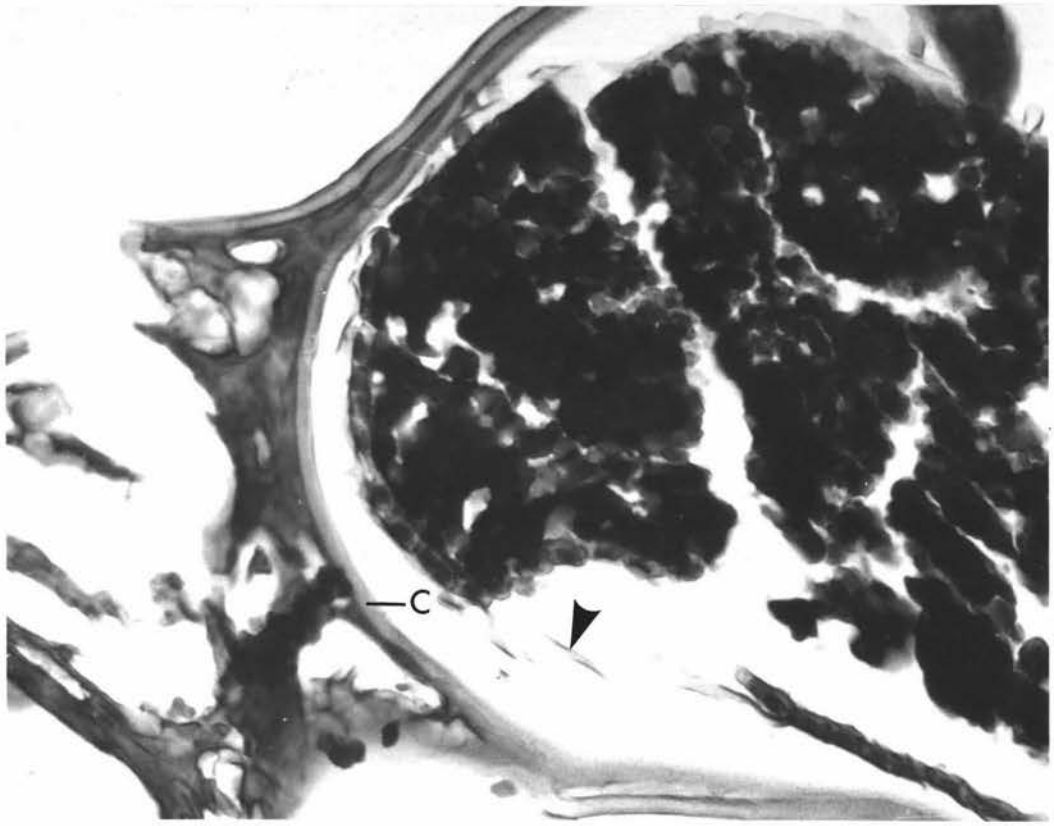
The chorion stained only weakly but component one of the cement, along with the yolk, stained intensely purplish-red. The vitelline membrane gave a moderate staining reaction (arrow).

Fig. 4.14 The Sakaguchi-dichloronaphthol method for arginine

Component one of the cement and the yolk stain strongly (orange-red).

C chorion

See Figs. 4.9 and 4.10 for a fuller explanation of structures.



100μm

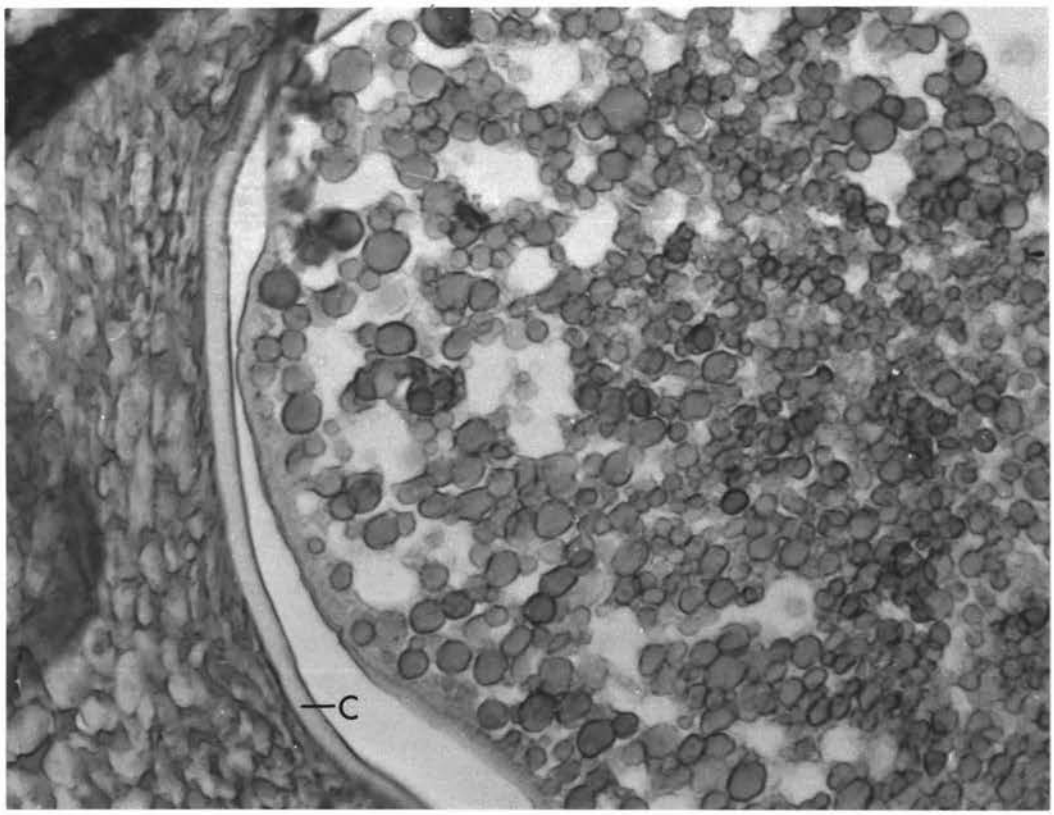
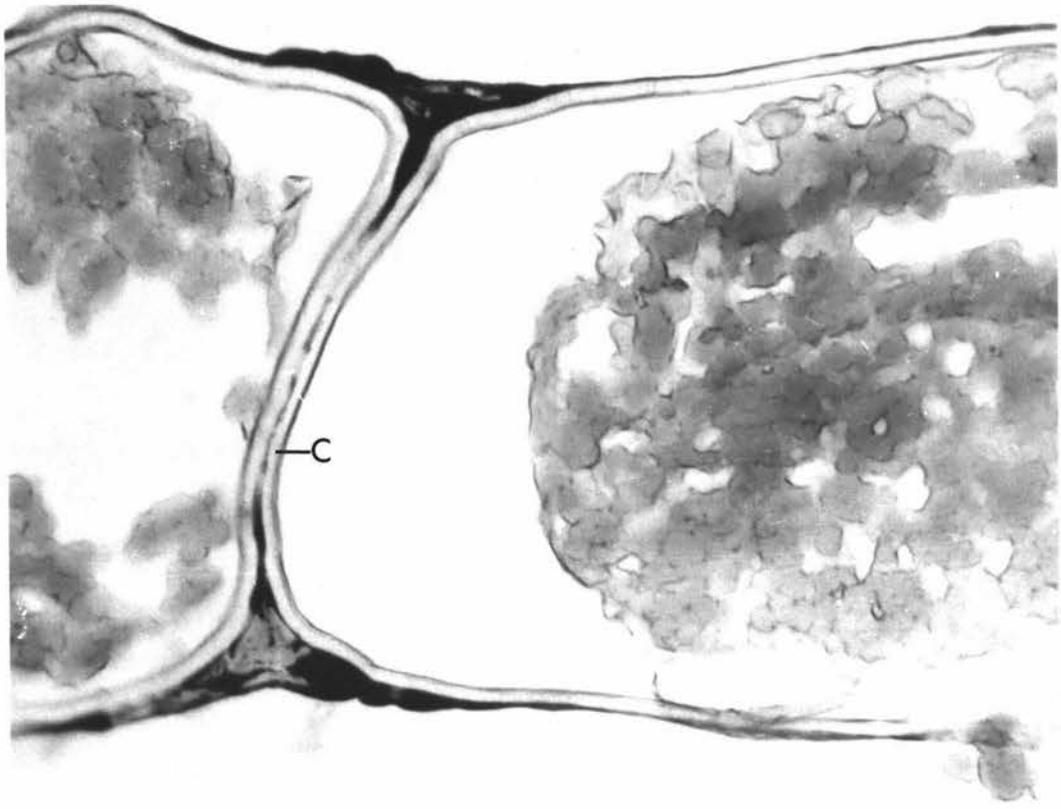


Fig. 4.15 The DMAB-nitrite method for tryptophan

Component one of the cement stains intensely dark blue, the yolk pale blue and the chorion not at all.

C chorion

See Figs. 4.9 and 4.10 for a fuller explanation of structure.



100µm

S. frequens Selys (Odonata, Libellulidae), but Pant and Sharma (1972) found relatively low levels of sulphur containing amino acids in the shell of A. mylitta, and Wilson (1966) found only traces of cystine and/or cysteine in the egg shell of D. melanogaster. Smith et al. (1971) found that both disulphide and hydrogen bonds were largely responsible for the strength of the chorion at H. cecropia, while in Gryllus mitratus Burmeister (Orthoptera, Gryllidae) disulphide bonds are similarly important but linkages involving divalent metal cations were also significant (Kawasaki et al., 1971b). The latter situation may be similar to that in Acleta domesticus L. (Orthoptera, Gryllidae) (McFarlane, 1962; Kawasaki et al., 1971b). Tanning of protein was found to be important in the chorion of R. prolixus (Beament, 1946a) whereas thick layers composed almost entirely of calcium salts, were considered to provide the strength of the chorion in B. libanicus by Hoscona (1950). Nevertheless, in spite of these exceptions, it seems that disulphide linkages are of considerable importance in the structure of the chorion of many insects and that this applies in G. leucoloma. Further evidence of this lies in the observation that the rupturing of the disulphide bonds during the DDD procedure for cystine resulted in a slight distortion of the chorion and that this did not occur with other procedures. However, the possibility of other types of bonding, such as hydrogen bonding, being important in chorionic structure in G. leucoloma has not been eliminated.

The strong reaction given by aspartic and/or glutamic acid agrees with the high levels of these amino acids detected in the chorions of Oryctes rhinoceros L (Coleoptera, Scarabaeidae) Schistocerca gregaria Forsk. (Orthoptera, Acrididae) (Furneaux and Mackay, 1972), A. domesticus (Furneaux, 1970), A. mylitta (Pant and Sharma, 1972; Kawasaki et al., 1972), a number of other silkmoths (Kawasaki et al., 1971a, 1972), and D. melanogaster (Wilson, 1960). Tyrosine and arginine levels were in somewhat lower but variable proportions in these insects although tyrosine was often present at higher levels than was arginine. Tryptophan was not mentioned except by Pant and Sharma (1972) who reported it in trace levels in the egg shell of A. mylitta, and by McFarlane (1962) who recorded it as "present" in a qualitative analysis of the egg shell of A. domesticus. Cystine was also present in all the species listed above but the level varied considerably, being highest in B. mori and lowest in S. gregaria.

The histochemical demonstration of lipid, protein and carbohydrate

in the inner layer and in particular of large amounts of acid mucopolysaccharides in the outer layer of the chorion of D. melanogaster by King and Koch (1963) as already mentioned contrasts with the findings in the present study and although Kawasaki et al. (1971a,b, 1972, 1974) also found sugars in various egg shells these were usually at a fairly low level, and were not acidic.

4.3.2 The Vitelline Membrane

The vitelline membrane in G. leucoloma is a tough, transparent membrane approximately 0.3 - 0.5µm thick which consists of three layers as seen under the electron microscope (Fig. 4.8). The outermost layer is less than 10nm thick and is just visible in Fig. 4.8. Of the other two the innermost is the thinnest but the two layers vary considerably in their relative proportions and the innermost layer may occupy the entire width of the membrane (Fig. 4.bb).

Under the light microscope the vitelline membrane appeared as a dark line even in unstained sections (Fig. 4.9); it frequently did not section well and there was usually a densely staining region closely adhering to its inner surface. For these reasons, the degree of staining was frequently difficult to ascertain but careful examination, particularly at high magnifications has enabled some conclusions to be drawn.

The vitelline membrane contained both proteins and acid mucopolysaccharides. Tyrosine was clearly present and cystine, tryptophan, arginine and glutamic and/or aspartic acids were probably also present, whereas it was difficult toascertain whether cysteine was present. The membrane was PAS negative and stained black with the PAD reaction; it stained lightly with Alcian Blue in the presence of 1.0M $MgCl_2$ at both pH 2.5 and pH 5.7 but only weakly at pH 2.5. The carbodiimide reaction appeared to give a positive reaction, indicating the presence of carbohydrate bound carboxyl groups, but the modified PAS reaction for uranic acids was negative or only very weakly positive. Hyaluronidase and neuraminidase did not alter its staining properties with Alcian Blue.

Weakly acidic sulphated mucosubstances, hyaluronic acids and sialomucins stain with Alcian Blue at pH 2.5 and strongly acidic mucosubstances do not, but the reverse is true at pH 1.0 (Pearse, 1968). With the critical electrolyte concentration method at pH 5.7, hyaluronic acid, sialomucins and some weakly acidic sulphomucins are not stained at or above 0.1M - $MgCl_2$ while most sulphated mucosubstance stain strongly at 0.2M. The various sulphated mucosubstance lose Alcianophila at different

levels with increasing $MgCl_2$ concentration, and only highly sulphated forms such as keratin sulphate and some heparins stain at the 1.0% level (Pearse, 1968). At pH 2.5 staining persists at somewhat higher levels of $MgCl_2$ (Scott and Dorling, 1965). The latter observation contrasts sharply with the results obtained in this study, since when staining differed between pH 2.5 and pH 5.7, it was weaker at pH 2.5 in both the vitelline membrane and cement (see below). No explanation could be suggested to account for this but the staining with Alcian Blue at pH 5.7 appeared to be more consistent than that at pH 2.5 and Pearse (1968) has based his interpretations on staining at pH 5.7, so that at pH 2.5 has been largely disregarded.

Staining of the vitelline membrane with Alcian Blue at pH 1.0 and at pH 5.7 in the presence of 1.0% $MgCl_2$ indicates the presence of a strongly acidic sulphated acid mucopolysaccharides such as keratan sulphate, heparin (Pearse, 1968), or a similar substance. The positive carbodiimide reaction also indicates the presence of carboxyl groups and since heparin possesses carboxyl groups while keratan sulphate does not (Pearse, 1968) the presence of heparin or a histochemically heparin-like substance is implicated. An alternative explanation is that two acid mucopolysaccharides are present, one of which behaves like keratan sulphate and the other is possibly a chondroitin sulphate. Heparin is found mainly in mammalian circulatory tissues and its main role, though at present poorly understood, appears to be as an anticoagulant (Brimacombe and Webber, 1964; White, Handler and Smith, 1968; Phelps, 1972), so its occurrence in the present situation is unlikely, but since Alcian Blue staining relies on the type and density of negatively charged groups (sulphate esters and carboxyls) (Pearse, 1968) a substance which behaves in the same way histochemically as does heparin could quite possibly be present. Acid mucopolysaccharides fulfil diverse functions in animals (Brimacombe and Webber, 1964) and among these a mechanical function is considered to be important (Brimacombe and Webber, 1964; Barrett, 1971) and it is possible that those in the vitelline membrane of G. leucoloma play a structural role.

King and Koch (1963) found that the vitelline membrane of D. melanogaster contained at least five classes of compounds: a protein, two lipids and two polysaccharides (one neutral and one acidic) and Beament (1946a) found that the vitelline membrane in R. prolixus would stain with water soluble protein stains. Kawasaki et al. (1974) found that dityrosyl and trityrosyl residues stabilised the structure of the vitelline membrane in

S. infuscatum and S. frequens and that this was further reinforced by "self-tanning", which resulted in a darkening of the membrane. The possibility of such elements occurring in G. leucoloma eggs has not been investigated but should not be rejected, particularly in view of the clearly positive reaction obtained for tyrosine.

The vitelline membrane was resistant to HCl, NaOCl and cold NaOH, but the eggs dissolved completely in hot 10% NaOH. It is therefore more chemically resistant than the chorion or the cement, since both of these dissolved rapidly in NaOCl. However, only the cement dissolved in cold 10% NaOH. Preliminary work regarding fixation for histochemistry and electron microscopy showed that the vitelline membrane prevented the entry of osmium tetroxide and picric acid (in Bouin's fixative), that alcoholic Bouin's fixative penetrated slowly, and that hot Bouin's fixative (60°C) penetrated rapidly, as did Carnoy's fixative.

The fact that the eggs of G. leucoloma are very resistant to dessication has already been demonstrated (Chapter Two). Dechorionated eggs, however, after only 10 minutes treatment with octane, collapsed within 15 minutes when exposed to the air and rapidly returned to their original state if placed in distilled water, while untreated dechorionated eggs remained apparently normal after more than 12 hours in either air or water. When partially developed eggs, with visible embryos, were placed in distilled water after treatment with octane, the embryo slowly swelled and distorted, and one egg in a sample of six ruptured violently after about 10 minutes. If dechorionated eggs were left in NaOCl, the contents slowly degenerated and the vitelline membrane eventually ruptured, after which the contents dissolved, but if the eggs were treated with octane first, the degeneration in NaOCl was much more rapid. The membrane, however, did not dissolve even after 24 hours in hypochlorite, though it did appear thinner and more transparent than an untreated membrane with the contents merely washed out. This latter observation indicates that while part of the membrane is resistant to hypochlorite, part (presumably the inner layer detected under the electron microscope) may not be. Nevertheless, this observation is somewhat subjective and must be treated with caution, as the membrane is very thin and quite transparent even when intact. Permeability of octane treated chorions to other materials, such as osmium tetroxide and picric acid, was not tested.

The effect of octane on dechorionated G. leucoloma eggs parallels the results obtained by Limbourg and Zalokar (1973) who found that dechorionated D. melanogaster eggs became highly permeable after eight minutes in

octane. This is consistent with the presence of a layer of lipid on the surface of the vitelline membrane which is responsible in large part for the impermeability of the egg shell to water and any substances dissolved in it. It is also probable that the lipid layer and the membrane itself are mainly responsible for the resistance of the egg shell to chemical attack, although the chorion is undoubtedly also important in this respect. Certainly, lipid layers have been found to be responsible for waterproofing of the eggs of a number of insects (Beament, 1946b, 1948; Slifer, 1948; Wigglesworth, 1972), although the eggs of some species are not at all resistant to drying as in Ocypus olens Müller (Coleoptera, Staphylinidae) (Lincoln, 1961) and appear to lack such a lipid layer.

It must also be noted that the extremely thin layer detected on the outer surface of the vitelline membrane of G. leucoloma is unlikely to be the lipid layer since the solubility of the latter in octane indicates that it would probably have been dissolved by the propylene oxide during preparation for electron microscopy.

4.3.3 The Cement

The cement surrounds the eggs, binding them together and to the substrate on which they are laid. It occasionally appears frothy, but it usually fairly solid, even when seen under the electron microscope (Figs. 4.4 - 4.7).

The cement was less chemically resistant than the chorion or vitelline membrane, and dissolved rapidly in 1% NaOCl and hot 10% NaOH, and slowly in 10% NaOH, but it did not dissolve in 10% HCl. It consisted of two components, "component one" and "component two", and the latter could be further subdivided into two types, "A" and "B" which occurred in different clumps or sometimes in separate areas of the same clump. The appearance of the cement was such that component two appeared to be suspended as globules or streaks in component one. The relative proportions of components one and two also varied widely.

4.3.3.1 Component One

This component consists mainly of, or possibly entirely of, protein. It gave strong reactions for tyrosine (Fig. 4.13), arginine (Fig. 4.14) and tryptophan (Fig. 4.15), weaker reactions for cystine and cysteine (Figs. 4.11 and 4.12), and glutamic and/or aspartic acid were not detected (Fig. 4.10). It also gave a weak, possibly non-specific reaction with the carbodiimide reagent, as did the chorion, and it was PAS negative

and did not stain with the PAD reaction or the modified PAS reaction for uronic acids or with Alcian Blue.

4.3.3.2 Component Two

This stained intensely with the PAS reaction (Fig. 4.16) and Alcian Blue at pH 2.5 (Fig. 4-18) and staining with the latter was not affected by neuraminidase. With the PAD reaction, it stained grey-brown after 7 hours but after 24 hours the stain was almost black (Fig. 4.20). It also stained lightly with the modified PAS reaction for uronic acids (Fig. 4.17) and with the carbodiimide reaction (Fig. 4.21). However, staining with Azure A and Toluidine Blue was inconsistent and will not be considered further, although it can be noted that similar problems were experienced by Ashhurst and Costin (1971a).

The staining properties described so far for component two were common to types A and B which were distinguished mainly on the basis of Alcian Blue staining; type B staining at pH 1.0 while type A did not (Fig. 4.19). Type B also stained at pH 5.7 and pH 2.5 in the presence of 1.0M $MgCl_2$ while staining of type A did not occur at pH 2.5 in the presence of 0.05M $MgCl_2$, was weak at pH 5.7 in 0.1M $MgCl_2$ and abolished altogether at 0.2M. Incubation with hyaluronidase did not affect staining at type A but it reduced staining of type B with Alcian Blue at pH 1.0. Both the PAS and modified PAS reactions were slightly weaker in type B than in type A. Treatment with methanolic thionyl chloride for 30 minutes abolished staining at pH 2.5 but four hours was required to abolish staining at pH 1.0. Pearse (1968) stated that methylation by this process abolishes basophilia due to carboxyl groups in 20 minutes and that due to sulphatedaacid mucopolysaccharides in 4 hours. Both the diaminobenzidine and tetrazonium methods for sulphate groups gave weak staining with no difference between types A and B.

It is concluded that type A contains a neuraminidase stable sialomucin and hyaluronic acid, the latter at relatively low levels, so that if any reaction occurs with hyaluronidase it is not detected because of the intense staining of the sialomucin element with Alcian Blue at pH 2.5. The presense of sialoglycans is indicated on the basis of their Alcian Blue staining properties and the assumption that they are the only PAS positive polysaccharides which are likely to be present (Pearse, 1968). The black colouration which resulted from the PAD reaction indicates the presence of periodic acid unreactive elements, which would be provided by hyaluronic acid. Weak staining with Alcian Blue at pH 5.7 in the presence of 0.1M

Fig. 4.16 The PAS technique

Only the yolk and component two of the cement stain red. Note the different appearance of the two types of cement: component two is present in greater proportions in type b cement and appears as globules rather than streaks as in type a cement. This difference was frequently, but not always apparent.

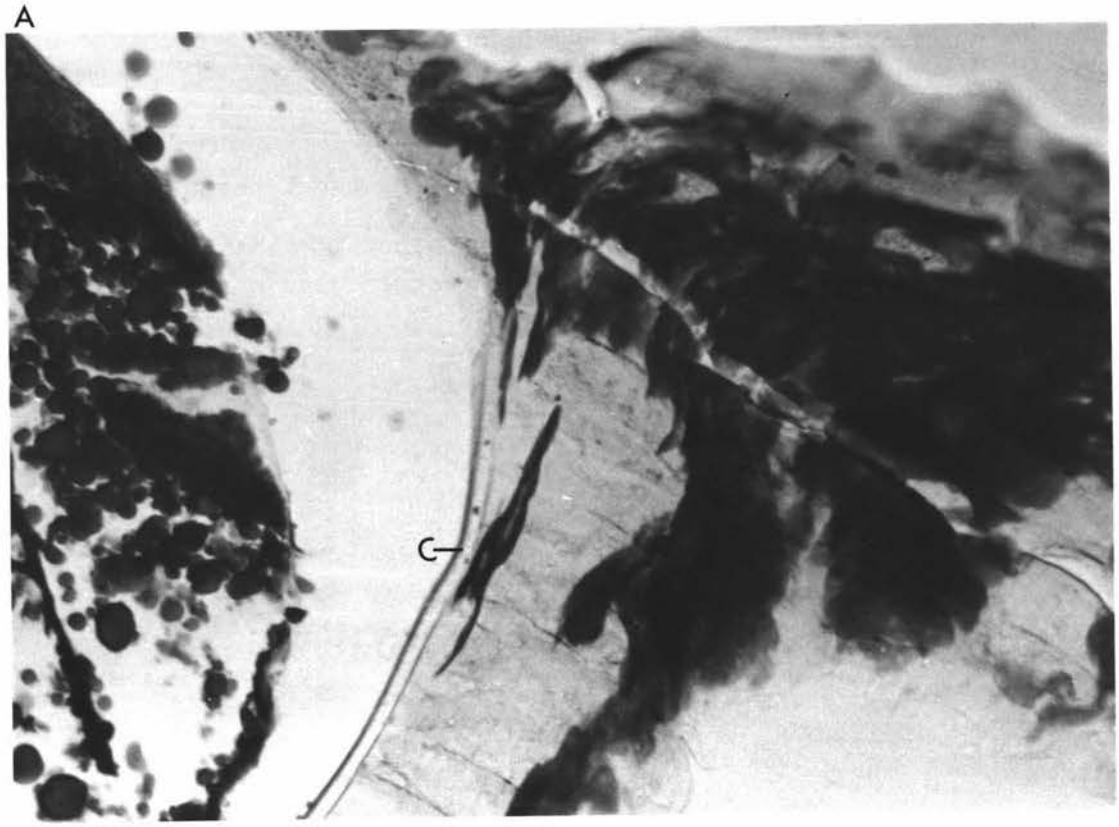
C chorion

See Figs. 4.9 and 4.10 for a fuller explanation of structures.

A) Type a cement

B) Type b cement

A



100µm

B

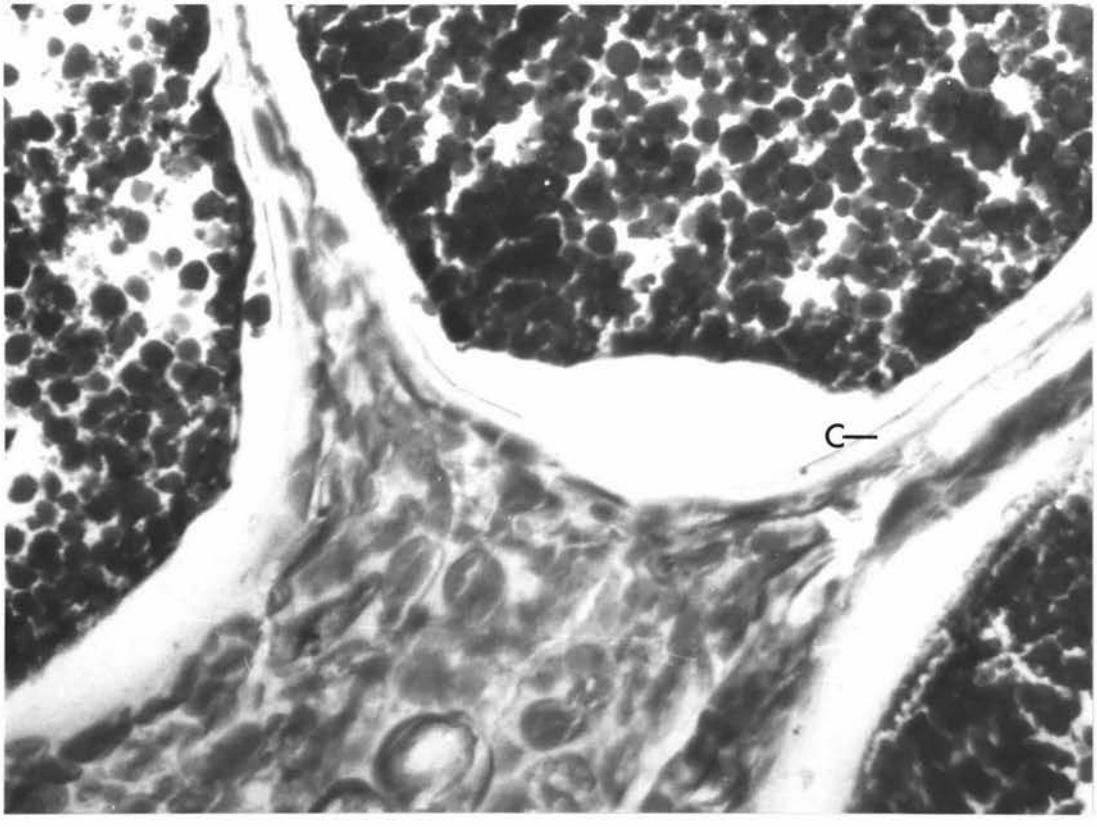


Fig. 4.17 The modified PAS method for uronic acid-containing glycosaminoglycans

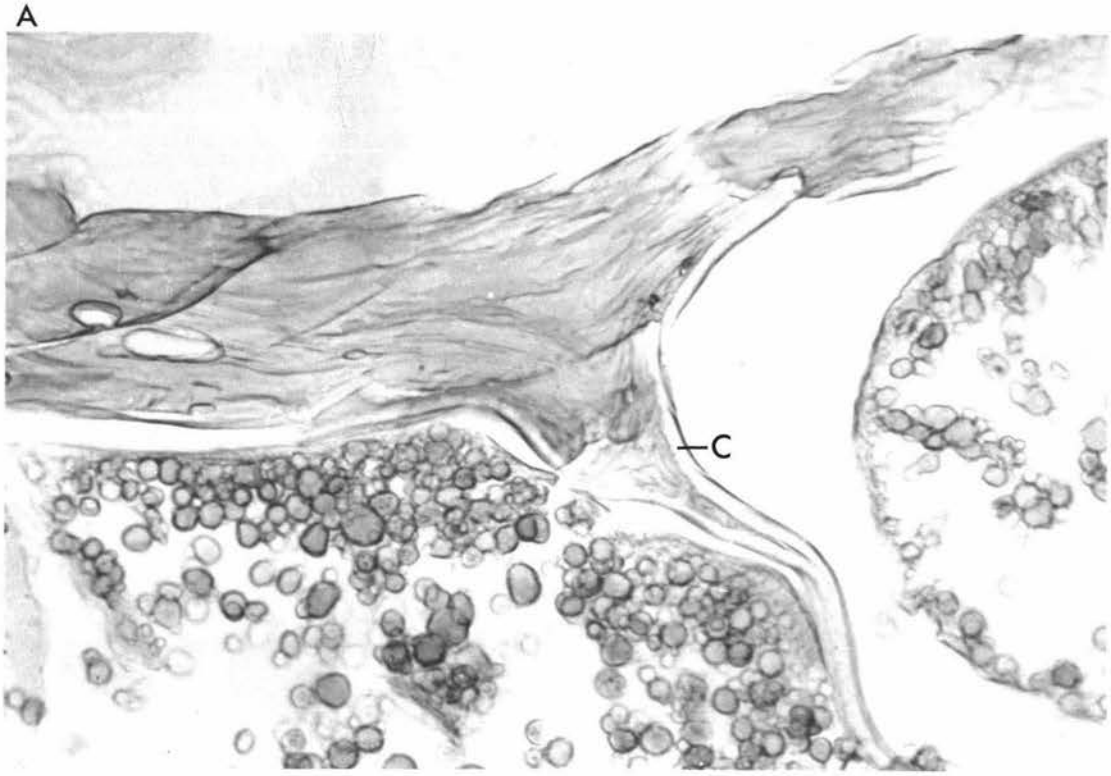
The yolk stains more deeply (pink) than does the cement.

C chorion

See Figs. 4.9 and 4.10 for a fuller explanation of structures.

A) Type a cement.

B) Type b cement.



100 µm

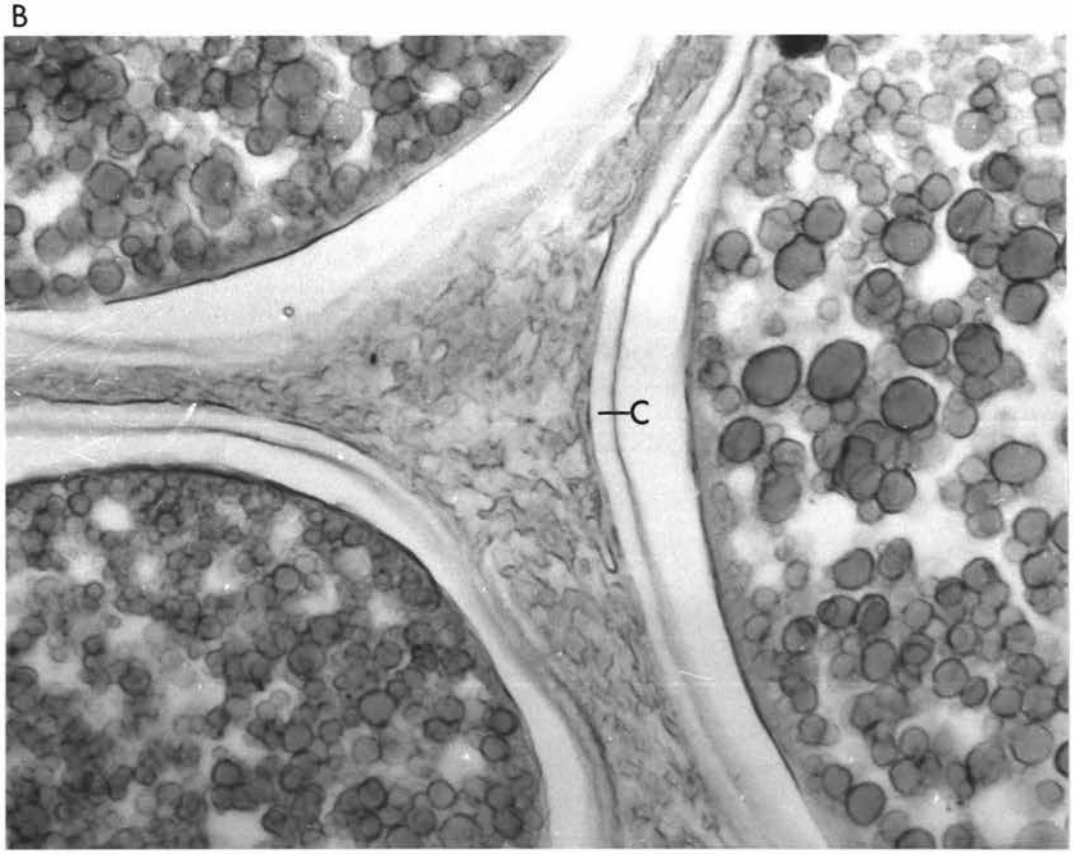


Fig. 4.18 Alcian Blue staining at pH 2.5

The yolk does not stain, but component two of the cement stains strongly blue.

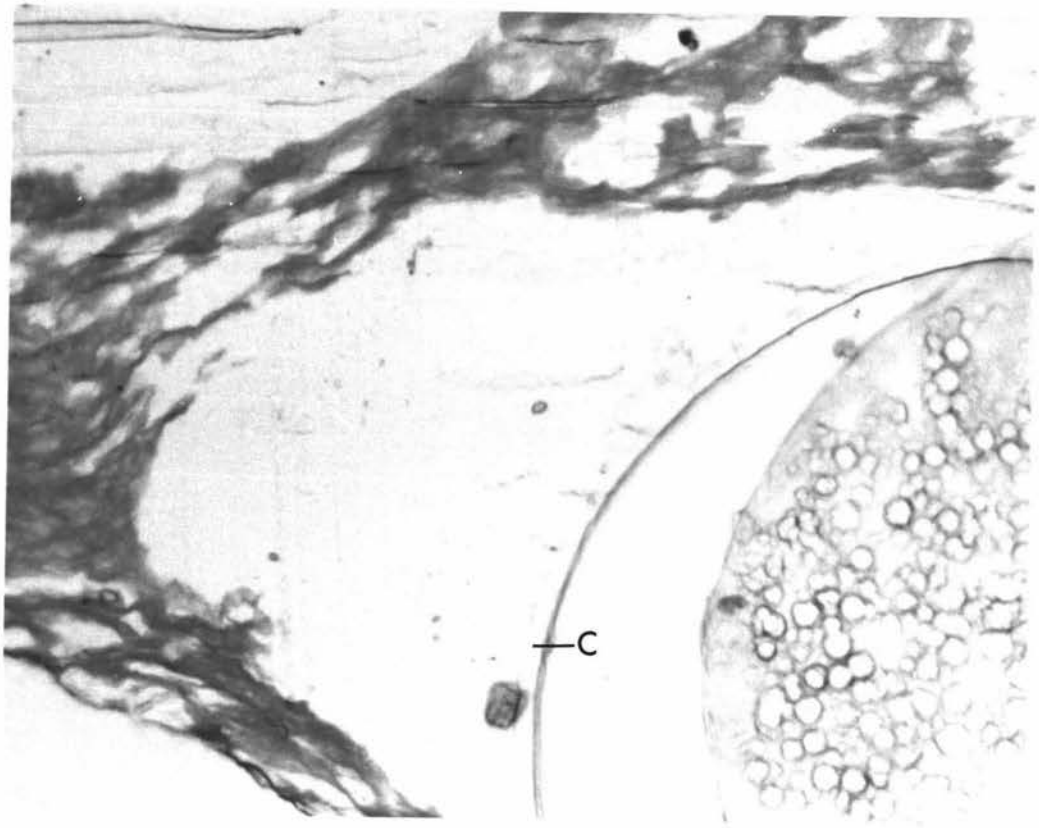
C chorion

See Figs. 4.9 and 4.10 for a fuller explanation of structures.

A) Type a cement

B) Type b cement

A



100µm

B

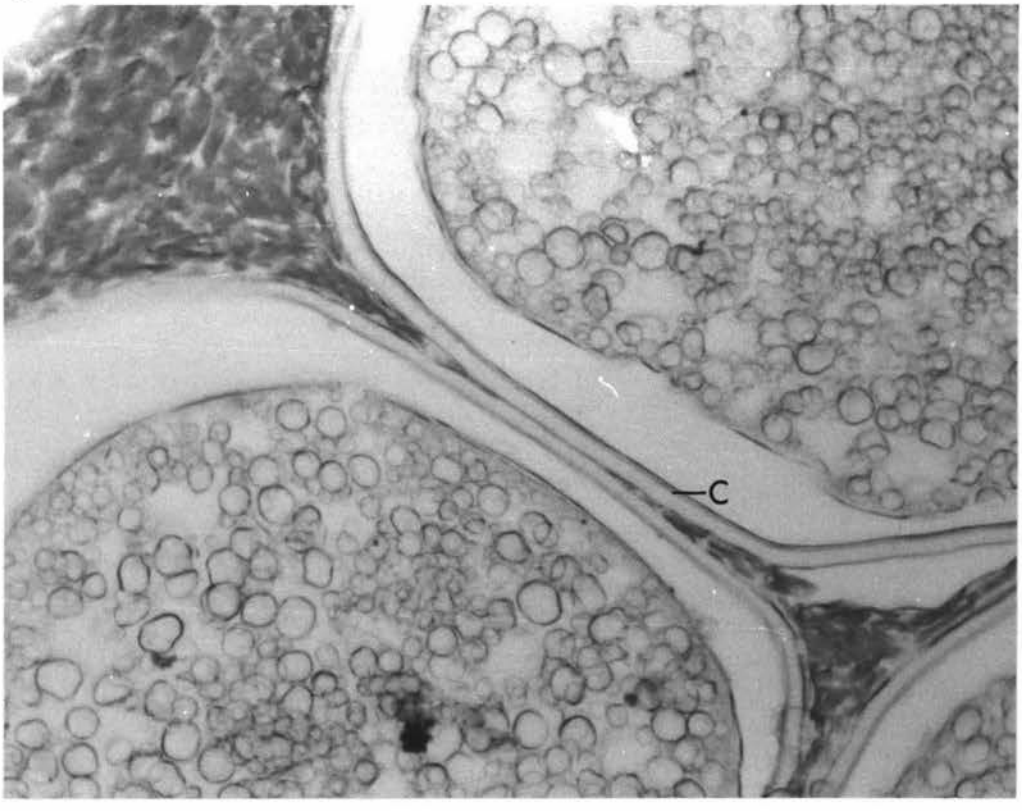


Fig. 4.19 Alcian Blue staining at pH 1.0

The yolk stains moderately to strongly (blue). Component two type b stains blue while type a does not.

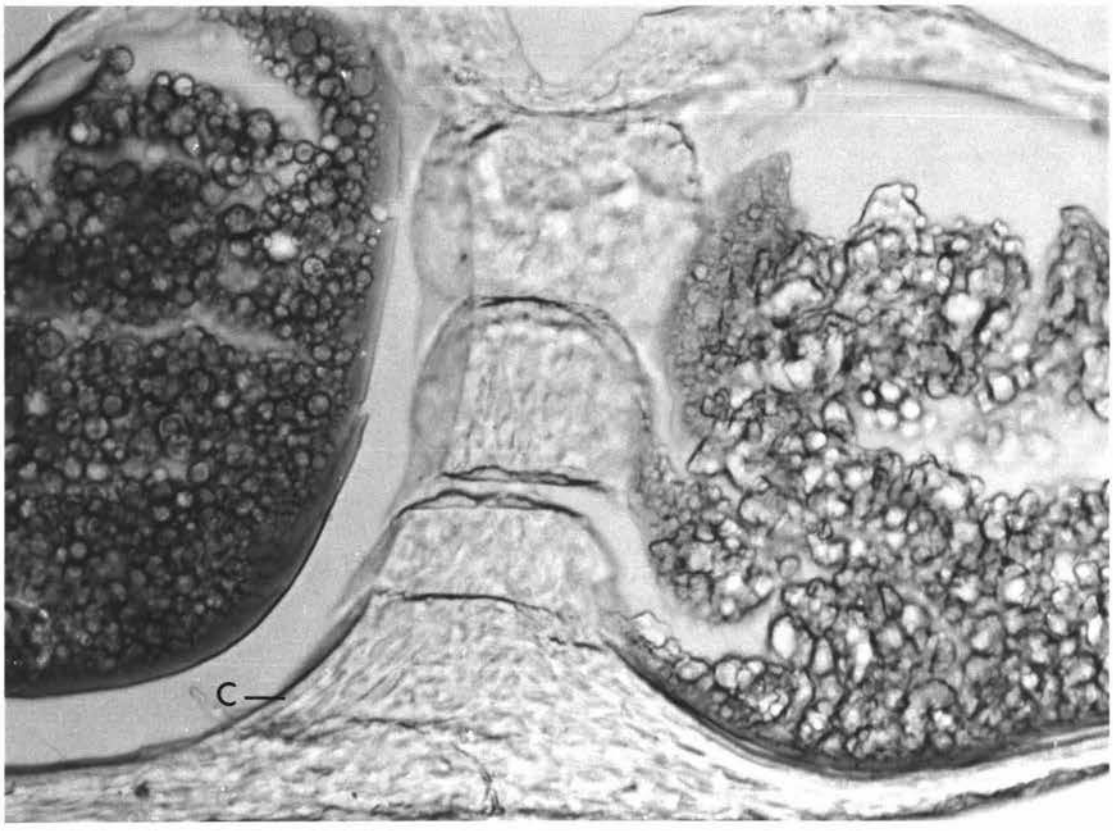
C chorion

See Figs. 4.9 and 4.10 for a fuller explanation of structures.

A) Type a cement

B) Type b cement

A



100 µm

B

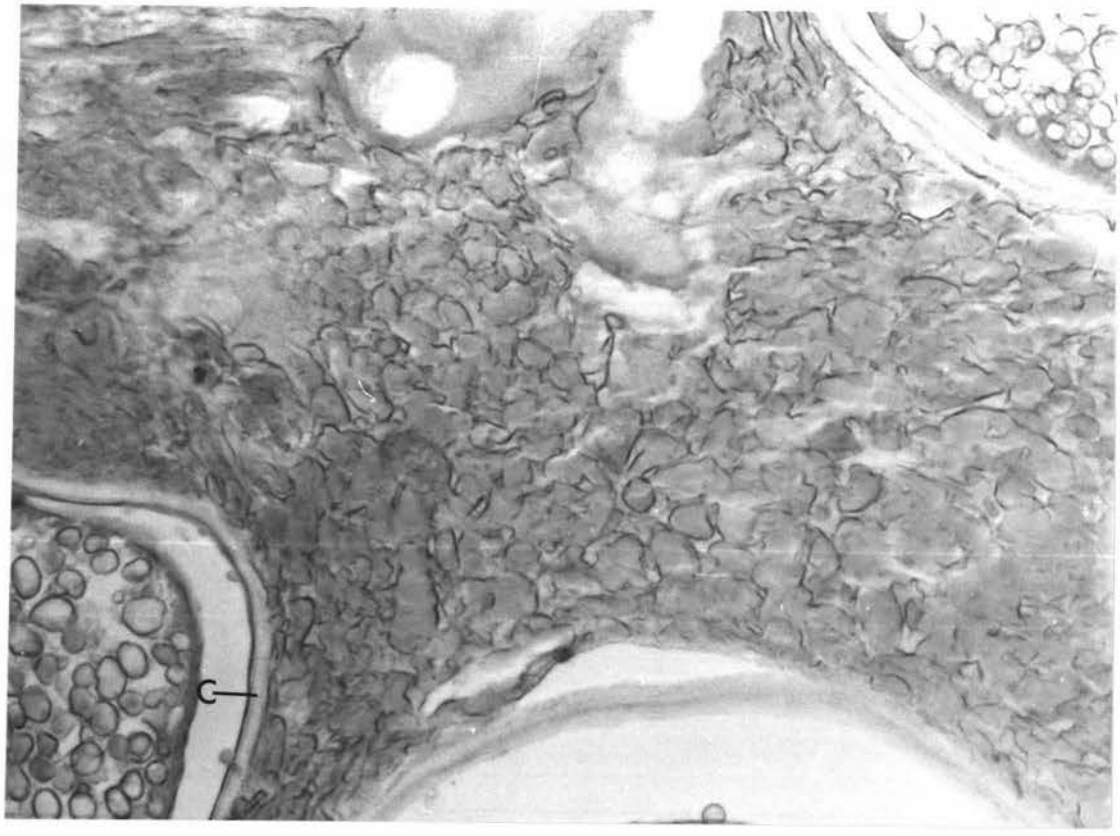


Fig. 4.20 The PAD technique

A) After 7 hrs staining.

The yolk and component two of the cement are stained brownish grey.

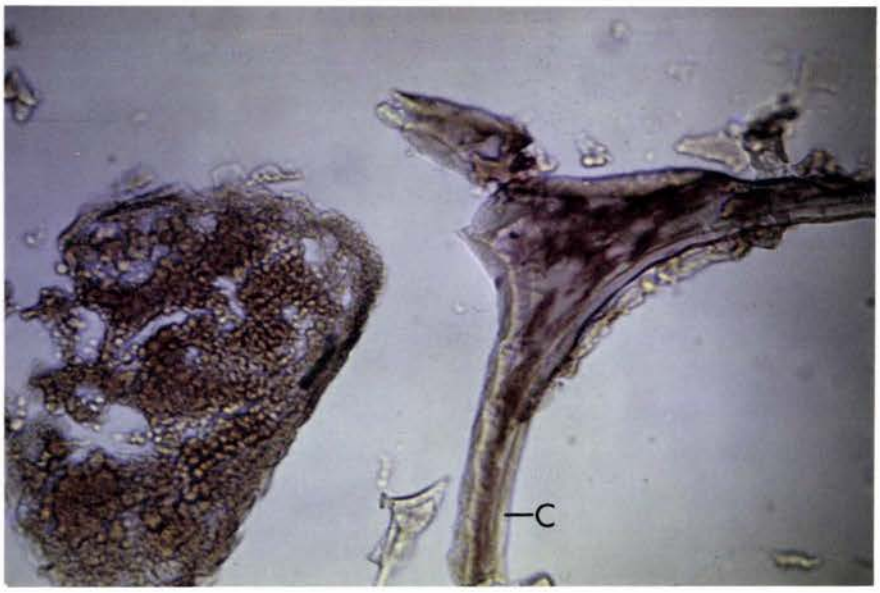
B) After 24 hrs staining.

Component two of the cement stained mostly black, with small areas of brown, while the yolk remained brown or brownish grey.

C chorion

See Figs. 4.9 and 4.10 for a fuller explanation of structures.

A



250µm

250µm

B

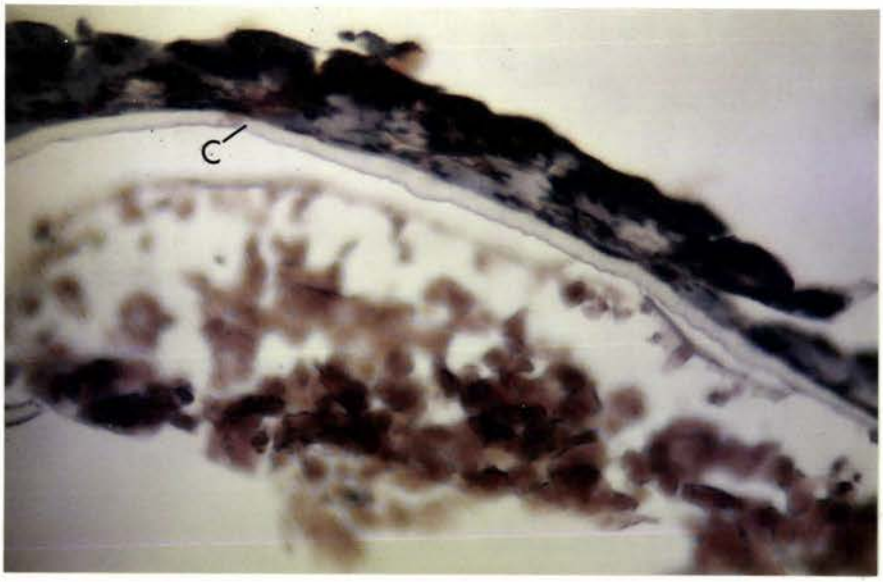


Fig. 4.21 The carbodiimide reaction for carbohydrate-bound carboxyl groups

Component two stains brown-black and the vitelline membrane appears to have stained intensely as well (arrow). The cement stains only moderately.

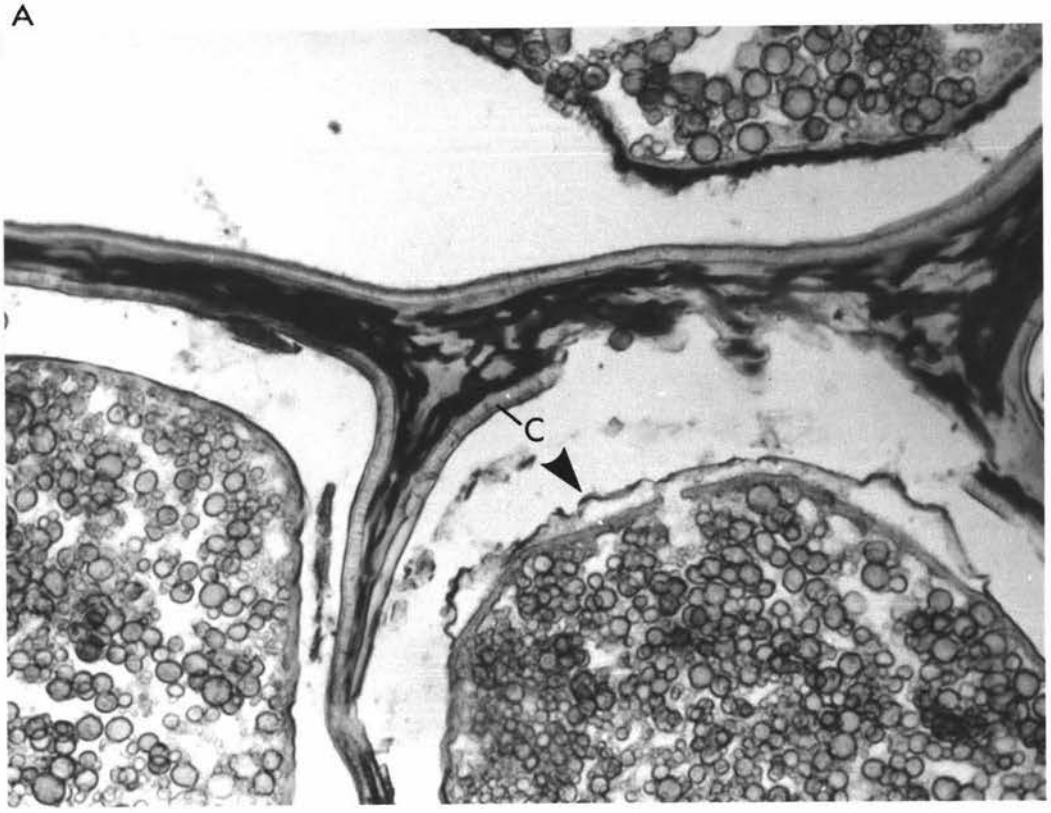
C chorion

See Figs. 4.9 and 4.10 for a fuller explanation of structures.

A) type a cement

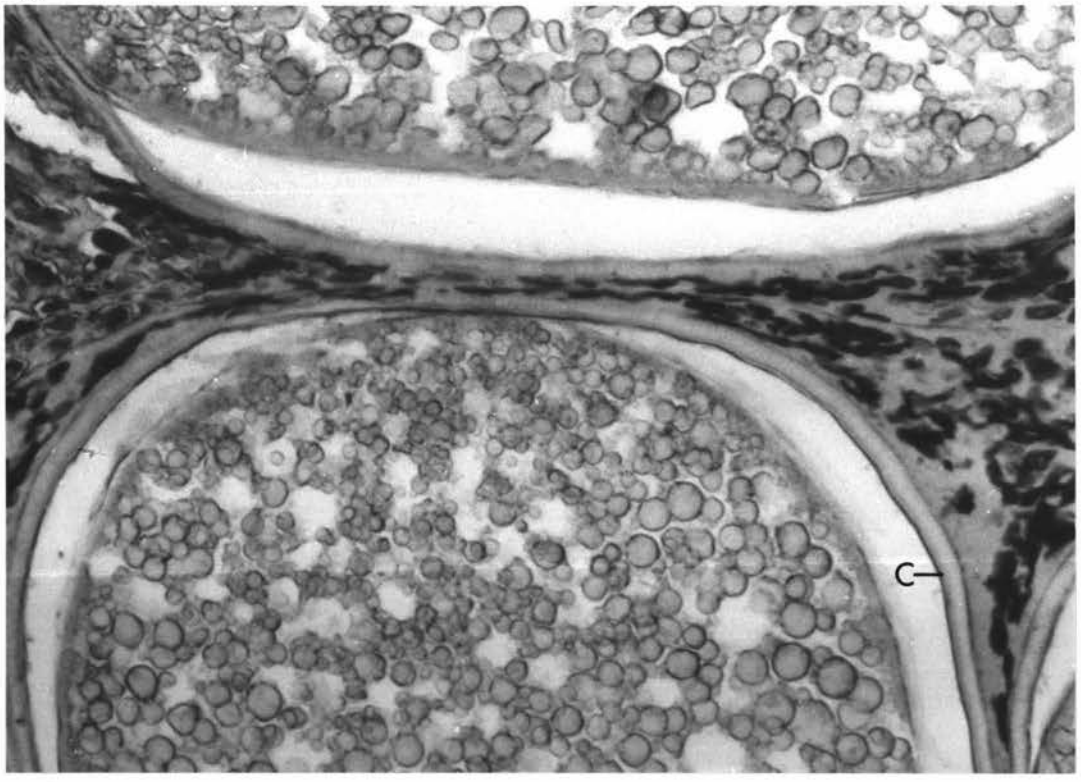
B) type b cement

A



100µm

B



$MgCl_2$ indicates that a third, weakly sulphated acid mucopolysaccharide may also be present at very low levels, and this could explain the staining of type A with the DAB reaction. It may also be noted that when rat trachae were treated with the DAB method only weak staining resulted, even though this tissue gave a strong reaction with Alcian Blue at pH 1 and it can be expected to contain considerable amounts of sulphated acid mucopolysaccharides, since these occur at high levels in horse and cattle trachae (Brimacombe and Webber, 1964).

Component two type B appears to consist of type A with additional elements. Keratan sulphate or a histochemically similar substance is indicated on the basis of staining with Alcian Blue in the presence of 1.0% $MgCl_2$, and the reduction in staining with Alcian Blue at pH 1.0 after treatment with hyaluronidase suggests that a second substance is present and this may be a chondroitin sulphate or something histochemically similar (Pearse, 1968).

Using the classification of Pearse (1968) the type A component of the cement contains S mucin (sap) BM or CG mucin (the sialomucin component) and u-mucopolysaccharide (hyaluronic acid) and possibly another compound from the "epithelial sulphomucins" category of this classification, while type B contains in addition S-mucopolysaccharide A (1.0 $MgCl_2$) (keratan sulphate or heparin) and S-mucopolysaccharide B2.0T or B4.0T (chondroitin sulphates) or similar substances.

The sialic acid components can be separated on the basis of the effect of saponification or resistance to neuraminidase, but this reaction was not attempted, and the chondroitin sulphates may be partly distinguished on the basis of staining with Azure A and Toluidine Blue, which gave unreliable results in this instance. It must be noted that this classification is a histochemical one and that at present histochemical studies provide relatively little chemical information. For some time to come, therefore, biochemical and histochemical classifications must remain separate (Pearse, 1968).

Component two of the cement gave a positive chloramine T-schiff reaction, showing that protein was also present. It appeared to contain cystine (Fig. 4.11) and cysteine (Fig. 4.12), possibly tyrosine (Fig. 4.13) and aspartic/glutamic acids (Fig. 4.10) but tryptophan (Fig. 4.15) and arginine (Fig. 4.14) were not detected. With respect to other insects a micilagenous secretion is often deposited over the eggs: this has been shown to be PAS positive in Melanoplus differentialis Thomas (Orthoptera,

Acrididae (Slifer and Sekhon, 1963), while Beament (1948) found the cement which adheres the eggs of R. prolixus to the substrate gave a positive stain for protein. Acid mucopolysaccharides have not previously been reported from the extrachorionic layers of insect eggs though they have been found in the chorion and vitelline membrane of D. melanogaster (King and Koch, 1963), as already noted in sections 4.3.1 and 4.3.2. They have also been found to occur widely in post-embryonic stages of insects (Mustafa and Kamat, 1970, 1972; Vadgama and Kanat, 1971; Ashhurst and Costin, 1971a,b,c; Sharief, Perdue, and Dobrogosz, 1973; Höglund, 1976a,b).

The unexpected complexity of the cement prompts some consideration of its function. May (1966) states that the cement helps the eggs to withstand drought, but this is highly unlikely to be important as it appears that a lipid layer on the vitelline membrane fulfils this role. Nevertheless, in covering many of the aeropyles the cement may reduce the area from which diffusion of water vapour can occur. Slifer and Sekhon (1963) regard mucus-like coating on the surface of M. differentialis eggs as possessing a lubricating function while the eggs are being laid, and this is a probable function in G. leucoloma. However, the coating on M. differentialis eggs rapidly disappears (Slifer and Sekhon, 1963) but the somewhat more copious cement around G. leucoloma eggs does not. It also binds the eggs together in clumps and to the substrate on which they are laid and this clumping may provide extra protection from physical damage and predation. Barrett (1971) regards the acid mucopolysaccharides as playing a structural role in many cases, and this may be a major role in the present situation, in that the cement may provide extra support and strengthening for the relatively thin and flexible chorion. The high affinity of acid mucopolysaccharides for water (Barrett, 1971) tempts the suggestion of a further role. They may absorb water and retard its evaporation, but the cement was observed to dry out rapidly and, as indicated above, this is not considered to be an important function. However, the cement may help in the wetting and softening of the chorion after the end of a dry period, thus indirectly facilitating hatching. Nevertheless, the highly complex nature of the cement cannot be fully explained at present, and it would be worthwhile examining other insect species to determine the extent of such extrachorionic secretions. Any correlation or otherwise of the nature of these secretions with the ecology of the insect may aid in a fuller interpretation of their role.

4.3.4 The Yolk

Although the aim of this work was not to investigate the composition of the yolk which, since it contains a developing embryo, together with its food supply, will be both extremely complex and constantly changing, a brief description of observations made on newly laid eggs is considered to be of some value.

The yolk gave a positive reaction for all the amino acids tested and was strongly PAS positive. Glycogen granules were observed under the electron microscope (Fig. A1.1, Appendix One) and this substance is probably a major component, in view of its importance as a storage compound in animals. The PAD reaction produced a brown stain after 7 hours but grey-brown after 24 hours, and the yolk stained with Alcian Blue at pH 1.0 but not at pH 2.5. When it occurred Alcian Blue staining was most intense in a narrow band of material adhering closely to the inside of the vitelline membrane, though some staining occurred throughout. With the critical electrolyte method at pH 5.7 the yolk stained strongly in the presence of 0.4M $MgCl_2$ but at a reduced level at 0.6M which persisted at 1.0M. Treatment with hyaluronidase reduced staining at pH 1.0 to levels similar to that at pH 5.7 with 0.6M or more $MgCl_2$. The carbodiimide reaction gave only a weak reaction, indicating a relatively low level of carboxyl groups.

The conclusions reached were that glycogen is present in large quantities along with two sulphated acid mucopolysaccharides. The persistance of Alcian Blue staining in the presence of 1.0M $MgCl_2$ coupled with the low carboxyl levels suggests that one is keratan sulphate or something similar while the reduction of Alcianophila by 0.6M $MgCl_2$ or hyaluronidase suggests that a chondroitin sulphate or something similar is also present.

4.4 SUMMARY AND CONCLUSIONS

The chorion of the egg of G. leucoloma is a flexible structure 4 - 9μm thick which is composed of protein in which disulphide linkages are probably important in the maintenance of structural integrity, although other types of linkages may well be involved. The egg possesses a very large number of aeropyles which are distributed over the entire outer surface and which communicate with a narrow trabecular layer near the inner surface of the chorion. This allows for gaseous exchange while providing a relatively small volume which water vapour can occupy, thereby possibly helping to reduce water loss. It appears that the aeropyles may provide a sufficiently large air-water interface for plastron respiration to occur if the egg was submerged in aerated water, although such a function has not so far been demonstrated. The large number of aeropyles and their distribution suggest that this is a mechanism ensuring that enough of them are exposed to the atmosphere to allow respiration to occur both in and out of water, since the eggs are held together in clumps and surrounded by a copious cement. This cement is a complex, variable and heterogeneous mixture of proteins and acid mucopolysaccharides. Apart from the functions of lubrication during laying and the binding of eggs together it may also provide extra protection from physical damage and while it probably has a minor role in water conservation, it may be involved in the uptake of water after the end of a dry period, helping to soften the egg shell and therefore indirectly aiding hatching. There is no micropylar apparatus, and this is related to the parthogenetic nature of the insect, which obviates the need for such a system.

The vitelline membrane is a tough structure 0.3 - 0.5μm thick which is composed of both proteins and acid mucopolysaccharides. It is highly resistant to chemical attack and is responsible for the major part of the waterproofing of the egg, this latter function being served by a thin layer of lipid on the outer surface of the membrane. This lipid layer is probably also largely responsible for the impermeability of the egg shell to a range of water soluble substances. The basis of the high degree of chemical stability of this membrane has so far not been determined but it undoubtedly contains moderate levels of tyrosine, and this may indicate that tanning may be involved, although no darkening was observed. Tanning of protein has been implicated in other species, as noted above. These studies indicate that to penetrate the shell, an ovicide would need to have a moderately high lipid solubility (but not too high as it might then dissolve in the lipid layer and stay there) and may need to be a relatively

small molecule.

Comparative studies of the structure of the egg shell of parthenogenetic and sexually reproducing weevils may provide some insight into the evolution of the lack of a micropylar apparatus, while comparisons of composition of the cement, with the extrachorionic secretions of other insects may be useful in establishing the extent and role of such complex mixtures of substances as occurs in G. leucoloma. Biochemical analyses of the envelopes are also needed to more precisely determine the nature of the compounds present.

APPENDIX ONE

MISCELLANEOUS OBSERVATIONS

A1.1 Resistance of Larvae to Dessication

About 20 larvae were collected from dry, sandy soil on which rain had not fallen for several weeks during the summer of 1973/74, and were placed in a jar with the same soil. No water was added for about three months, after which the soil was very difficult to wet. Food was provided in the form of an occasional carrot, which shrivelled within two days. In spite of this treatment, two adults emerged about eight months after collection, demonstrating that the larvae possess considerable resistance to dessication.

A1.2 Observations on the Relationship between Larval Populations and Crop Damage

During preliminary work a crop of rape (Brassica Napus) at Rangiotu in the Manawatu was observed in which the relationship between crop loss, larval damage and other factors appeared to be complex. The plants in one half of the field died early in a drought but supported a very low larval population, while the other half was more healthy, even though it was infested with a high population of G. leucoloma larvae. Later, however, as the soil dried out further large areas died, leaving small patches of healthy plants. Few larvae were found in the healthy patches but many occurred around the dead and dying plants.

A1.3 Jumping by Newly Hatched Larvae

Newly hatched larvae were observed to be capable of leaping for several centimeters by curling the body into a circle and suddenly straightening it. They were thus capable of easily escaping from the petri dishes used during the first season's work, but very few escaped unless they were left for several days, because their normal reaction was to crawl beneath the filter paper. A similar ability to jump has been found in the related species Asynonychus cervinus Boheman (Dickson, 1950).

A1.4 Cannibalism by Larvae

While pupae were being collected from site three (see Chapter Three) a few larvae were found with their heads buried in pupae, and were apparently devouring them. This site had by far the highest population density. In addition, larvae from all sites were generally very aggressive

and if two were placed together one or both would soon be bitten and the body wall punctured. Similarly, pupae accidentally dropped in with larvae in the ice-cube trays were frequently bitten and punctured before they could be removed. Both larvae and pupae usually died after being bitten.

A1.5 Duration of the Pupal Stage

The pupal period of a sample of 34 individuals, which were collected as larvae, was found to be 19.6 ± 0.3 days with a standard deviation of 1.7 days and a range of from 15 to 23 days at 20 ± 1.5°C.

A1.6 Drinking of Water by Adults

Several adults were observed drinking water from the drop placed in the container. This was most noticeable when the insects had just laid large clumps of eggs with considerable quantities of cement.

A1.7 Deformed Adults in the Field

One teneral adult was found in site three in which the elytra had failed to expand and this indicated that this deformity, which was the commonest occurring in the laboratory, could also occur naturally.

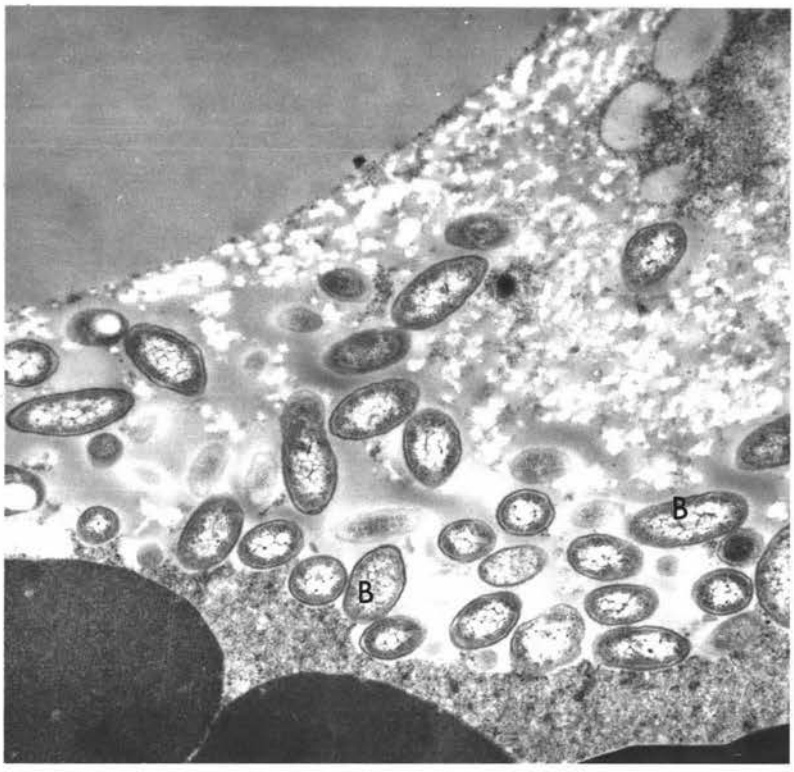
A1.8 Bacteria in the Eggs

During examination of sections of eggs with the transmission electron microscope a number of rod shaped bacteria were observed in the yolk (Fig. A1.1a,b). These were usually in distinct clusters, and where they were not there was evidence of poor infiltration with resin. (As already noted in Chapter Four, infiltration of these eggs proved to be difficult.) While these bacteria may have been pathogenic it is also likely that they are normally present, as symbiotic bacteria are frequently transmitted in the egg (Wigglesworth, 1972).

Fig. A1.1 Transmission electron micrographs of the yolk showing the rod-shaped bacteria which were observed in the eggs.

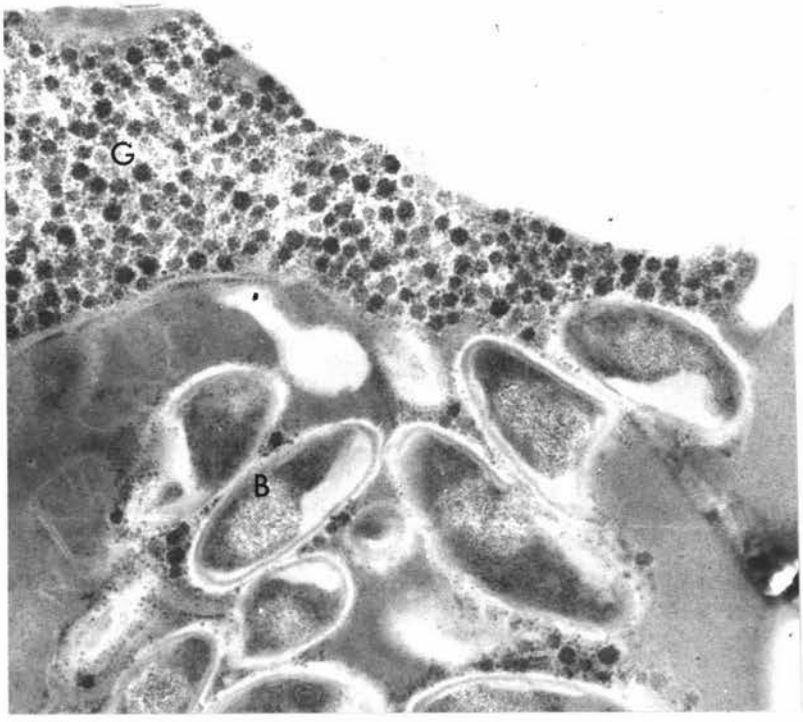
B bacteria

G glycogen granules



1µm

1µm



APPENDIX TWO

ARTIFICIAL DIET FOR THE ADULT WHITE-FRINGED WEEVILS (Barnes and Bass, 1973)

Ascorbic acid	4 g
Brewers yeast	14 g
Cellulose	5 g
Vitamin diet fortification mixture (NBC)	15 g
Casein	15 g
Lucerne leaf meal	14 g
Cane sugar	10 g
Maize oil	2 ml
Distilled water	430 ml
Agar	11 g

The agar plus water were heated to boiling and the dissolved agar placed in a blender. The dry ingredients and oil were added, mixed together thoroughly, poured into petri dishes, allowed to solidify, and then placed in the refrigerator.

APPENDIX THREE

HISTOLOGICAL AND HISTOCHEMICAL METHODS

A3.1 Transmission Electron Microscopy

1) Fix in a mixture of 3% glutaraldehyde and 2% formaldehyde in 0.1M phosphate buffer, pH 7.2, for 3-5 hrs at 4°C.

2) Wash in three changes of buffer.

3) Post fix in 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.2, for 4 hrs at 4°C.

4) Wash in three changes of buffer.

5) Dehydrate in a graded ethanol series (25% stages).

6) Propylene oxide, two changes.

7) Infiltrate in a propylene oxide-resin series to complete resin (Fluka epoxy resin).

8) Cure in fresh resin in oven at 60°C for 3 days.

9) Cut sections and place them on supporting grids.

10) Stain for 7 min in a saturated solution of uranyl acetate in 50% ethanol.

11) Wash in 50% ethanol, then in water.

12) Stain for 7 min in 0.1 - 0.4% lead citrate in approximately 0.1N sodium hydroxide (Venable and Coggeshall, 1965).

13) Wash in three changes of distilled water, and allow to air dry.

14) Examine.

A3.2 Light Microscopy

A3.2.1 Embedding of eggs

1) Fix in appropriate fixative, wash and dehydrate in a graded ethanol series.

2) Place in 1% celloidin in methyl benzoate for 24 hrs.

3) Place in fresh celloidin solution for 48 hrs.

4) Place in a third change of celloidin solution for 72 hrs if tissues are not clear.

5) Three washes in benzene - 4, 8 and 12 hr respectively.

6) Infiltrate with wax (60°C Paraffin + ceresin, 3 changes, 20 min each, in vacuum) and embed.

A3.2.2 Histochemical methods

All non-aqueous preparations were mounted in Xam.

(a) Alcian Blue 2.5 procedure (Pearse, 1968)

Method. (1) Bring sections to water.

(2) Stain in freshly filtered Alcian Blue 8GX, 1 percent in 3 percent acetic acid, 30 minutes (pH 2.5).

(3) Wash in running water for 5 minutes.

(4) Dehydrate in alcohols, clear in xylene and mount.

Result. Weakly acidic sulphated mucosubstances, hyaluronic acids and sialomucins, dark blue. Strongly acidic sulphated mucins are stained weakly or not at all.

(b) Alcian Blue 1.0 procedure (Pearse, 1968)

Method. (1) Bring sections to water.

(2) Stain for 30 minutes in 1 percent. Alcian Blue 8GX in 0.1 N-HCl (pH 1.0).

(3) Blot dry with fine filter paper (Whatman No. 50).

(4) Dehydrate in alcohols, clear in xylene and mount.

Result. Only sulphated mucosubstances stain.

(c) Alcian Blue - critical electrolyte concentration method (Scott and Dorling, 1965; Ashhurst and Costin, 1971a).

Stain. a) 0.1% Alcian Blue 8GX in 0.05M acetate buffer, pH 5.7, with the addition of magnesium chloride ($MgCl_2$) at the following concentrations: 0.05M, 0.1M, 0.2M, 0.4M, 0.6M, 0.8M and 1.0M.

b) 0.1% Alcian Blue 8GX in 3% acetic acid, pH 2.5, with magnesium chloride added as for the stain at pH 5.7.

Method. (1) Bring sections to distilled water.

(2) Stain for 18 hr at room temperature in the appropriate stain solution.

(3) 3 washes (5 min each) in the same buffer plus salt solution in which the stain was dissolved. This avoided the possibility that replacing the buffer-plus-electrolyte solution with water might alter conditions such that excess dye could then bind with previously unstained areas (Ashhurst and Costin, 1971a).

(4) Dehydrate and mount.

(d) Azure A staining procedures (Ashhurst and Costin, 1971a)

Method. (1) Bring sections to water.

(2) Stain for 30 min in the following:

a) 0.02% Azure A, pH 1.5, in 30ml 0.1N hydrochloric acid and 20ml 0.1M potassium dihydrogen phosphate.

b) 0.02% Azure A, pH 3.5, in 48ml distilled water, 1.4ml 0.1M ci citric acid and 0.6ml 0.2M disodium hydrogen phosphate.

c) 0.02% Azure A, pH 4.0, in 48ml distilled water, 1.25ml 0.1M citric acid and 0.75ml 0.2M disodium hydrogen phosphate.

(3) Examine preparations mounted in distilled water.

(e) Toluidine blue

(1) Bring sections to water.

(2) Stain in 0.5% aqueous Toluidine Blue.

(3) Examine as wet mounts.

(f) The periodic acid-Schiff (PAS) technique (Pearse, 1968)

Method. (1) Bring sections to water.

(2) Oxidize for 10 minutes in 1.0 percent aqueous periodic acid.

(3) Wash in running water for 5 minutes.

(4) Immerse in Schiff's reagent 10 minutes.

(5) Wash in running water for 5 minutes.

(6) Dehydrate in alcohol, clear in xylene and mount.

Result. Hexose-containing mucosubstances stain in various shades of purplish-red. Glycogen stains deeply.

(g) Modified PAS test for uronic acid-containing glycosaminoglycans (Scott and Dorling, 1969; Ashhurst and Costin, 1971a,b)

Method. (1) Bring sections to water.

(2) Treat with cetylpyridinium chloride (CPC) for 30 min at 30°C. CPC forms insoluble complexes with glycosaminoglycans, and was used because hyaluronic acid becomes depolymerised and highly diffusable when exposed to periodate for long periods (Ashhurst and Costin, 1971b).

(3) Oxidise in 2% aqueous sodium meta periodate for 60 min at 30°C.

(4) Treat with 1% aqueous sodium borohydride for 3 min at room temperature to block the periodate engendered aldehyde groups.

(5) Oxidise in 1% aqueous sodium metaperiodate for 24 hr at 30°C.

(6) Follow standard Schiff procedure (above) from step (3).

Control sections were taken after both the first periodate oxidation and the subsequent borohydride treatment and treated as for step (6).

(h) Periodic acid-paradiamine method (PAD) (Pearse, 1968)

Reagents. Add 50 mg. N,N-dimethyl-p-phenylenediamine HCl, just before use, to 50ml citrate-phosphate buffer (0.1M-Citric acid, 4.8ml, 0.2M disodium phosphate, 7.2ml, distilled water 38 ml).

Method. (1) Bring slides to water.

(2) Oxidize in 1 percent periodic acid for 10 minutes.

(3) Rinse in running water for 10 minutes.

(4) Immerse in paradiamine solution for 7, 24 or 48 hours.

(5) Differentiate in 1 percent. HCl in 70 percent alcohol for 8 seconds (after 24 hours' stain) or for 10 seconds (after 48 hours' stain).

(6) Wash in water for 5 minutes.

(7) Dehydrate through the alcohols, clear in xylene and mount in a synthetic medium.

Result. Neutral mucopolysaccharides, brown; periodate-reactive polymers, purple or grey-brown; periodate unreactive mucosubstances, black.

(i) Methylation, using methanolic thionyl chloride

Add 1ml $SOCl_2$ slowly to 50ml methanol. Allow to stand overnight before use. Sections are treated with the reagent for 4-6 hours at 22°. All tissue basophilia is abolished in this time. Cytoplasmic pasophilia due to COOH and RNA disappears in 30 minutes, and that due to sulphated mucopolysaccharides in 4 hours. After 6 hours the extraction of the phosphate groups of nucleic acids begins. It is complete in 24 hours. Extraction of mucopolysaccharides is negligible up to 12 hours. Primary amines are methylated in 30-60 minutes.

(j) Tetrazonium method for sulphate groups (Pearse, 1968)

Method. (1) Bring paraffin sections to water.

(2) Treat with acidic Fast blue B solution for 10-30 minutes (50mg Fast blue B salt in 10ml 5 percent acetic acid).

(3) Rinse for 30-60 seconds in cold (0°-5°) distilled water.

(4) Treat for 2-5 minutes in a cold (0°-5°) saturated solution of 1-naphthol in borax buffer at pH 9.4.

(5) Wash in distilled water and mount in glycerine jelly, or other watery medium.

Result. Tissue sulphate esters, and sulphonic acid groups induced by oxidation or sulphation, are stained reddish violet.

(k) Diaminobenzidine (DAB) method for sulphate groups (Bussolati, 1971)

Method. (1) Bring sections to water.

(2) Wash in two changes of 2% boric acid (30 sec. each).

(3) Treat for 30 min. in a freshly prepared 1% solution of 3,3'-diaminobenzidine hydrochloride dissolved in 5% boric acid.

(4) Wash in three changes of 2% boric acid (30 sec. each).

(5) Apply a drop of 1% osmium tetroxide to the sections.

(6) Dehydrate and mount.

The presence of sulphate groups is indicated by a brownish-black colour which develops immediately.

(l) Modified carbodiimide reaction for carbohydrate-bound carboxyls (Geyer, 1971)

Carbodiimide-thiocarbohydrazide solution:

Dissolve 100mg I-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-p-toluene-sulphonate and 50mg thiocarbohydrazide in 20ml 10% glycerol in 0.2M borax buffer at pH 7.4.

Hexamine-silver solution:

Stock solution A: 25ml 3% hexamethylene tetramine and 1.25ml 5% silver nitrate.

Stock solution B: 5% sodium tetraborate.

For use mix 25ml of solution A and 3ml of solution B with 22ml of distilled water.

Method. (1) Dewax paraffin sections and bring to water.

(2) Incubate for 2 hr at 30°C in the carbodiimide-thiocarbohydrazide solution.

(3) Rinse in distilled water for 30 min at 50°C.

(4) Treat with hexamine-silver solution (heated for 30 min at 60°C) for 2-10 min at 60°C.

(5) Wash in distilled water.

(6) Immerse in 5% sodium thiosulphate for 1 min.

(7) Wash in water.

(8) Dehydrate, clear and mount.

Result. Sites of carbohydrate-bound carboxyl groups stain dark brown or black.

(m) Hyaluronidase digestion (Ashhurst and Costin, 1971a)

Method. (1) Bring slides to water.

(2) Incubate for 2 hr at 37°C in an 0.05% solution of testicular hyaluranidase (in this case ovine type III, original activity 610 NF units/mg (Sigma Chemical Corporation)) in 0.1M phosphate buffer, pH 5.5. Incubate controls in buffer only.

(3) Stain with Alcien blue at pH 1.0 and 2.5 by the standard method.

(n) Neuraminidax digestion

Add 0.5ml of Vibrio cholerae neuraminidase (initial activity 500 units/ml) to 2ml of 0.05M acetate buffer, pH 5.5, containing 1% sodium chloride and 0.1% calcium chloride.

Method. (1) Bring sections to water.

(2) Incubate for 18 hr at 39°C in the enzyme solution.

(3) Incubate controls in buffer only.

(4) Stain with Alcian blue 8GX at pH 2.5 by the standard procedure.

(o) Diazotization-coupling method for tyrosine (Pearse, 1968)

Method. (1) Bring paraffin sections to water.

(2) Nitrosate (in the dark) for 18-24 hr at 3° in a mixture containing 6.9g $NaNO_2$, 5.8ml acetic acid, distilled water to 100ml.

(3) Rinse in four changes of ice-cold distilled water.

(4) Treat for 1 hr at 3° with a mixture containing 1g S-acid (8-amino-1-naphthol-5-sulphonic acid). 1g KOH, 1g ammonium sulphamate and 100ml 70 percent alcohol. (This part of the reaction should also be carried out in the dark since the reaction product is light-sensitive.)

(5) Wash in three changes of 0.1 N-HCl, 5 minutes in each.

(6) Wash in running water, 10 minutes.

(7) Dehydrate, clear, and mount in neutral synthetic resin.

Result. Tyrosine-containing proteins stain purplish-red to pink.

(p) The DMAB-nitrite method for tryptophan (Pearse, 1968).

Method. (1) Bring sections to absolute alcohol and allow them to become just dry in the air at room temperature.

Alternatively,

(2) Remove from alcohol and coat with a thin film of celloidin (0.25 percent); proceed to the next stage without drying.

(3) Immerse sections in 5 percent p-dimethylaminobenzaldehyde in conc. HCl (S.G. 1.18) for 1 minute.

(4) Transfer to an approximately 1 percent solution of $NaNO_2$ in conc. HCl for a further minute.

(5) Wash for 30 seconds in tap water.

(6) Rinse in 1 percent acid-alcohol.

(7) Dehydrate, clear and mount.

Result. (Fig. 47 and Plate Ia). Tryptophan-containing proteins are shown in varying intensities of deep blue.

(q) Tryptophan method for formalin-fixed tissues (Pearse, 1968)

Method. (1) Bring sections to absolute alcohol.

(2) Dip sections into the following solution: glycerol, 5ml; 60 percent ferric chloride, 1ml; conc. H_2SO_4, 5ml; distilled water, 9ml; methylated alcohol, 80ml (this solution keeps for several months in a stoppered bottle).

(3) Grip slide in forceps, tip off excess fluid, ignite in a small flame. While burning, hold slide horizontal with the section uppermost. Repeat process 3-6 times.

(4) Wash in absolute ethanol.

(5) Rinse until clean in glacial acetic acid/ethanol (1:1). Clear in xylene and mount.

Result. A mauve pigment indicates sites containing tryptophan. Examine (and photograph) within 24 hours as the pigment is not stable.

(r) The Sakaguchi dichloronaphthol reaction for arginine (Pearse, 1968)

Preparation of Reagents:

Immediately before use prepare: (a) 4 percent barium hydroxide (filtered), (b) 1 percent sodium hypochlorite, (c) 1.5 percent 2,4-dichloro-a-naphthol in tert butanol.

Method. (1) Bring slides to water, using 2 changes of distilled water. Blot slides and place in an empty staining jar.

(2) Pour 5 parts $Ba(OH)_2$, 1 part NaOCl and 1 part dichloronaphthol into a flask in succession. Agitate after each addition. Pour contents of the flask into the staining jar.

(3) Allow reagent to act for 10 minutes at 22°.

(4) Transfer slides to three 5 second changes of tert butanol; agitate vigorously in each change.

(5) Transfer to two changes of xylene (30-60 secs) containing 5 percent tri-N butylamine.

(6) Drain and mount in immersion oil (1.515)* containing 10 percent tri-N-butylamine.

Result. An orange-red colour indicates sites containing arginine. (If a $BaCO_3$ precipitate forms on the underside of the slide it can be removed with cotton wool soaked in dilute acetic acid.)

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* The original method called for Shillaber's oil (ref. index, 1.580) but this was not available for the present study.

(s) The DDD method for SH groups (Modified from Pearse, 1968)

Method. (1) Bring sections to water.

(2) Incubate for 1 hour at 50°C in a solution containing 35ml 0.1M-veronal acetate buffer (pH 8.5) and 15ml absolute ethanol in which has been dissolved 25mg of the DDD reagent (2,2'-dihydroxy-6,6'-dinaphthyl disulphide).

(3) Cool to room temperature.

(4) Rinse briefly in distilled water.

(5) Wash for 10 minutes in two changes of deionised distilled water acidified to pH 4 - 4.5 with acetic acid (deionised water was used in this case because the distilled water at Massey University had a pH below 4). This step converts the sodium salt of the reagent, and of the unwanted reaction product (6-thio-2-naphthol), to free naphthols.

(6) Extract the free naphthols by passage through a graded series of alcohols including two changes of absolute ethanol, and wash three times in absolute ether for 5 minutes in each wash (Note: this washing schedule is increased over that outlined by Pearse, 1968).

(7) Wash in distilled water.

(8) Stain for 2 minutes at room temperature in a freshly prepared solution of 50mg tetrazotized diorthoanisidine (Fast blue B salt) in 50ml 0.1M-phosphate buffer at pH 7.4.

(9) Wash in running tap water.

(10) Dehydrate in alcohols, clear in xylene and mount.

Result. Blue staining indicates a high concentration of SH groups; red staining areas contain lower concentrations. Without any other treatments, this method demonstrates cysteine.

(t) DDD method for SS groups (Pearse, 1968)

This requires the blocking of pre-existing SH groups (cysteine) (in this case with N-ethyl maleimide) and reduction of SS groups (cystine) (with thioglycollate in this instance.

Maleimide block: Treat sections for 4 hours at 37° with 0.1 M-N-ethyl maleimide in 0.1 M-phosphate buffer at pH 7.4. Follow this by washing in 1 percent acetic acid and then in tap water.

Thioglycollate reduction: Cover sections with a thin layer of 0.5 percent celloidin. Incubate for 4 hours at 37° in 0.5 M-thioglycollic acid (freshly made) titrated to pH 8 with 0.1 N-NaOH. Afterwards wash in tap water, rinse briefly in 1 percent acetic acid and wash again in water.

(u) Mixed anhydride method for side-chain COOH (Pearse, 1968)

Method. (1) Remove wax from paraffin sections with light petroleum.

(2) Allow to dry and then wash in glacial acetic acid, 2 minutes.

(3) Incubate sections for 1 hour at 60° in a mixture of equal parts of acetic anhydride and anhydrous pyridine (redistilled over barium oxide).

(4) Rinse in glacial acetic acid and wash in absolute alcohol.

(5) Incubate for 2 hours at 22° in 0.1 percent 2-hydroxy-3-naphthoic acid hydrazide. (50mg hydrazide in 2.5ml warm glacial acetic acid, to which is added 47.5ml of 50 percent ethanol).

(6) Wash in 3 changes (10 minutes each) of 50 percent ethanol.

(7) Incubate for 30 minutes in 0.5 N-HCl at 22°.

(8) Rinse in distilled water and then in 3 changes of 1 percent sodium bicarbonate.

(9) Rinse in several changes of distilled water.

(10) Transfer slides to a solution containing equal parts of 0.06M Sorensen's phosphate buffer (pH 7.6) and absolute ethanol, to which has been added 1 mg/ml tetrazotized diorthoanisidine (Fast blue B salt). Colour develops in 2-5 minutes.

(11) Wash in distilled water.

(12) Dehydrate, clear, and mount in a synthetic medium.

Result. Protein-bound side-chain carboxyl groups give a deep reddish-purple colour.

(v) Chloramine-T Schiff method for protein-bound NH_2 (Pearse, 1968)

Method. (1) Bring paraffin sections to water.

(2) Treat with 1 percent aqueous chloramine-T (pH 7.5) for 6 hours at 37°.

(3) Wash briefly in distilled water.

(4) Treat with 5 percent aqueous solution thiosulphate for 3 minutes.

(6) Rinse in 10 percent aqueous sodium bisulphite.

(7) Wash in tap water.

(8) Dehydrate in alcohols, clear in xylene and mount in DPX.

Result. Protein-bound NH_2 groups stain pink or reddish-magenta.

APPENDIX FOUR

REAGENTS AND STAINS USED IN THE PRESENT STUDY

Reagent	Synonyms	Grade, type or purity	Purchased from
Alcian Blue 8GX			G.T. Gurr
8-amino-1-naphthol-5-sulphonic acid	S-acid		Sigma
Basic fuchsin		?	BDH
1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-p-toluene sulphonate		99%	Aldrich
3,3' diaminobenzidine tetra hydrochloride	3,4,3',4'-tetra aminobiphenyl hydrochloride	Spot test reagent	BDH
2,4,dichloro-α-naphthol		Laboratory reagent	BDH
2,2'-dihydroxy-6,6'-dinaphthyl disulphide (DDD)	bis(2-hydroxy-6-naphthyl) disulphide		Sigma
p-dimethylamino-benzaldehyde (DMAB)		Analar	Hopkin & Williams
N,N-dimethyl-p-phenylenediamine hydrochloride	p-amino dimethyl-aniline hydrochloride	Purified Grade III	Sigma
N-ethyl-maleimide			Sigma
Hexamethylene tetramine	Hexamine, methenamine	Laboratory reagent	BDH
Hyaluronidase		From ovine testis Type III. Activity 610 NF units/mg	Sigma
2-hydroxy-3-naphthoic acid hydrazide			Koch-light

1-naphthol		Analar	BDH
Neuraminidase	N-acetylneuraminate glycohydrolase	From *Vibrio cholerae*	BDH biochemicals
Tetrazotized diorthoanisidine	Fast blue B salt		Sigma
Thiocarbohydrazide		Purum	Fluka
Toluidine Blue		Standard stain	BDH

APPENDIX FIVE

A list of insect species referred to in the text, and their taxonomic position.

Acheta domesticus L.	Orth., Gryllidae
Adelges piceae Ratzeburg	Hem., Phylloxeridae
Amanthes c-nigrum L.	Lep., Noctuidae
Anobium punctatum Degeer	Col., Anobiidae
Antheraea mylitta Drury	Lep., Saturniidae
Anthonomis grandis Boheman	Col., Curculionidae
Anthonomis signatus Say	Col., Curculionidae
Atrachya menetriesi Faldermann	Col., Chrysomelidae
Bacillus libanicus Uvarov	Phasmida, Bacteriidae
Bombyx mandarina Moore	Lep., Bombycidae
Bombyx mori L.	Lep., Bombycidae
Bruchus obtectus Say	Col., Bruchidae
Calliphora erythrocephala Meigen	Dipt., Calliphoridae
Cerura vinula L.	Lep., Notodontidae
Cimex lectularius L.	Hem., Cimicidae
Conoderus vespertinus Fabr.	Col., Elateridae
Costelytra zealandica White	Col., Scarabaeidae
Diabrotica	Col., Chrysomelidae
Diatraea grandiosella Dyar	Lep., Pyralidae
Drosophila melanogaster Meigen	Dipt., Drosophilidae
Galleria mellonella L.	Lep., Pyralidae
Graphognathus leucoloma Boheman	Col., Curculionidae: Brachyderinae
Graphognathus minor Buchanan	Col., Curculionidae: Brachyderinae
Graphognathus peregrinus Buchanan	Col., Curculionidae: Brachyderinae
Gryllus mitratus Burmeister	Orth., Gryllidae
Haematobia irritans L.	Dipt., Muscidae
Hyalophora cecropia L.	Lep., Saturniidae

Hylobius pales Herbst	Col., Curculionidae
Hypera brunneipennis Boheman	Col., Curculionidae
Hypera postica Gyllenhal	Col., Curculionidae
Laspeyresia pomonella L.	Lep., Tortricidae
Lasiorhynchus barbicornis Fabr.	Col., Brentidae
Lissorhoptrus oryzophilus Kuschel	Col., Curculionidae
Listronotus oregonensis Le Conte	Col., Curculionidae
Locusta	Orth., Acrididae
Lytta viridana Le Conte	Col., Meliodae
Melanoplus differentialis Thomas	Orth., Acrididae
Ocypus olens Müller	Col., Staphylinidae
Oncopeltus fasciatus Dallas	Hem., Lygaeidae
Oryctes rhinoceros L.	Col., Scarabaeidae
Otiorhynchus sulcatus Fabr.	Col., Curculionidae
Plodia interpunctella Hübner	Lep., Pyralidae
Ptinus tectus Boield	Col., Ptinidae
Rhodnius prolixus Stähl	Hem., Reduviidae
Schistocerca gregaria Forsk.	Orth., Acrididae
Sitona lineata L.	Col., Curculionidae
Sitophilus granarius L.	Col., Curculionidae
Sitophilus oryzae L.	Col., Curculionidae
Sympetrum frequens Selys	Odonata, Libellulidae
Sympetrum infuscatum Selys	Odonat, Libellulidae
Tetrix	Orth., Tetrigidae
Thermobia domestica Packard	Thysanura, Lepismatidae
Tribolium confusum Duval	Col., Tenebrionidae
Trichoplusia ni Hübner	Lep., Noctuidae

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