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DOCTOR OF PHILOSOPHY

The biology and control of Riccadoella limacum (Schrank), a mite pest of farmed snails.

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The

Biology and Control

of

Riccardoella limacum (Schrank),

a Mite Pest of

Farmed Snails

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A thesis submitted to the University of Wales

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in candidature for the degree of
Philosophiae Doctor

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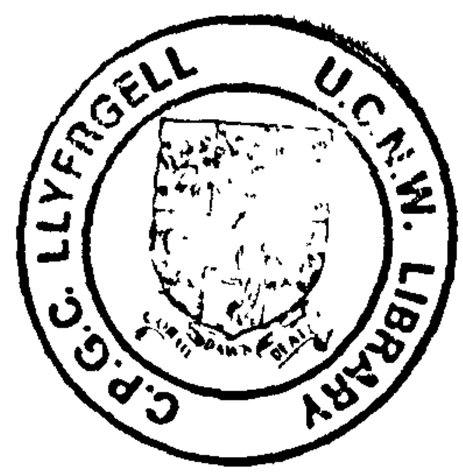

"INTO THE ARK WITH NOAH WENT ONE PAIR, MALE AND FEMALE OF ALL BEASTS, CLEAN AND UNCLEAN, OF BIRDS AND OF EVERYTHING THAT CRAWLS ON THE GROUND. . ."

GENESIS 6:8

For

Alexis,

one of the bravest

and

most determined

women

I have known...

and

Mum and Dad

for their

love and support

Fi

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SUMMARY

The differences between the similar species, *Riccardoella limacum* and *Riccardoella oudemansi* were examined using SEM techniques. The two species may be separated by differences in setal structure and number. The internal anatomy of *R. limacum* was examined using plastic sections for light microscopy and found to be similar to previous descriptions. Computer-aided three dimensional reconstruction was carried out on serial sections and the relative positions of various organs noted. The life cycle of *R. limacum* was confirmed as having a hexapod larva and three nymphal stages. The occurrence of an overwintering egg was observed in hibernating snail hosts and there was no relationship between hatching and snail hibernation. No such egg is present in *R. oudemansi*, perhaps related to the death of their hosts during the winter. Mites were found to spread rapidly to colonise a new host and quickly laid numerous eggs, large numbers of mites have been seen to build up inside a snail.

R. limacum was found to contain a substance which reacted with keyhole limpet haemocyanin antibodies, this was likely to be haemocyanin. The mite obtains haemocyanin as a form of nourishment from the hosts blood, though the possibility that mucus is also a source of food cannot be eliminated in this study. The effect of different mite numbers on the snail was examined. High mite infestations cause poor quality shells, slow growth and delayed reproductive maturation. A low number of mites can be tolerated seemingly with little ill effect except on reproduction. The concentration of proteins and glycogen/free glucose in the snail tissues was the same in snails with and without mites. However, as the final weights of snails were dramatically different, the total amount of protein and glycogen/free glucose in each snail was also different.

Some interest in *R. limacum* was shown by the predatory mite, *Macrocheles muscaedomesticae* though it is not certain whether deaths of *R. limacum* can be attributed to the predator. *R. limacum* is attracted to certain stimuli more than others, snail odour being the preferred stimulus. In the light of the present study recommendations for controlling *R. limacum* on a commercial snail farm have been made.

CONTENTS.

Page No.

CHAPTER 1: GENERAL INTRODUCTION.....1

CHAPTER 2: MAINTENANCE OF EXPERIMENTAL ANIMALS.....
2.1 Mite Culture.....7
2.2 Snail Culture Aquaria.....10

CHAPTER 3: COMPARISON OF TWO CLOSELY RELATED SPECIES OF THE GENUS: *RICCARDOELLA*.....
3.1 Introduction.....12
3.2 Materials and Methods.....15
3.3 Results.....17
3.3.1 Description of *Riccardoella limacum*.....17
3.3.2 Main Differences Between *Riccardoella limacum* and *Riccardoella oudesmansi*.....29
3.4 Discussion.....31

CHAPTER 4: WHOLE MOUNTS, INTERNAL ANATOMY AND THREE-DIMENSIONAL RE-CONSTRUCTION.....
4.1 Introduction.....32.
4.1.1 Histological Investigations of Internal Anatomy.....32.
4.1.2 Whole Mounts.....32
4.1.3 Computer-aided Three-Dimensional Re-construction.....34
4.2 Materials and Methods.....37
4.2.1 Preparation for Light Microscopy.....37
4.2.2 Preparation of Whole Mounts.....40
4.2.3 Computer-aided Three-Dimensional Re-construction.....41
4.3 Results.....43
4.3.1 Histological Observations.....43
4.3.1.1 The Reproductive System.....43
4.3.1.2 The Digestive System.....53
4.3.1.3 The Muscular System.....54
4.3.1.4 The Nervous System.....54
4.3.2 Whole Mount Observations.....55
4.3.3 Computer-aided Three Dimensional Re-construction.....55
4.4 Discussion.....67

CHAPTER 5: SOME ASPECTS OF THE LIFE CYCLE AND DISPERSAL.....
5.1 Introduction.....70
5.2 Materials and Methods.....75
5.2.1 Collection from Wild Populations.....75
5.2.2 Effects of Temperature, Humidity and Snail Physiology on Mite Populations in Wild Snails.....76
5.2.2.1 Effect of Influencing Snail Activity by Manipulating Humidity.....76
5.2.2.2 Effect of Temperature.....77

5.2.3 Life Cycle Observations in the Laboratory.....78
5.2.3.1 Addition of Larvae.....78
5.2.3.2 Addition of Adults.....78
5.2.3.3 Use of Culture Cells.....79
5.2.4 Dispersal of Mites.....79
5.2.5 Proportion of Life Stages in the Population.....79
5.2.6 Observations on Internal:External Proportions.....80

5.3 Results.....81
5.3.1 Monthly Population Sample.....81
5.3.2 Effects of Temperature, Humidity and Snail Physiology Influencing the Mites.....86
5.3.3 Life Cycle Observations in the Laboratory.....88.
5.3.3.1 Addition of Larvae.....88.
5.3.3.2 Addition of Adults.....88.
5.3.3.3 Culture Cells.....92.
5.3.4 Dispersal of Mites.....92.
5.3.5 Proportion of Life Stages in the Population.....93
5.3.6 Observations on the Internal:External Proportions....93
5.5 Discussion.....100

CHAPTER 6: OBSERVATIONS ON THE FOOD AND NUTRITION OF *RICCARDOELLA LIMACUM*.....
6.1 Introduction.....105
6.2 Materials and Methods.....110
6.2.1 Location of Mites Feeding *in situ*.....110
6.2.2 Immunological Techniques.....110
6.2.2.1 Raising Antiserum.....110
6.2.2.2 Testing for Cross Reactivity with *Helix haemocyanin*.....111
6.2.2.3 SDS PAGE.....112
6.2.2.4 Western Blotting of SDS PAGE.....117
6.3 Results.....120
6.3.1 Location of Mites Feeding *in situ*.....120
6.3.2 Immunological Tecniques.....120
6.3.2.1 Raising Antiserum.....120
6.3.2.2 Testing for Cross Reactivity with *Helixhaemocyanin*.....120
6.3.2.3 SDS PAGE.....120
6.3.2.4 Western Blotting of SDS PAGE.....123
6.4 Discussion.....127

CHAPTER 7: LONG TERM EFFECTS OF *RICCARDOELLA LIMACUM* LIVING IN THE PULMONARY CAVITY OF *HELIX ASPERSA*.....
7.1 Introduction.....131
7.2 Materials and Methods.....137
7.2.1 Experiment A: Effect of High and Low Incidence of Mites on Isolated Snails.....137
7.2.2 Experiment B: Effect of High Incidence of Mites on Communally Reared Snails.....140
7.3 Results.....142
7.3.1 General Observations.....142

7.3.2 Experiment A....142
7.3.2.1 Final Weights....147
7.3.2.2 Shell Characteristics....147
7.3.2.3 Histological Observations....147
7.3.2.4 Condition of Lung....155
7.3.3 Experiment B....155
7.3.3.1 Final Weights....155
7.3.3.2 Shell Characteristics....158
7.3.3.3 Albumen Glands....158
7.3.3.4 Histological Observations....158
7.3.3.5 Condition of Lung....162
7.3.4 Statistical Analysis....162
7.3.4.1 Experiment A....162
7.3.4.2 Experiment B....163
7.4 Discussion....164

CHAPTER 8: BIOCHEMICAL ANALYSIS OF SNAILS INFESTED WITH MITES....
8.1 Introduction....168
8.2 Materials and Methods....172
8.2.1 Preparation of Samples....172
8.2.2 Protein Assay....172
8.2.3 Glycogen/Free Glucose Assay....174
8.3 Results....176
8.3.1 Protein Assay....176
8.3.2 Glycogen/Free Glucose Assay....176
8.3.3 Statistical Analysis....176
8.3.3.1 Protein Assay....176
8.3.3.2 Glycogen/Free Glucose Assay....177
8.4 Discussion....183

CHAPTER 9: FACTORS AFFECTING THE CONTROL OF *RICCARDOELLA LIMACUM*....
9.1 Introduction....185
9.2 Materials and Methods....189
9.2.1 Use of a Predatory Mite....189
9.2.2 Agents Attractive to *Riccardoella limacum*....190
9.3Results....193
9.3.1 Use of a Predatory Mite....193
9.3.2 Agents Attractive to *Riccardoella limacum*....194
9.4 Discussion....195

CHAPTER 10: GENERAL CONCLUSIONS AND DISCUSSION....
10.1 General Conclusions....198
10.2 General Discussion....202

APPENDIX....209
REFERENCES....212

CHAPTER 1

GENERAL INTRODUCTION

Man has exploited snails as a source of food for many years. As long ago as 50 BC the Romans "farmed" *Helix pomatia* and indeed introduced it to Britain (Cooper & Knowler, 1991) although they did not breed their snails, but merely kept them in an enclosure and fattened them ready for use when required.

In regions such as Africa, snails are widely used as a common everyday food; the large Achatinidae e.g. The Giant African Land Snail, *Achatina fulica* provides an important source of protein (Monney, 1992). Also, in Thailand, the same snail has been collected in such quantities as to seriously reduce the "natural" population although the locals dislike of its light coloured flesh means that most of the collected snails are exported to other countries (Upatham *et al.*, 1988). Closer to home, the smaller helicid snails enjoy much acclaim as a delicacy, *H. pomatia, Helix lucorum* and *Helix aspersa* (Müller) being widely farmed and collected throughout Europe to supply the huge demand that has recently arisen, France being the major consumer. *H. aspersa* has two common varieties; the smaller, grey garden snail, *H. aspersa normalis* that are traditionally collected for consumption throughout France and *H. aspersa maxima*, the larger of the two, that is more commonly farmed. In countries such as France the demand far exceeds the supply of both commercially farmed and locally collected snails and consequently this traditional snail eating country must now import some of its requirement from other places (Curry, 1991). Presently snails are collected in Spain, Portugal, Greece, Turkey, Austria, Germany,

Poland, Hungary, Rumania, Switzerland and France, some countries having companies dealing solely with the import of snails either for their own consumption or for re-export to another country depending on the demand, (Welch & Pollard, 1975). The demand for snails has caused a total ban on their collection in Holland and temporary bans in Switzerland, there is also legislation governing the time of the year when collection of wild populations of snails can take place and the minimum size allowed. This varies from country to country but for *H. pomatia* is approximately 32 mm in Switzerland and 28 - 38 mm in Hungary (Welch & Pollard, 1975).

In Africa the eating of achatinid snails is seasonal because they can only be freely collected in the rainy season with only the rich able to afford them in the dry season as a luxury item (Monney, 1992). This coupled with the obvious demand for *Helix spp.* in Europe has opened the way for a snail farming industry to develop. At first the only animals to be kept artificially tended to be *Achatina spp.* and these only in small private collections as pets, in zoos or as laboratory animals. These animals were kept in vivaria varying in size depending on the age of the snail. Rees (1950) suggests 3" of garden humus as a substrate, Pawson and Chase (1984) use a crushed shell substrate, while Nisbet (1974) preferred 9" of sterilized soil. Nisbet (1974) also states that the previous trend for keeping upwards of 500 adult animals that occurred in the 1950's at the time of Rees's work, is excessive and results in poor quality stock. He recommends that approximately 200 animals are a suitable number for one worker to keep in a healthy condition. Farms come in many forms, some in France are like the original Roman enterprises where snails are merely fattened for consumption. On other farms the snails are bred and reared in outside runs with nesting boxes provided. They may also be kept in plastic tunnels, although these have the disadvantage of wide temperature fluctuations and a limited life span (3-7

years). Using these methods snails may take 18 months to reach a marketable size (Cooper & Knowler, 1991), and possibly another year to reach sexual maturity (Lazaridou-Dimitriadou & Saunders, 1986). Intensive indoor farming with high snail numbers within a controlled environment and maintained at 80-90% humidity and 20°C has only really taken off in Britain in the last 10 years, before this snails were again collected from the wild when required (Groves, 1991). Snails farmed under these conditions are usually *H. aspersa maxima*, they will not hibernate and with continuous growth reach a marketable size in about five months and sexual maturity at about six or seven months. Very few of these snails are required to maintain a breeding population and most are fattened ready for sale to the consumer.

The first attempts at farming in the UK were not very successful. Electric heating tended to be used (Groves, 1991) and although power cuts would not affect so harshly those who farmed *Helix spp.*, many farmers experienced catastrophic losses of their faster growing achatinid snails which cannot stand large temperature fluctuations. Another common fault was to try to build too large an operation and many of these more ambitious farmers have since gone out of business. Groves (1991) who set up the Snail Centre in Colwyn Bay to advise people on these matters, suggests that a standard unit of 400,000 snails per year is a good number to start with. Obviously one of the major concerns of a farmer is to have a healthy breeding stock that will give him a good annual turnover with reasonable profits. To establish such a farm the farmer must understand many aspects of snail biology. The type of food, for example, is crucial to building healthy animals as they, like other animals, have requirements that must be met. Their diet must contain the vitamins A, B complex and possibly D (Howes, 1937) and they certainly need a good supply of calcium with which to build strong shells. However the greater weight of snails given a calcium supplement, compared with controls was attributed not to the shell

but to the actual flesh and Crowell (1973) suggested that a supply of calcium gives healthier snails that grow more successfully. Aside from the feeding programme, environmental parameters must be assessed as these can have a major effect on the reproductive cycle of snails (Owiny, 1974). The light regime is important and Hodasi (1982) working on *Achatina (Achatina) achatina* found that a continuous light source or a reversed light/dark cycle promoted better and faster growth, while natural light cycles gave the best results for reproduction. Stephens & Stephens (1966) working on *H. aspersa* found that the snails needed at least 15 hours of light in order to induce egg laying. Temperature is also known to have a direct effect on reproduction (DeWitt, 1954); too high a temperature can be detrimental to both oocytes and sperm with oocytes being more seriously affected (Lüsis, 1966) although Bank (1931) states that the sperm of *H. pomatia* is more vulnerable to higher temperatures than oocytes. Low temperatures delay the development of both and low humidity causes the development of more oocytes, (Bouillon, 1956; Lüsis, 1966). Temperature is also known to affect the snail's eating habits, Mason (1970) found that in *H. aspersa* assimilation rate of food was dependant on the temperature between 5-15 °C and on a well-run farm temperature must be maintained within narrow limits. The snails will also begin to slow their activity and become dormant if the humidity falls to a low enough level, even at an adequate temperature (Lazaridou-Dimitriadou & Saunders, 1986). Hibernation is a natural part of the snail's life cycle and does not damage them in any way. Darbishire (1889) reports a specimen of *Helix aperta* having woken from hibernation after three years and Baker (1934) states that *Oxystyla capax* has lasted up to 23 years. Cases such as this are undoubtable rare and a farmer will no doubt be advised to try to prevent hibernation as it interrupts the growth of his stock. To keep the snails growing optimally it is necessary to clean them regularly and avoid too high a stocking density as a build up of mucous will slow overall growth, although the actual

physical constraints of numbers do not appear to affect growth (Dan & Bailey, 1982). This can be a very laborious business and must surely have been the downfall of many small enterprises. It is very important that not just the mucus is removed but also any dead animals, as these encourage the build up of bacteria and attract flies which can become a nuisance (Annandale, 1919). Cabaret *et al.* (1988) state that three hours a day is spent on farm maintenance in the breeding season and recommend that the floor, breeding cages and instruments be washed more than twice a week using antiseptics and insecticides. They also suggest a quarantine period for new snails brought in from outside to help minimise parasitic infections.

Another problem affecting farmed snails is a bacterial infection of the foot caused by the soil-water living *Aeromonas*. This results in blistering of the foot, may cause sterility and, in the most severe cases, death (Runham, 1991). The extent of the damage to the snail depends upon its general wellbeing and hence how well organised the farm is. The all too common use of copper water pipes and steel rivets in the modern automatic cleaning systems, now used to minimise the work load, has created many problems associated with toxicity of metals. Copper, zinc and manganese are all known to inhibit growth and in extreme cases cause death. Metal toxicity can be remedied by the addition of two plastic filters the second of which is fitted with a cation exchange resin to remove all the metals from the water supply. Unfortunately this will also remove calcium that is required in the building of healthy shells and therefore necessitates the addition of calcium in the diet (Runham, 1991). The remaining problems which afflict farmed snails tend to be parasite related, trematodes and nematodes being particularly common, (Cabaret *et al.*, 1988; Morand, 1985, 1988) but the one that has inspired this study is the snail mite; *Riccardoella limacum* Schrank. This mite frequently infects the snails, in farms particularly, as in general there are no quarantine periods to establish whether or not the snails are

infested before introducing a new batch to the existing animals.

The lack of quarantining for new stock animals is probably partially to blame for the introduction to farms of the mite *R. limacum*. *R. limacum* is a prostigmatid mite of the family Ereynetidae, it has a characteristic integument, patterned with lines, long sensory hairs and in the adult form is white with a pale pinkish tinge. It is visible to the naked eye on the terrestrial land pulmonate molluscs that are its hosts where it can readily be seen running over the surface of the body and frequently entering the pulmonary cavity via the pneumostome. What it actually does once inside the lung of the host has long been debated in the literature, and there have been many reports of its occurrence over the years. However there has been confusion over the identification of the mite and its close relation *Ricardoella oudemansi* which is found on slugs, indeed the following authors have wrongly named the mite they have been working on; Baker, (1945); Turk & Phillips, (1946); White, (1959); Baker, (1967, 1970a, 1970b & 1973); Arutyunyan, (1972); and Mienis, (1984). Those who correctly named the mite found on its snail host are fewer and include; André & Lamy, (1930); Thor, (1932); Fain & Van Goethem, (1986); and Fain & Klompen, (1990). This confusion will be dealt with in Chapter 3.

It is not certain whether the mite feeds on mucous secretions of the lung or whether it punctures the skin and drinks the blood of the snail for nourishment. If the latter is true, it is not certain whether this has a detrimental effect on the growth of the snail, and therefore the profit of the farmer and this aspect will also be dealt with in Chapter 7. It is known for certain, however, that *R. limacum* has a high incidence on snail farms, in fact Morand (1985) puts it in the top five parasites found on *H.aspersa*. One of the purposes of this work was to discover to what extent, if any, the mite affects the snail and to advise on its importance to the snail farmers.

CHAPTER 2

MAINTENANCE OF EXPERIMENTAL ANIMALS.

2.1 Mite Culture.

In vitro culturing of mites has been studied for many years with a variety of species, particularly those of some economic importance. Banks (1915) is one of the earliest to comment that keeping mites is not all that easy to accomplish, he suggests that free living forms may be kept quite successfully on a mixture of decaying leaf litter but that the parasitic types are more difficult. He says that the Oribatidae and Parasitidae may be kept in a cell formed by placing a piece of cut rubber tubing onto a microscope slide and covered with a microscope coverslip, these cells must not be allowed to dry out and to accomplish this he adds a small piece of damp blotting paper.

Much research has been carried out on the so-called "chiggers", which belong to the family Trombiculidae and are important vectors of disease in the Far East (Keay, 1937; Michener, 1946), and several workers around this period have tried to develop culture methods. The main advantage that these researchers had was that chiggers can be fed quite successfully while in the larval stage on vertebrate surrogate hosts such as chicks, snakes, turtles and lizards (Jenkins, 1947) and then transferred, when fully engorged, to a rearing jar containing a mixture of soil and chicken faeces (Melvin, 1946) to develop into further stages. Melvin notes the appearance of

nymphs in his culture bottles after nine to eighteen days and says that a second generation of larvae appeared shortly after this. This is contrary to later reports which state that it had not been possible to rear the mite through more than one generation and that even in the first generation there were very high mortalities, making it impossible to establish a breeding colony in the laboratory, (Jenkins, 1947). These mites were kept at a relatively high temperature, between 28-30 °C, and a relative humidity of 86-100%. This was maintained by the use of wet plaster of Paris in the rearing jars which served to keep the humidity levels up without the formation of too much condensation which would drown the young mites. It was necessary to use a layer of activated charcoal over the plaster of Paris to cover the toxic plaster, (Jenkins, 1947). Using this type of arrangement Melvin (1946), Michener (1946) and Jenkins (1947) have all had some success rearing *Eutrombicula batatasis* and maintaining it in the laboratory. This method was not updated until Jones (1950a), working on the harvest mite *Trombicula autumnalis* modified the equipment used to raise the mite from the larval stages. He used a small perspex cell lined with filter paper and fed the nymphs on a mixture of chicken faeces, agar, yeast and molasses. This method succeeded in rearing the mite through to the adult stage, whereas a similar method employed by Keay (1937) using airtight glass dishes only managed to rear nymphs, no adults being obtained. Cleat (1952) reports a similar success with *Scheloribates laevigatus* using a further modification of the filter paper cell in which one of the layers of filter paper was omitted.

However a return to the type of cell previously used by Banks (1915) was favoured by Baker (1967) when he attempted to culture *Riccardoella limacum (=Riccardoella oudemansi)*, and get it to feed artificially away from its molluscan host, a task which he admitted was extremely difficult as so little was known about the natural food of this species. The cells used by Baker were made by drilling holes of 1.5cm

diameter and 2mm depth in a piece of perspex which was then placed on a microscope slide. The larval mite was placed inside and a coverslip applied over the top with a film of water on the under side. In his first experiments in 1967, Baker attempted to get the mites to feed on a variety of foods including: fresh yeast, eggs from *Anopheles (Cellia) gambiae* (mosquito), chopped slug, slug haemolymph, slug homogenate and surface slug mucous. He first collected his mites from their hosts and then starved them for several days before transferring them to the culture cells along with the test food medium. Unfortunately despite satisfying the criteria favoured by all previous workers for using relatively airtight equipment, in order to minimise dehydration by evaporation, and therefore maintaining a high relative humidity in which the mites should feel comfortable, he could not get them to feed. None of the test foods proved suitable and the mite could not therefore be kept for any length of time. In later work Baker (1970b) used the same type of culture cell to look at the stages in the life cycle of the mite. He took specimens straight from the slug hosts, (again probably *R. oudemansi* and not *R. limacum*) and placed them in the cells for observations. He succeeded in rearing all the stages from larva to adult but not from the same mite, it was only possible for the mite to moult once before it died, presumably from starvation, but much valuable information was obtained in this way.

For the purposes of the present study it was obviously necessary to have a permanently accessible supply of mites and as all previous attempts at the long term culture of *Riccardoella spp.* by other workers proved fairly fruitless, another solution was required. It was decided that the best method to ensure a readily available supply of mites at all stages was to keep them *in situ* on their snail hosts; this meant that an easy and reliable method of keeping a relatively small quantity of snails was required.

2.2 Snail Culture Aquaria.

There are a few instances in the literature of workers keeping small scale cultures of terrestrial gastropods in the laboratory. These state very clearly that it is of the utmost importance that the tanks that are used are large enough to give ventilation whilst maintaining a high humidity level (Stephenson, 1961; Gray *et al.*, 1985). Stephenson (1961) used an organic loam substrate but Gray *et al.*, (1985) also tried vermiculite, these both hold moisture adequately but can be difficult to keep clean. Another problem associated with these substrates was the attraction of flies to the vermiculite and the presence of free living soil mites in the loam, though if any dead animals are removed along with all spoiled food and the sides of the tanks cleaned regularly, these problems are minimised (Gray *et al.*, 1985).

The snails were therefore housed in two large plastic aquaria, fitted with lids made from small aperture wire mesh on wooden frames. At first a loam mixture was used as a substrate for the animals as suggested in the literature but on closer inspection it was found to contain a high proportion of soil mites. These mites were identified as *Macrocheles muscaedomesticae* (Evans & Browning, 1956) and were observed on occasion to be carrying the mite *R. limacum* in their chelicerae. As there was some concern that *M. muscaedomesticae* may have harmed the culture population an uninfested substrate was preferred. The replacement was a highly absorbent cellulose wadding (medicinal gamgee tissue) which was placed in the bottom of the aquarium and moistened with water, keeping the humidity at an acceptable level for the snails. The snails *(H. aspersa)* were collected from several local sites and given commercial snail food, Winstay Cereal Compound Mixture, to eat. They were cleaned on a weekly basis, though spoiled food was removed more frequently, and sprayed daily with water to keep the humidity high. These aquaria were kept in the laboratory at

22°C which, provided the humidity was maintained, prevented hibernation. Collected snails were checked for the presence of mites, more snails were gathered and any mites carefully removed with a fine paint brush and transferred to the culture snails and thus a laboratory culture of mites was established and maintained using their natural hosts. Snails kept in this manner were healthy and lived up to three years supplying an almost constant stream of mites for this study.

This study not only required a reliable source of mites but also one of snails that had no mites. As it was not possible to tell from looking at a snail whether it was completely free of mites (although externally they may be mite free, the pulmonary cavity invariably contains several) it was necessary to set up a "clean culture" of snails. Juvenile snails approximately eight weeks old with a shell diameter of 1cm were obtained from a local commercial snail farm; L'Escargot Anglais, Hereford. They were housed in large plastic boxes with a wire mesh lid lined with netting, which prevented escape and helped to keep the humidity high. These boxes were kept in a room at 25 °C with a 16:8 hour light:dark cycle, a humidifier was installed to maintain the high humidity and encourage the snails to remain active and growing. The snails were cleaned and fed daily on commercial snail food. Periodically, random dissection of a few snails was made in order to check that they had not somehow become infected with mites. Snails were maintained in this manner quite successfully throughout the study until they were required for experiments.

CHAPTER 3

COMPARISON OF TWO CLOSELY RELATED SPECIES OF MITE OF THE GENUS; *RICCARDOELLA*

3.1 INTRODUCTION

Much of the available literature concerning the mite *Riccardoella limacum* was produced at, or before the beginning of this century. Consequently this research lacks many of the benefits of modern technology that could help explain the confusion arising between mites of this particular genus. There is also very little information concerning the commercial farming of snails or the relationship between the mites and the snails. The available literature on pests of farmed snails usually deals almost exclusively with a common nematode parasite with the effect of *R.limacum* on this industry largely ignored.

The first person to mention these "snail mites", was Rèamur in 1710 (In: Fain & Van Gothem, 1986) who described "insectes des limacons" which he collected from the snail host, *Helix pomatia*, but it was not until 1776 that Shrank (In:Fain & Van Gothem, 1986) named this mite *Acarus limacum*. In 1923 Berlese (In: Fain & Van Gothem, 1986) coined the modern generic name *Riccardoella* that was also used by Oudemans in 1929 when he described a new mite; *Riccardoella oudemansi*. Also in 1930, André & Lamy (1930) gave an account of the mite parasites that they found "sliding" around on the mucous secreted by the snails. They described how

the adults enter the lung chamber by way of the pneumostome when it opens and deposit their eggs there. The eggs then hatch to hexapod larvae which leave the lung, again via the pneumostome, and take up the parasitic lifestyle of their parents on the body of the snails, eventually becoming octopod nymphs and then adults. In 1932 Thor re-described the species, *R. oudemansi* and in 1933 published a large work in which he was the first to compare the two similar species and recognise the fact that they had different hosts, i.e. *R. limacum* living on snails and *R. oudemansi* on slugs.

The next major study was undertaken in 1946 by Turk and Phillips who compiled the "Monograph of the slug mite", although in this work they actually described *R.limacum* that lives on snails. In this paper they mentioned the fact that they found mites resembling both *R. limacum* and *R. oudemansi* and decided that along with another closely related mite; *R. jenynsi*, they must in fact be different stages in the life cycle of the same animal, with the name "*limacum*" taking preference over the other two. This study also contains work on the life cycle of the mite, it describes the different stages but does not say for how long they survive; this may be accounted for by the difficulty in keeping the mites alive once removed from the snail to a study environment. A basic study of the internal anatomy is also included although the accompanying pictures and diagrams are a little unclear.

White (1959) worked on the mite infestations of slugs that he also called *R. limacum* (the snail mite). Baker (1967, 1970a, 1970b, 1973) similarly collected mites from slugs, figured *R. oudemansi* in his publication but again wrongly named his mites *R.limacum*. Baker (1970a) also attempted to define the life cycle by placing mites in small culture cells made of plastic and trying to get them to feed off various mixtures of ground up snail, yeast and/or blood in an attempt to rear them through

their entire cycle. As well as work on the life history, Baker (1970b) wrote about the nutrition of the mite. Using light microscopy he obtained sections which did not show the presence of mucus in the gut when stained for mucus, but showed cells in the gut caecae similar to slug amoebocytes thus indicating blood feeding. Despite numerous attempts at clarifying this it is still unclear whether the mite feeds from the mucus covering the body of the snail and/or tissue cells or blood. Turk and Phillips (1946) favour the theory that the mite feeds only on the mucus and does not in any way harm the snail as its chelicerae are too blunt to pierce the skin. Other authors such as Jones (1950b) who worked on a related species (*Trombicula autumnalis*), have noticed a "histio-syphon" which pierces the tissues gradually and draws body fluids and cells into the mite. Lawrence (1951) worked on *R. eweri* and noted that although it was possible that the mite fed on mucus in the nasal cavities of the toad *Bufo regularis*, the gut contents, when examined closely, appeared similar to those of mites known to be blood feeders. This mite has similar mouthparts to *R. limacum* and therefore if it does feed on the toad's blood there is no reason why *R. limacum* should not also be a blood feeder, an option which many authors have dismissed as unlikely due to the nature of its feeding apparatus. Oldham (1931) states that a mite of the family Ereynetidae (to which *R. limacum* also belongs) infected nesting-boxes of *Arianta arbustorum* and caused many of the young to die producing serious shell deformations in some of the adults. It is unknown whether this was due to the mites feeding on their blood, or to some other effect of the mites.

Arutyunyan (1972) repeated the mistake of calling the slug mite, *R. limacum* as did Mienis (1984) so it was not until more recently that *R. limacum* and *R. oudemansi* and their respective hosts were finally separated. This was in work by Fain and Van Goethem (1986) in which detailed drawings pointed out the differences between the anatomy of the two species and their choice of host.

3.2 MATERIALS AND METHODS

Mites were removed from the surface of their snail host's (from the laboratory culture) body using a fine paint brush (Banks, 1915), this method caused less damage than using a pipette or suction. They were then placed in a small aluminium foil dish floating on iced water, this gradually slowed their activity to a standstill, and in many cases the legs were stretched out and displayed well. At this point they were then treated for microscopical examination in one of several ways, the most successful being the following:

1. A small drop of isopentane, which had been cooled in liquid nitrogen, was placed on the mites in the foil dish.
2. The dish was then gently touched on the surface of the liquid nitrogen.
3. Meanwhile a freeze-dryer was cooled to -70°C and the foil dish placed in the vacuum at this temperature and left for 24 hours.
4. After 24 hours the mites were removed from the freeze-dryer, mounted on stubs using Sellotape glue and coated with gold for 20 minutes in a Polaron sputter coater.
5. At this stage they were examined using a Hitachi scanning electron microscope SE520 (SEM) and pictures of all the main distinguishing features taken after closely examining both species.

This method was found to be better then the harsher methods of fixing in gluteraldehyde and dehydrating prior to using a critical point dryer, or fixing in osmium, critical point drying and coating.

Simultaneously with this study several specimens were prepared as whole mounts to use as an initial aid to identification of the two species using light microscopy.

Mites were placed in a drop of polyvinyl lactophenol on a microscope slide and then warmed gently to help to clear them. They were then examined carefully for any noticeable differences.

3.3 RESULTS.

The whole mounts provided very basic information concerning the two species; it was possible, for example, to see the difference in setal shape characteristic of the two, though not in great detail. It was also possible to see the genital aperture in enough detail to establish the sex of the mite and to see the structures known as genital suckers.

However the results of the SEM study were far more fruitful and it was possible to clearly distinguish between the two species.

3.3.1 Description of *R. limacum.*

As a member of the Ereynetidae *R. limacum* (and *R. oudemansi*) has palps with three joints, no eyes and few hairs on the idiosoma. The chaetotaxy of the legs and body is well known from work done by Fain and Van Goetham (1986) and confirmed by the electron microscope pictures.

Numbers of setae on leg joints of *R. limacum.*

Number of hairs	Leg 1.	Leg 2.	Leg 3.	Leg 4.
Tarsus	12	9	8	7/8
Tibia	5	3	3	3
Genu	4	4	3	3
Femur	4/5	3	2/3	3
Trochanter	1	1	0	0
Coxa	1	1	2	1

The tarsus of the pedipalp has three hairs and a small thumb-like sensillus, which can

PLATE 1

a). *Riccardoella limacum* showing narrow palpal hairs (EH) and a smaller apotele (A) compared to that of *R. oudemansi* and also hairs of the gnathosoma (AH) & (PH). Scale bar = 9.9μm.

b). *R. oudemansi* showing the shorter, more swollen hairs (SH) and slightly larger apotele (A), and the hairs of the gnathosoma (AH) & (PH). Scale bar = 7.9μm.

c). *R. limacum,* which has the following number of hairs on the coxae (C); 1 1 2 1. Scale bar = 26.8μm.

d). *R. oudemansi*, which has hairs; 2 1 2 1 or 2 1 3 1, the extra hair on the coxa is indicated by the arrow. Scale bar = 33.3μm.

e). *R. limacum;* elongated hairs (EH) ending with a fine extension. Scale bar = 15.6μm.

f). *R. oudemansi*; swollen hairs (SH) especially the two apical hairs which are swollen dorso-ventrally and compressed laterally. Scale bar = 33.3μm.

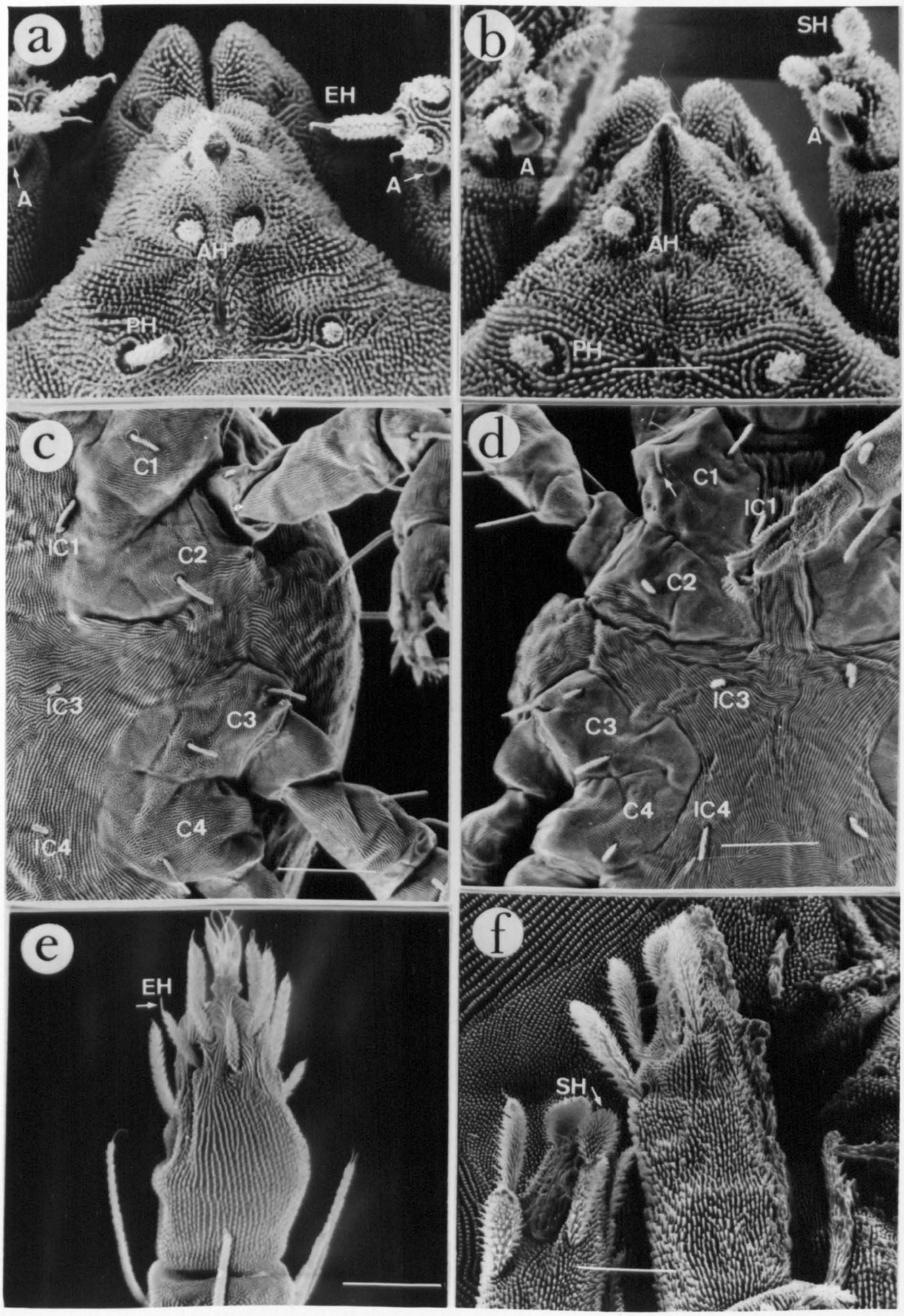

a
EH
A
A
AH
PH
b
SH
A
A
AH
PH
c
C1
IC1
C2
IC3
C3
C4
IC4
d
C1
IC1
C2
IC3
C3
C4
IC4
e
EH
f
SH

PLATE 2

a). *Riccardoella limacum* showing that "d1" is longer than "vi". Scale bar = 46.9μm.

b). *R. oudemansi* showing that "d1" is shorter than "vi". Scale bar = 50μm.

c). *R. limacum* female showing the arrangement of hairs in the genital region (1-5 & 1-5) this is the same in *R. oudemansi.* GA = genital aperture, AE & AI = anal hairs. Scale bar = 24.5μm.

d). *R. oudemansi* male showing the arrangement of hairs in the genital region, this is the same in *R. limacum.* GA = genital aperture, B = bristles inside the vestibule, P = penis, AE & AI = anal hairs. Scale bar = 15.3μm.

e). *R. limacum* deutonymph showing the two characteristic patterned regions (PR). Scale bar = 15.4μm.

f). Hexapod larva of *R. limacum* showing the dorsal arrangement of hairs "d1" - "d4", "ve", "vi", "sci", "sce", "l1" & "l4". Scale bar = 36.9μm.

g). Adult female *R. limacum* in the process of depositing an egg. E = egg, GA = genital aperture. Scale bar = 25μm.

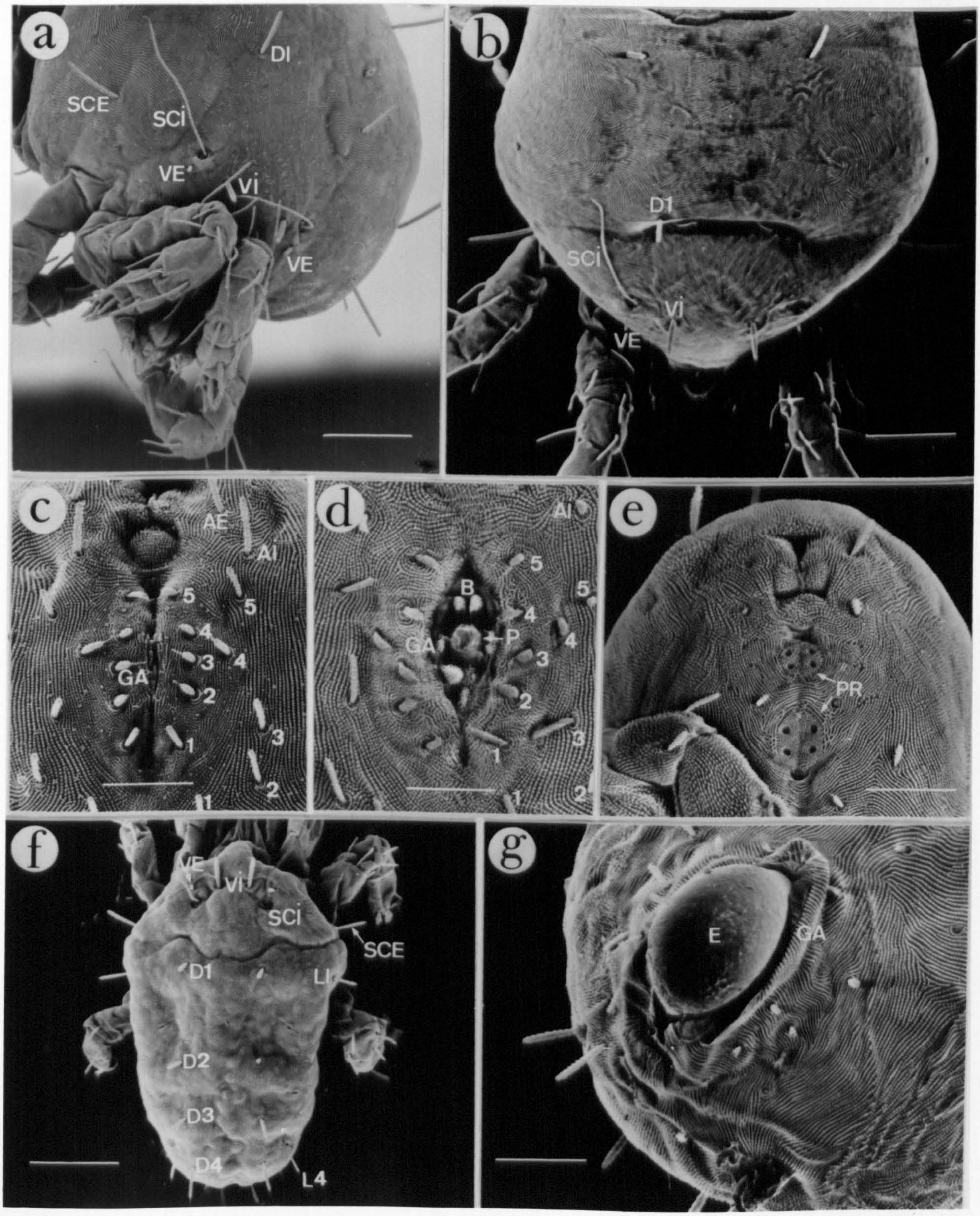

a
DI
SCE
SCi
VE
Vi
VE
b
D1
SCi
Vi
VE
c
AE
Ai
5
5
4
4
GA
3
2
3
1
2
1
d
Ai
5
B
5
4
GA
P
4
3
2
3
1
2
1
e
PR
f
VE
Vi
SCi
SCE
D1
Li
D2
D3
D4
L4
g
E
GA

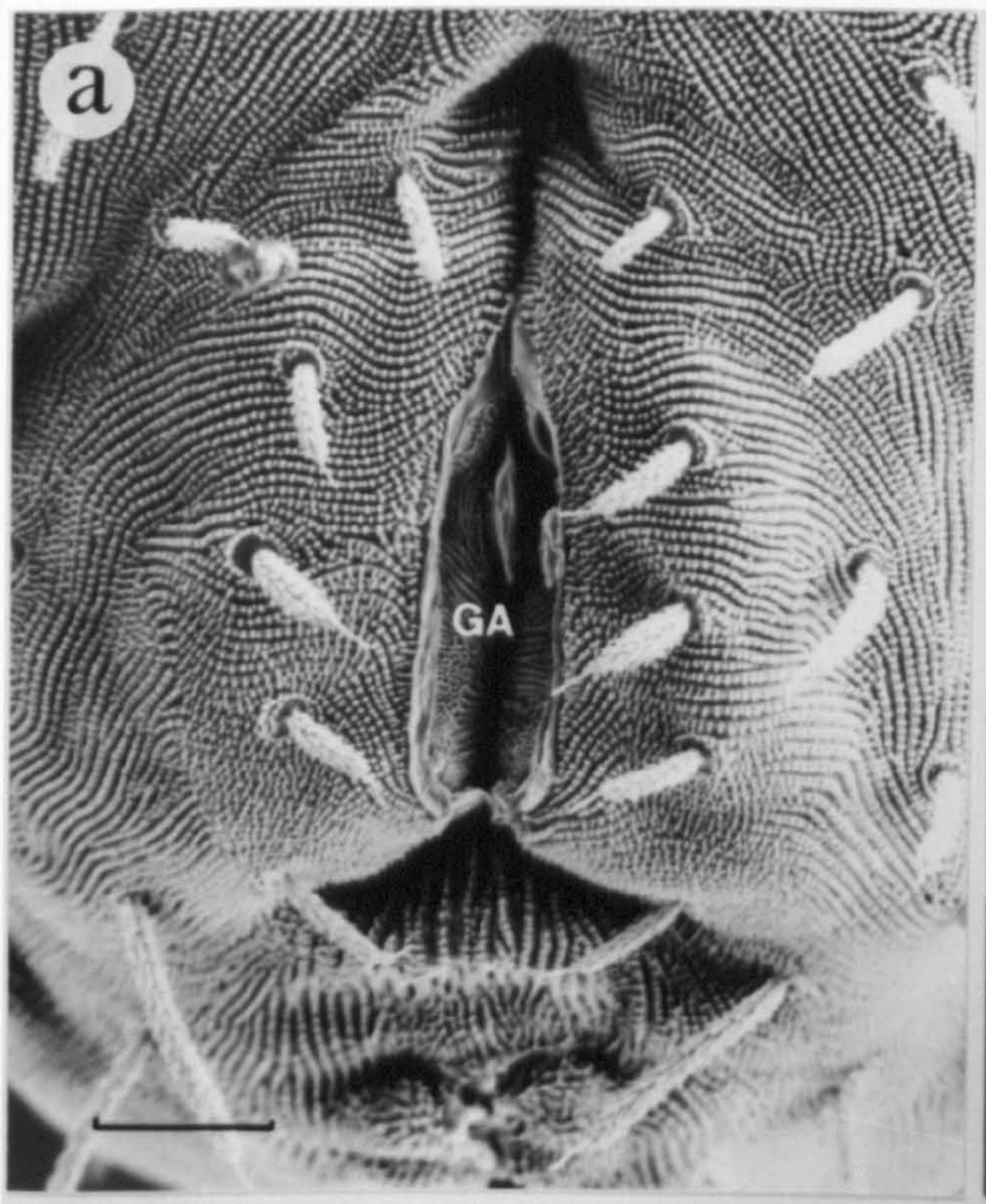
PLATE 3

a). *Riccardoella limacum* female with partially everted genital aperture (GA). Scale bar = 15μm.

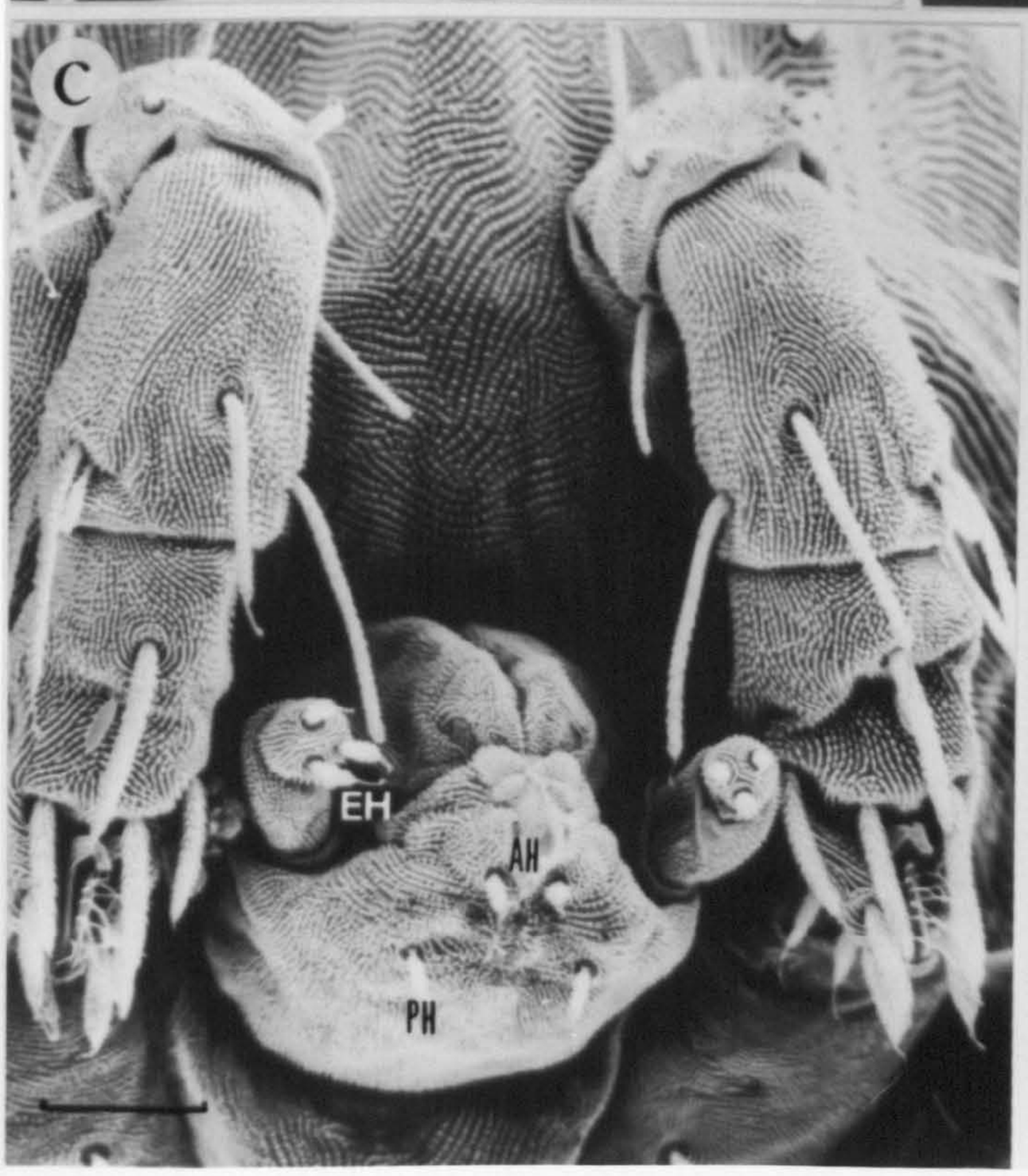
b). *R. limacum* female with everted genital aperture (GA), showing genital suckers (GS). Scale bar = 21.5μm.

c). *R. limacum* with characteristic elongated hairs (EH) and hairs of the gnathosoma (AH) & (PH). Scale bar = 17.8μm.

d). *R. limacum* showing some of the dorsal arrangement of hairs; "ve", "vi" & "sci". Scale bar = 15.35μm.


a
GA

b
GA
GS


c
EH
AH
PH

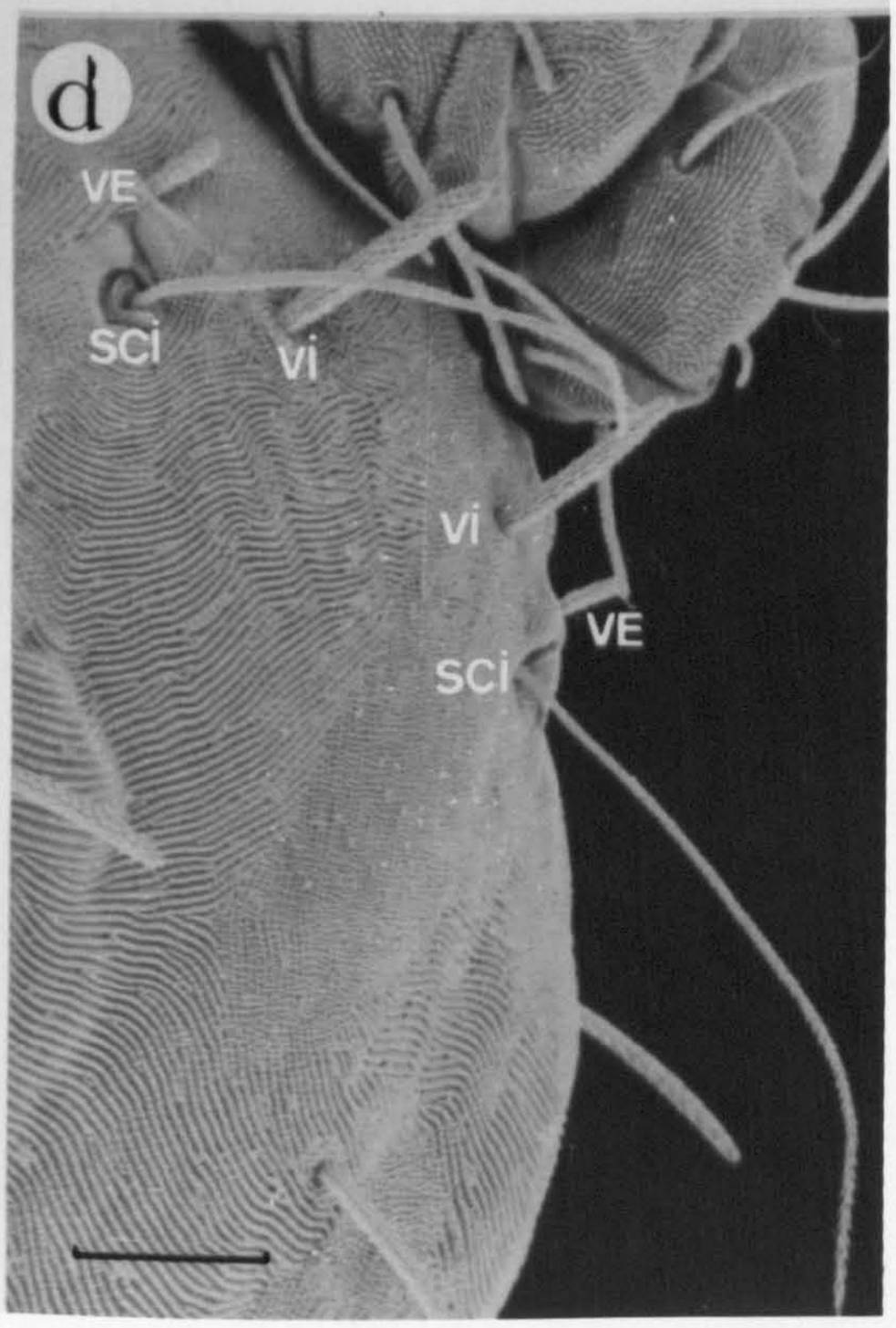

d
VE
SCi
Vi
Vi
VE
SCi

PLATE 4

a). Hexapod larva of *R. limacum* viewed from the front showing the chelicerae (CH) and palps (PP). Scale bar = 30μm.

b). Tarsus of first leg of *R. limacum* demonstrating the elongated hairs (EH) and characteristic "finger print pattern" of the integument (SP). Scale bar = 3.75μm.

c). Lateral view of the gnathosomic region (GN) of *R. limacum.* (PP) = palps, (AH) & (PH) = hairs of the gnathosoma. Scale bar = 20μm.

d). Gnathosoma (GN) of *R. limacum* showing elongated hairs (EH) and hairs (PH) & (AH). Scale bar = 12.95μm.

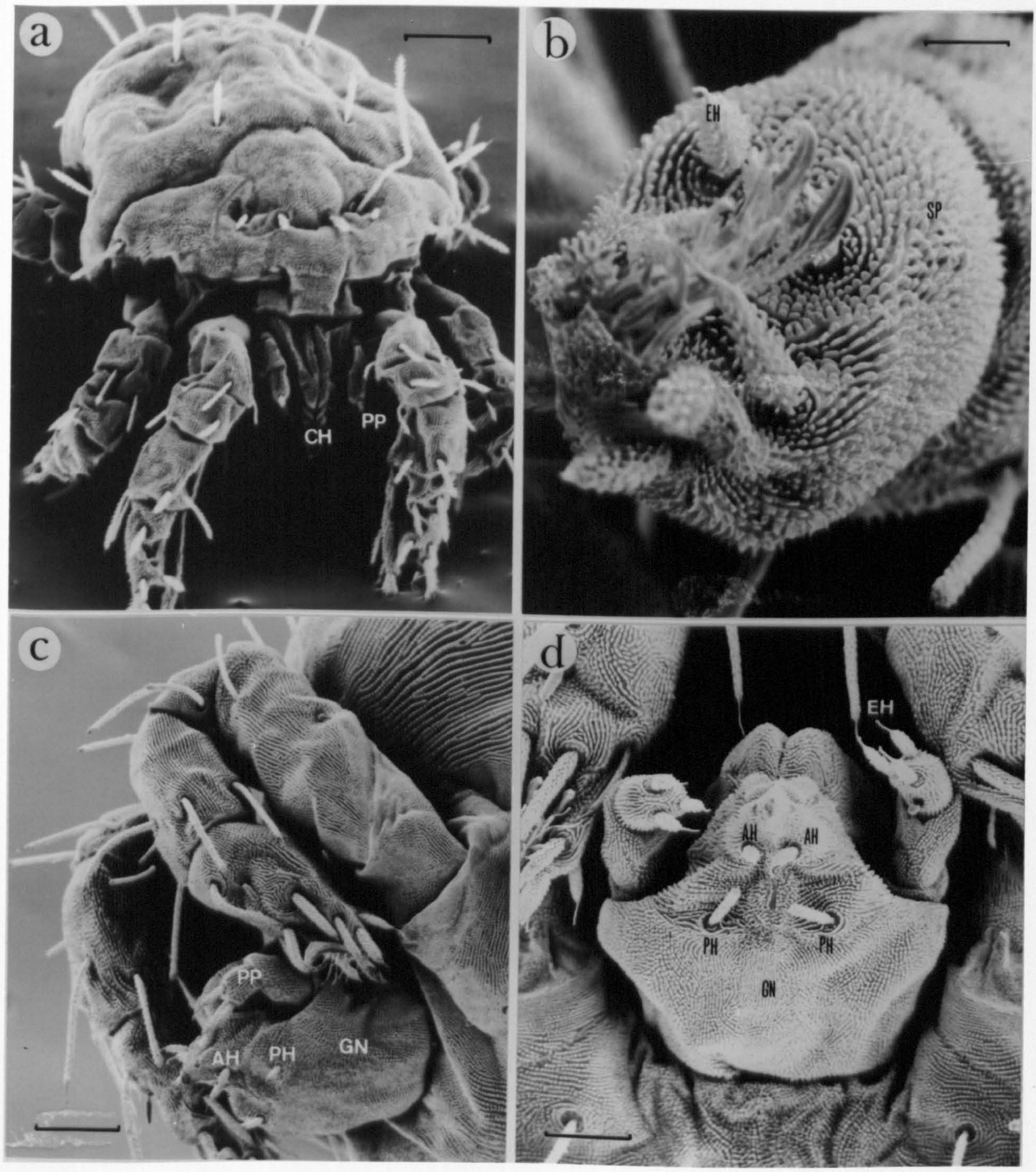

a
CH
PP
b
EH
SP
c
PP
AH
PH
GN
d
EH
AH
AH
PH
PH
GN

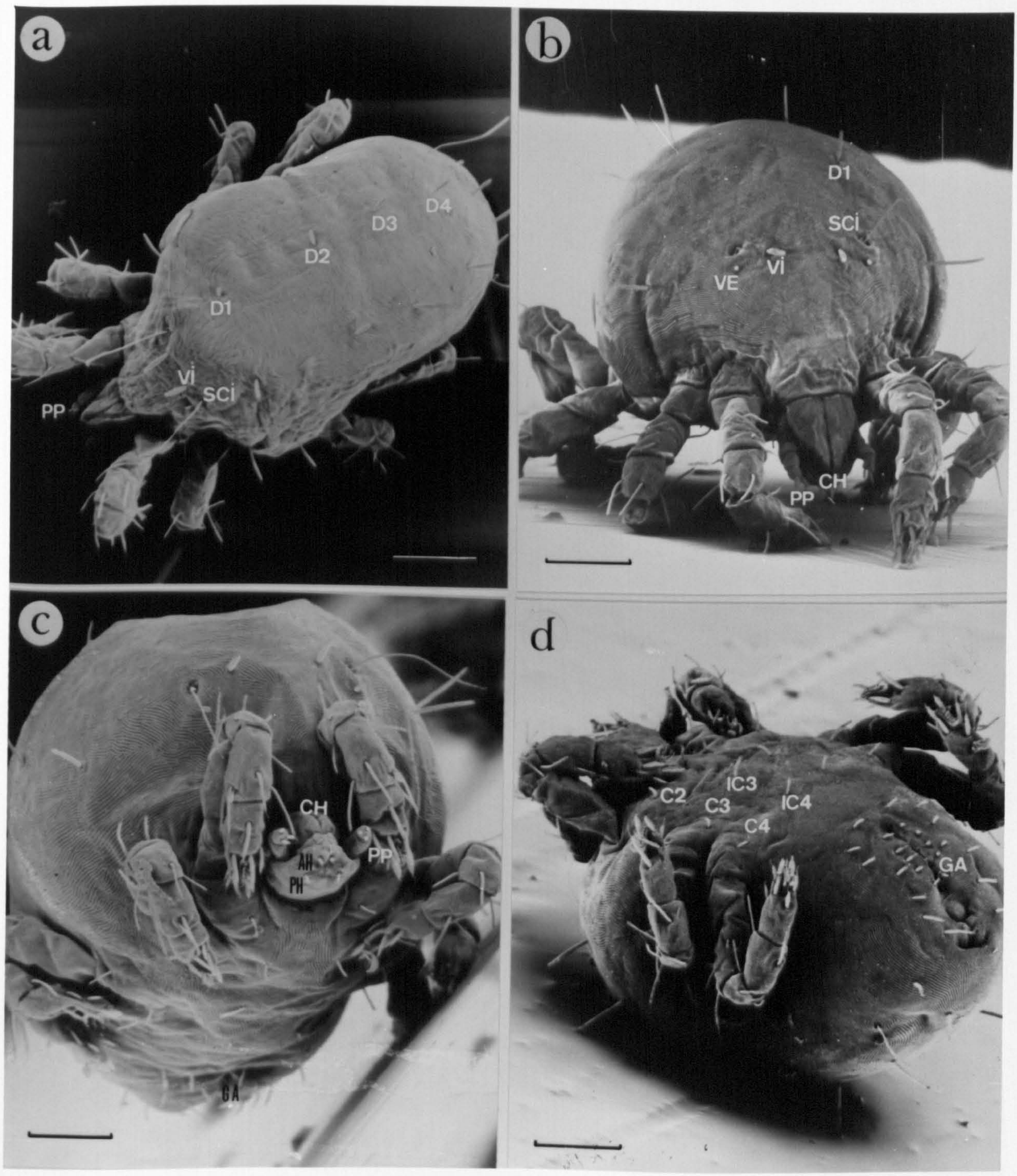
PLATE 5

a). *Riccardoella limacum* showing the dorsal hairs "d1" - "d4", "vi", "sci" and the palps (PP). Scale bar = 63.75μm.

b). *R. limacum*, frontal view of palps (PP) and chelicerae (CH), also some of the dorsal hairs. Scale bar = 36.45μm.

c). Frontal view of *R. limacum* from slightly underneath the mite showing position of the genital aperture (GA), palps (PP) and chelicerae (CH). Scale bar = 45μm.

d). Ventral view of *R. limacum* showing coxal hairs (C2, C3 & C4) and internal coxal hairs (IC3 & IC4) in relation to the genital aperture (GA). Scale bar = 50μm.


a
D1
D2
D3
D4
Vi
SCi
PP
b
D1
SCi
Vi
VE
CH
PP
c
CH
AH
PH
PP
GA
d
C2
C3
IC3
C4
IC4
GA

be seen in Plate 1a.

The setae of the dorsal surface of the body are generally easier to see than those of the legs as there are fewer of them. They consist of five pairs, termed "*dl* - *d5*" (Fain & Van Goethem, 1986), which run down the centre of the back (Plates 2b, 5a & 5b), two pairs, "*sc e*" and "*ll*" which are lateral to the others and are found at the widest part of the body (Plate 2a), and a shorter, thicker pair at the anterior of the body, "*vi*", next to a pair of extremely short ones to their right and left, "*ve*" (Plates 2a, 3d, 5a & 5b). Lastly there are two pairs of modified sensory hairs approximately twice the length of the "*d*" hairs, "*sci*", which are situated posterior to the hairs "*ve*"(Plate 2a, 3d & 5a) and hairs "*l4*" are at the rear of the mite in a lateral position (Plate 2f).

Ventrally the chaetotaxy is even simpler, being divided into the genital setae and three pairs of setae, found close to the bases of coxae 1,3 and 4, called "*icl*", "*ic3*" and "*ic4*" respectively (Plates 1c & 5d). It sometimes appears as though "*icl*" should be included with the hairs of the first coxa but they are in fact completely separate.

The genital chaetotaxy includes five pairs of inner and five pairs of outer setae that run in two almost straight lines down the sides of the genital slit with the inner ones starting slightly anterior to the outer ones (Plates 2c, 2d, 3a & 3b). Posterior to these are two pairs of anal setae "*ai*" and "*ae*" which are slightly longer than the others and surround the anus (Plate 2c & 2d).

Also ventrally are two pairs of small setae on the underside of the gnathosoma, with the posterior pair slightly longer than the anterior ones, (Plate 1a, 3c, 4c & 4d).

This general description of the chaetotaxy applies to both the male and the female except that the slightly smaller male has a very different genital region. In some of the females, part of the genital region had been partially everted and exhibited a characteristic striped appearance and the genital suckers observed previously in whole mounts were also visible (Plates 3a & 3b).

In the male the aperture is shorter and wider with a slightly gaping appearance that allows the presence of several pairs of short bristles to be seen inside the vestibule, it is also possible to make out other components of the sexual apparatus. The general appearance of the genital region of the male can be seen from the photograph of *R. oudemansi* (Plate 2d) and does not seem to differ from that of *R. limacum.*

From observations made during the winter months of 1991, the male does not seem to be very abundant, comprising approximately 1% of the population at this time.

3.3.2 Main differences between *R. limacum* and *R. oudemansi.*

There are several quite obvious differences with a few more subtle ones noted (Fain & Van Goethem, 1986), these having now been clarified using the SEM.

Firstly there is a hair on the third trochanter of *R. oudemansi* that is not present in *R. limacum.* There is also an extra hair on the first coxa although this can be confused with "*ic 1*" which is very close to the base of the coxa. The coxae may also extend further into the body of *R. oudemansi* thus compounding the problem, (Plates 1c & 1d). Another major difference between the two is that hair "*vi*" is longer than "*di*" in *R. oudemansi*, (Plates 2a & 2b).

A more subtle difference lies in the actual hair structure, which in *R. limacum* is

long, narrow, quite cylindrical and has an extremely fine, drawn out extension at the tip. In *R. oudemansi* the hairs are much shorter, quite fat and all end bluntly without the extension. These features are particularly obvious in the two apical hairs of tarsi 2-4 which are very swollen at the ends and slightly flattened in one plane, (Plates 1e & 1f). Although there is some variation between hairs the differences can quite easily be seen with the SEM (Plates 1a, 1b, 1e, 1f, 4b & 4d).

The hair arrangement in the genital regions varies very little between the two species and only the differences in the actual hair structure previously mentioned, appears to separate them (Plates 2c & 2d).

Using the SEM it was possible to see the "patterned" areas in the genital region of both protonymphs and deutonymphs prior to their final moult (Baker, 1970a), (Plate 2e). The "finger print" pattern characteristic of prostigmatous mites could also be observed at high magnification showing it to consist of many small projections in grooves all over the body. Slight variations in these patterns may be due to the method of preparation as they are very similar in both species (Plate 4b).

3.4 DISCUSSION

Mites are becoming a pest in snail farms. This is a growing industry and since it is possible that these mites may cause severe debilitation to the snails, it is very important to gain an understanding of their general biology.

The confusion between the two species is important since the biology of the mites on slugs and snails may show significant differences. With modern technology correct identification should no longer be a problem. More work is needed on the life cycles but it is now certain that the two different species are not just different stages in the life cycle of one animal (Turk & Phillips, 1946). These authors also claimed that the adult form is disappearing from the cycle, with the deutonymph becoming reproductively active. However adult females were seen, using the SEM, with an egg in the genital aperture about to be deposited (Plate 2g) and in several cases a partially everted vagina was noted (Plates 3a & 3b), this phenomenon has been reported in some prostigmatid mites as part of the oviposition process (Evans, 1992). In accompanying light microscope studies large numbers of fully formed eggs were observed within adult females. This would not be the case if they were non-fertile. No deutonymphs with eggs were observed. Fewer nymphs were collected than adults (observations in winter 1991), which suggests they are a fairly short lived stage. Hexapod larvae were also collected in slightly larger numbers than nymphs but were still less common than the adults. The chaetotaxy of the larvae (Plate 2f) and nymphs appears to be the same as the adults except for the genital region where progressively more pairs of hairs develop around the slit at successive moults (Baker, 1970a).

CHAPTER 4

WHOLE MOUNTS, INTERNAL ANATOMY AND THREE DIMENSIONAL RECONSTRUCTION OF *RICCARDOELLA LIMACUM*

4.1 INTRODUCTION

4.1.1 & 4.1.2 Whole mounts and histological investigation of internal anatomy.

As has already been noted in Chapter 3, there has been considerable confusion between the two closely related species of *Riccardoella*. This has only served to highlight the lack of work carried out on either species and this is particularly true with regard to their internal anatomy. Much of the previous work on internal anatomy was carried out many years ago without the benefits that up to date methods have brought with them and few authors have successfully sectioned and photographed these mites. Turk & Phillips (1946) carried out extensive research on *R. limacum*, (= *R. oudemansi*) having ascertained that the species was not well documented. They mention some of the difficulties that they faced when embarking on their study, among the main problems being the small size of the mite, the fact that it has a cloudy integument, that it is prone to rapid dehydration after death and is very easily damaged in the general course of handling. For these reasons most of their work relied on the dissection of whole animals; they state that section cutting was wholly unsuccessful. They reconstructed the internal anatomy as accurately as they could from the information gained in their studies. They describe aspects of

the digestive, nervous, respiratory, reproductive and muscular systems of the mite in extraordinarily good detail considering that microscope sections were not used at any point to facilitate the study.

Later work carried out by Lawrence (1951) on *Riccardoella eweri*, provided only limited information about the anatomy of this mite. The study was mainly concerned with external morphology and a small amount of work was carried out using whole mounts to identify elements of the tracheal system and the mouthparts (information about which was used to postulate the possible food of this mite). Some microscope work was carried out as mention is made of the gut contents, though no detailed information or illustrations are provided.

The bulk of the literature available concerning *Riccardoella spp.*, indeed mites of the family Ereynetidae in general, seems to have come from work carried out by Baker (1970b, 1971 & 1973), although it is the 1973 work which provides the most detail concerning the internal anatomy whilst the other two papers only touch on it in relation to feeding strategies. Baker cut paraffin wax sections, orientating the mite in a small block of liver to enable cutting to take place in particular planes, but the results were not very clear. The light microscope pictures were of poor quality partly due to the harsh nature of wax embedded sectioning and partly due to the fact that specific staining techniques were used in order to identify the food material in the gut, and these stains provided poor quality general information. Baker (1970b) did manage to obtain some usable photographs and gained information concerning the gut contents of *R. limacum* and was able to compare them to those of *Xenopacarus africanus* (Baker, 1971). He also described some of the major reproductive and digestive structures in the body of *R. limacum*, (Baker, 1973) and the digestive system and brain of the ereynetid mite *X. africanus* which lives in the nasal passages

of the African clawed toad, *Xenopus laevis* (Baker, 1971). However the pictures are not very clear and by no means conclusive of the actual structures present.

There do not appear to have been any further studies on the internal anatomy of *R.limacum* in the last 20 years. Some high quality electron micrographs have been produced by Witalinski *et al.* (1990) working with *Acarus siro* of the family Acaridae, these show the reproductive system of both the male and female mite in great detail. Even more recently Matsubara *et al.* (1992) have carried out detailed work on *Tetranychina harti* providing excellent transmission electron micrographs and fully describing both male and female reproductive systems. Evans (1992) also published an extensive work reviewing all aspects of the biology of both ticks and mites although no mention was made specifically of *R. limacum.*

It was therefore decided to use the much improved resources available at this present time to study the internal anatomy of *R. limacum.* In conjunction with the more traditional approach of using light microscope sections a study of the three dimensional aspects of the main organ systems was carried out using computer-aided reconstructions.

4.1.3 Computer-aided 3-dimensional Reconstruction.

Whilst it is important to gain a thorough knowledge of an organism on a cellular level, it is also necessary to be able to spatially relate all of the information gathered about different tissues in this way, to gain a full understanding of the whole organism. This can be accomplished to a degree using a series of whole mounts, especially with something as small as a mite. However as has already been stated there are some difficulties with this as mites tend to have quite thick, chitinised

exoskeletons which resist attempts to view internal structures to all but the most basic degree. Obviously section cutting provides a great deal of information about the internal anatomy and it is possible to use the imagination to reconstruct certain organs as solid objects but a more scientific approach is required. Gaunt & Gaunt (1978) reviewed the methods available for reconstructions and these fall under three headings:

A. Hand drawn graphic reconstruction.

This method was first used over a century ago and it basically involves using sets of parallel lines to mark the extremes of certain parts of the organism in relation to a previously cut section. Unfortunately this distorts the image, and the necessity for shading the final drawing in order to confer the impression of a three dimensional object with shadows, lends a very subjective element to the whole process. It also has the disadvantage of providing only a fixed view point for the worker (Gaunt & Gaunt, 1978; Sautto; 1987).

B. Reconstruction of a solid object.

This technique is also a very old one and again requires a good set of serial sections to begin with. Plates of wax (or a similar substitute) are cut into the shape of the whole organism or just a part of it, they are aligned, stacked and sealed using a hot implement to smooth the edges. This method is very time consuming, requires some artistic skill and whilst it does not have a limited view point or need any shading, it does not allow the inside of the organism or organ to be viewed unless "windows" are cut into the surface.

C. Computer-aided reconstructions.

This method is a relatively recent advance in the field of reconstruction, it was not

until the 1960's that it really developed as a viable research method. Serial sections are converted into computer data and a series of programmes create a computer generated image which has many advantages over the other methods. It is possible to view the image from almost any angle, internal organs can be plotted separately or viewed within the whole organism by making the outer surface transparent and it is even possible to "tile" surfaces giving them a much more solid look.

It was this last method which was employed in the present study to enable the viewing of the internal anatomy of *R. limacum* as a whole.

4.2 MATERIALS AND METHODS

4.2.1 Preparation for Light Microscope.

Mites were collected from the surface of snails (Plate 6a) in the laboratory culture using a soft brush as this was found to be the least damaging method (Banks, 1915). They were transferred to a test tube where they were kept until sufficient numbers were obtained for the investigation. In the past only paraffin wax sections have been available, but these have to be cut relatively thickly and do not give very good resolution. Therefore for the purpose of this study plastic (Historesin) was used as an embedding medium which enabled much thinner, clearer sections to be cut.

The first attempts at fixation were not wholly successful as the mites floated on the surface of the fixative and failed to be preserved. Piercing the body wall in order to let the fixative penetrate was too damaging and when it was time to change the fixative solution the mites were invariably sucked up the bore of the pipette. Both of these problems were solved by placing the mites in a small nylon bag made from a circle of cloth and closed with a drawstring around the circumference. This enabled the mites to be pushed beneath the surface of the fixative and also allowed the solution to be changed without the loss of any mites. The following method was found to be the most successful:

1. Fix in Heidenhain's Susa for 24 hours.
2. Place in cellosolve with a few grains of lignin pink* for 3 hours.
4. Change to pure cellosolve for 1 hour.
5. Place in xylene for 1 hour** in a fume cupboard.
6. Wash in cellosolve.

7. Place in 50:50 cellosolve:historesin infiltration solution for 24 hours in a fridge.

8. Change to 100% historesin infiltration solution 24 hours in a fridge.

9. Replace the historesin infiltration solution and leave for another 24 hours in a fridge.

10. Remove the bag and carefully transfer the mites one at a time on the tip of a cocktail stick to small pre-infiltrated blocks of agar*** (a hole for the mite is easily made by poking a hot metal seeker into the block).

11. These agar blocks were then embedded in a mixture of historesin infiltration solution and historesin hardener in the ratio 15:1 in small plastic moulds (specimen tube lids were used in this case).

12. The resin sets hard overnight and the agar blocks with the mites in them can be cut out with a hack saw and mounted on a perspex block using fast setting superglue.

13. The blocks were then trimmed with a razor blade to give two parallel edges and one corner removed to help orientate the mite and also to reduce the force hitting the cutting edge.

14. Blocks prepared in this manner were allowed to harden further overnight in an oven before 3 μm serial sections were cut using an LKB Ultracut microtome with a freshly made glass knife.

*Lignin pink stains the mites slightly and thus makes them easier to locate in the nylon bag when it is time to embed them.

** At first this stage was not carried out but when resulting slides were examined under a microscope it was found that the mites had fallen out of the sections due in part to the waxy nature of their cuticle. Treating them first with xylene to extract

any waxes prevented this.

***Placing the mite in a block of agar prior to embedding helped in the final orientation of the mite and made it possible to avoid for the most part cutting oblique sections. Agar can be made up freshly and whilst it is in the liquid form the mite is placed in it. This allows greater freedom to manoeuvre the mite but as the agar is aqueous the mite must go through an hydrating series to bring it on a par with the agar. Both mite and agar must then be re-dehydrated before infiltration and the time taken for the infiltration steps doubled. Alternatively once the agar has set, it may be cut into small blocks and infiltrated so that it is ready to use, as in this instance.

After the 3μm sections were cut they were allowed to toughen over night and were then mounted in strips on glass microscope slides using water and glycerine albumen as an adhesive. These were left to dry and then stained using a general polychrome stain (Blackstock, personal communication), as follows:

Polychrome Staining Schedule.

Tissue fixed in Heidenhains Susa must have mercury removed first, otherwise there will be small black deposits left in the sections, they are therefore pre-treated as follows:

1. Slides placed in 0.5% iodine in alcohol - 3 min.
2. Washed in running water - 1 min.
3. Bleached in 5% sodium thiosulphate until the sections are white.
4. Washed in running water - 3 min.

Then stained as follows:

1. Placed in 1% Alcian Blue in 3% acetic acid pH 2.5 - 30 min.
2. Placed in running water - 5 min.
3. *Placed in 1% periodic acid freshly prepared - 10 min.
4. *Rinsed in running water - 5 min.
5. *Placed in Schiff's reagent in the dark at room temperature - 10 min.
6. *Rinsed in running water - 5 min.
7. Placed in iron alum mordant - 30 min.
8. Rinsed in water.
9. Placed in Heidenhain's haematoxylin - 30 min.
10. Rinsed in water.
11. Differentiated with iron alum using microscopical control until only the nuclei were stained black.
12. Rinsed in water.
13. Placed in acid fuchsin/xylidine ponceau counter-stain - 5 min.
14. Rinsed in water.

*The timing of these steps is critical and must be strictly adhered to.

After staining, the slides were dried and then coverslips put on using DPX mountant, the slides were then examined and photographed.

4.2.2 Preparation of Whole Mounts.

As so many previous workers had achieved good results from whole mounts (Banks, 1915; Turk & Phillips, 1946; Jones 1946), it was decided that although of limited use compared to sectioning, some should be prepared. Jones (1946) used polyvinyl

lactophenol with picric acid as opposed to the more traditional methods which preferred gum-chloral and his method renders the chitinised cuticle clear after 24 hours. For this study a drop of polyvinyl lactophenol was placed on a clean microscope slide and a mite transferred live to this droplet and heated gently over a Bunsen burner. This speeded up the clearing process and encouraged the mite to extend its legs in a suitable position for examination. A cover slip was placed over the preparation which gradually hardened and became semi-permanent. The slide was then examined using a microscope.

4.2.3 Computer-aided Three-dimensional Reconstruction.

Firstly a good set of serial sections was obtained with no omissions, these sections were treated as already described for light microscope preparation and stained with the polychrome stain. The images of the slides were projected one at a time from a microscope, using a series of mirrors, to a light table which magnified the image and made it possible to trace, with a pencil, the outline of the whole organism and the main organ systems within the body onto high grade tracing paper. Once all of the sections in the series had been traced (in this study there were 53 sections in all) they were aligned by hand (it is possible to put reference marks into the block prior to sectioning which makes alignment easier) and reference marks drawn on to each. For ease of use later on it is advisable to use the same sized sheet of tracing paper for all of the drawings, even though in some cases the section will only occupy a small area of the page, this is so that the reference marks will remain constant throughout. The next stage after alignment was to input these pictures to the computer using a digitising system. This involved tracing around all of the previously drawn outlines using an electronic cross-wire tracer, for each section the reference marks also had to be input, (system designed and developed by Mr D A Davies for

UCNW). Each line drawing was converted into to a series of X Y co-ordinates which the computer stored as data files for each section. These files then had to undergo numerous transformations using software written by Mr D.A. Davies and software modified by Mr D. Roberts and Dr N.W. Runham of UCNW from that originally developed by Dr Houseman, University of Amsterdam.

The final product was a database which the reconstruction programme proper, Illus04, could read and use to produce a 3-D screen image. This programme allows the worker to rotate the reconstructed object and thus in effect view it from one of seventeen different vantage points. It is also possible to call up images of either the whole mite, the mite and internal organs or just the organs, the contours making up the image can also be colour filled or left empty in order to view the interior and see the relationship between the organs.

4.3 RESULTS

4.3.1 Histological Investigation Using Light Microscopy.

The results of this part of the investigation proved very satisfactory once the initial problems had been solved. The polychrome stain used showed up many specific features such as carbohydrates (periodic acid Schiffs technique), acid mucopolysaccarides (alcian blue) and nuclei (iron haematoxylin) as well as providing useful information on the general structures present. The 3µm plastic sections provided very good resolution, better than could be obtained by using thicker wax sections. Mites were sectioned in all planes; transverse, vertical longitudinal and horizontal longitudinal. The use of agar blocks to orientate the specimens was also very successful in that misleading oblique sectioning was almost eliminated. Information was obtained on all the major organ systems as follows.

4.3.1.1 The reproductive system.

During the three year study many mites were collected and invariably it was noticed that the female is by far the more common sex, the male form occupying no more than 1 or 2% of the population. During the histological work, although numerous specimens were collected and sectioned, no males were available for examination. This observation confirmed Turk & Phillips (1946) finding that the male was extremely rare and they subsequently failed to describe it, although they attributed this to their belief that the adult forms of both sexes were in atrophy. Baker (1973), on the other hand experienced no such difficulty in finding the male form and manage to successfully section both sexes. It must however be noted that he was probably working on *R. oudemansi*. The lack of male specimens is due to their rarity

(possibly at certain times of the year) and not to the adult forms dying out altogether, the only description of the male, albeit rather brief, remains that of Baker (1973)(= *R. oudemansi)*.

Female Reproductive System.

The female anatomy can be clearly divided into its constituent parts. During this study many females were examined giving a wide range of sections for comparison in all planes. Prominent in all the specimens examined was the ovary, which occupies a ventral position within the body at the posterior end (Plates 7a, 7b, 7d, 7e, 8a, 8d & 9f). The ovary is a four lobed structure with all the lobes loosely connecting to each other (Plate 8c). In most of the specimens examined there were a great many oocytes in various stages of development including several with fully mature oviducal eggs (Plates 7c, 7d, 7e & 8d). There appeared to be a maturation gradient from the younger ventral to the older dorsal surface. Another structure which occupies a large part of the body cavity and is particularly prominent in the vertical longitudinal sections, is the oviduct. This takes on a slightly leaf like shape when viewed from the side and extends over the top of the ovary in a curve following the contours of the dorsal surface of the body (Plates 7a, 7c, 7e & 8a). It tends to be darkly staining and comprises cuboidal epithelial cells with large nuclei which have the appearance of glandular secretory cells. In those specimens which have an egg present in the oviduct the cells are stretched thinly around it in a layer and the duct loses the leaf like shape (Plate 7d). To the rear of the mite the oviduct narrows to form a chitin lined vagina which exits to the outside via the genital slit. In the transverse sections and the horizontal longitudinal sections it is possible to see that the oviduct divides anteriorly into two tubes with a marked lumen, and in the horizontal sections the oviduct has a "Y"shape to it (Plates 9a, 9b, 9c & 9d). This arrangement was not observed from the side in the vertical longitudinal sections.

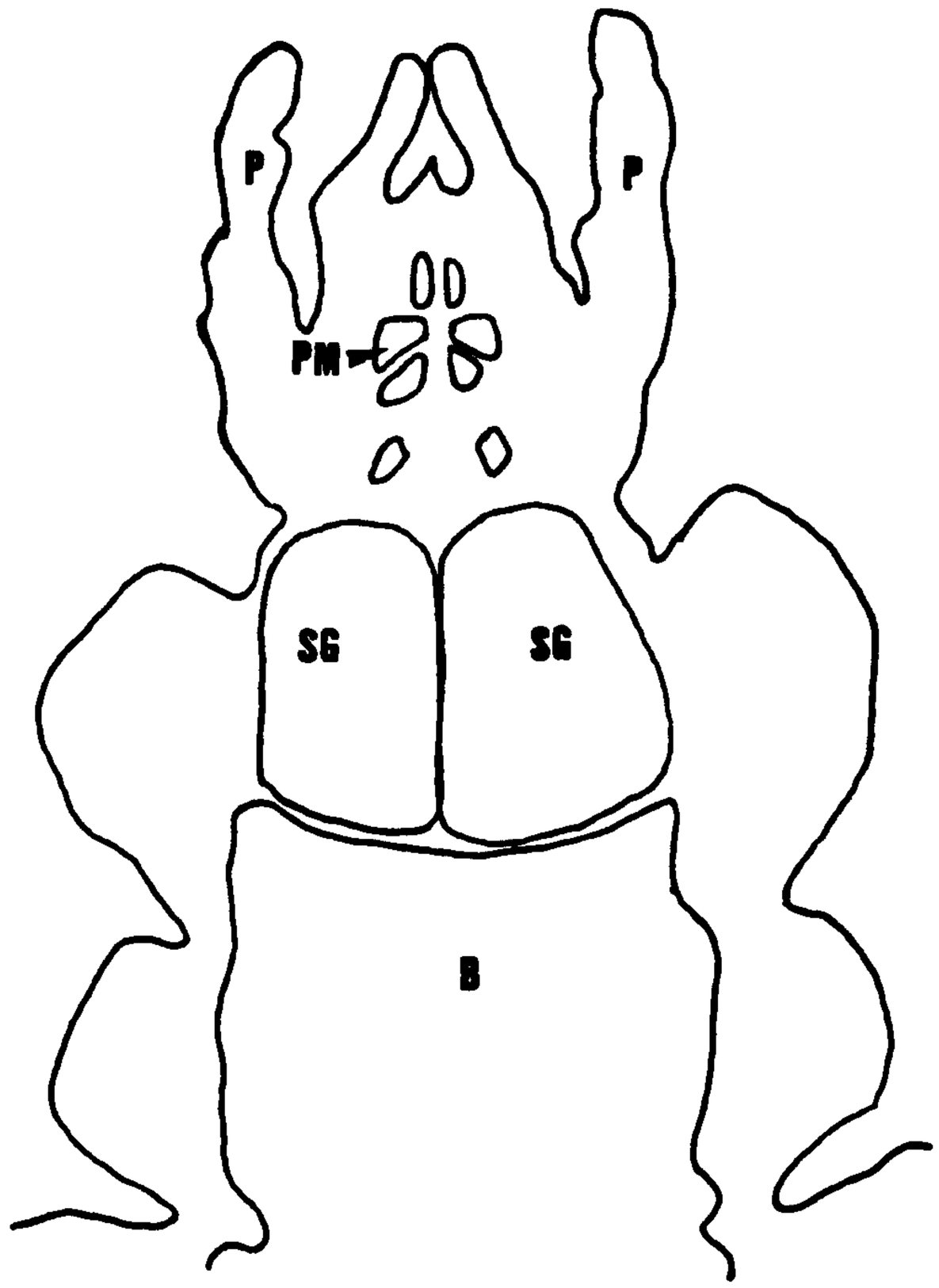
PLATE 6

a). *Helix aspersa* withdrawn into shell showing *Riccardoella limacum* (R) in region of the pneumostome. Scale bar = 4mm.

b). Whole mount of *R. limacum* showing legs, palps (P) and region of genital aperture (GA). Scale bar = 96.5μm.

c). Whole mount of *R. limacum* concentrating on the genital region showing the genital suckers (GS). Scale bar = 16.1μm.

d). Horizontal, longitudinal light microscope (LM) section (3μm) of *R. limacum* showing palps (P), muscles of pharynx in the gnathosoma (PM), salivary glands (SG), brain (B) and the bases of the first two pairs of legs (1 & 2). Scale bar = 16.1μm.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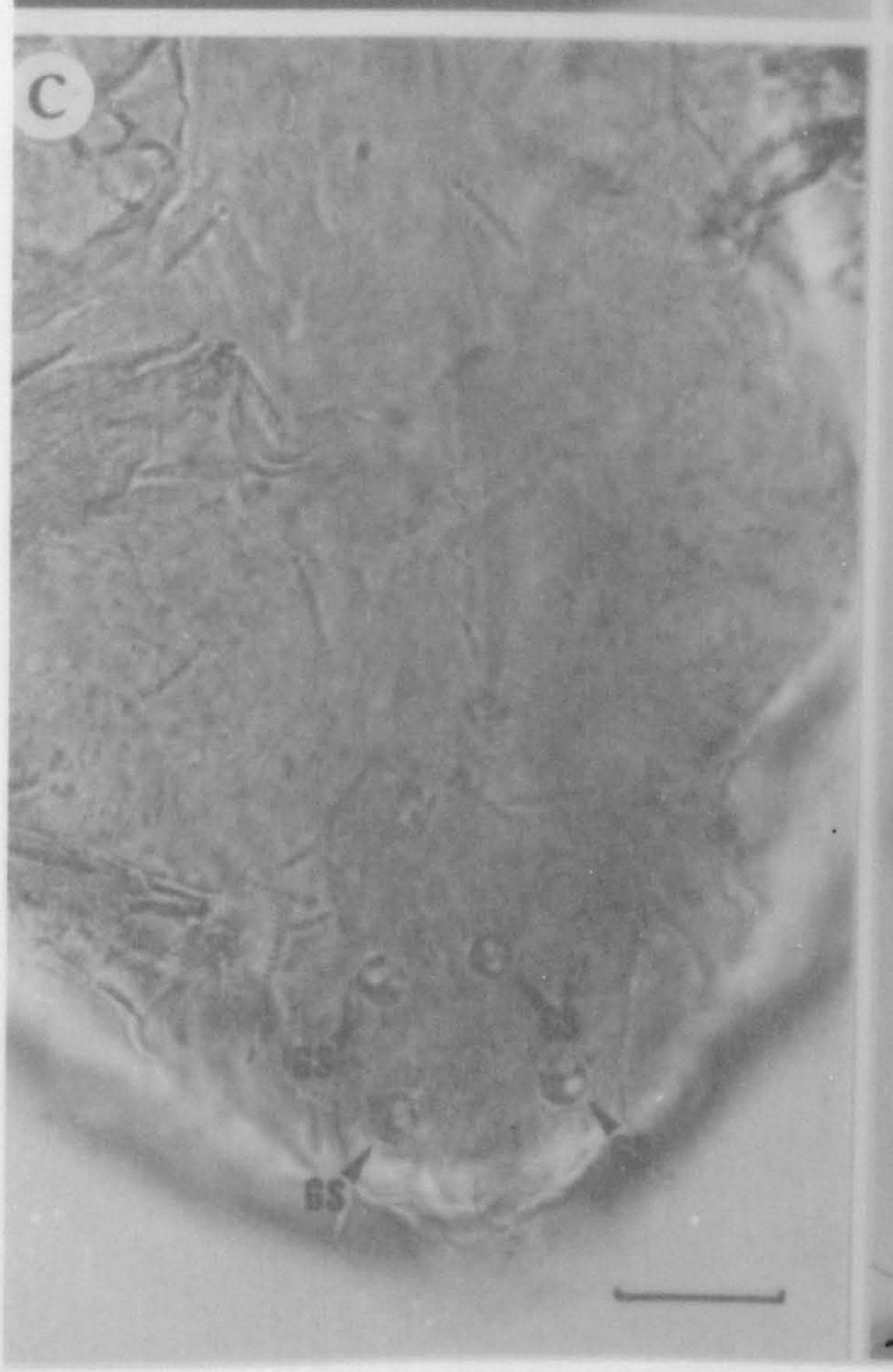
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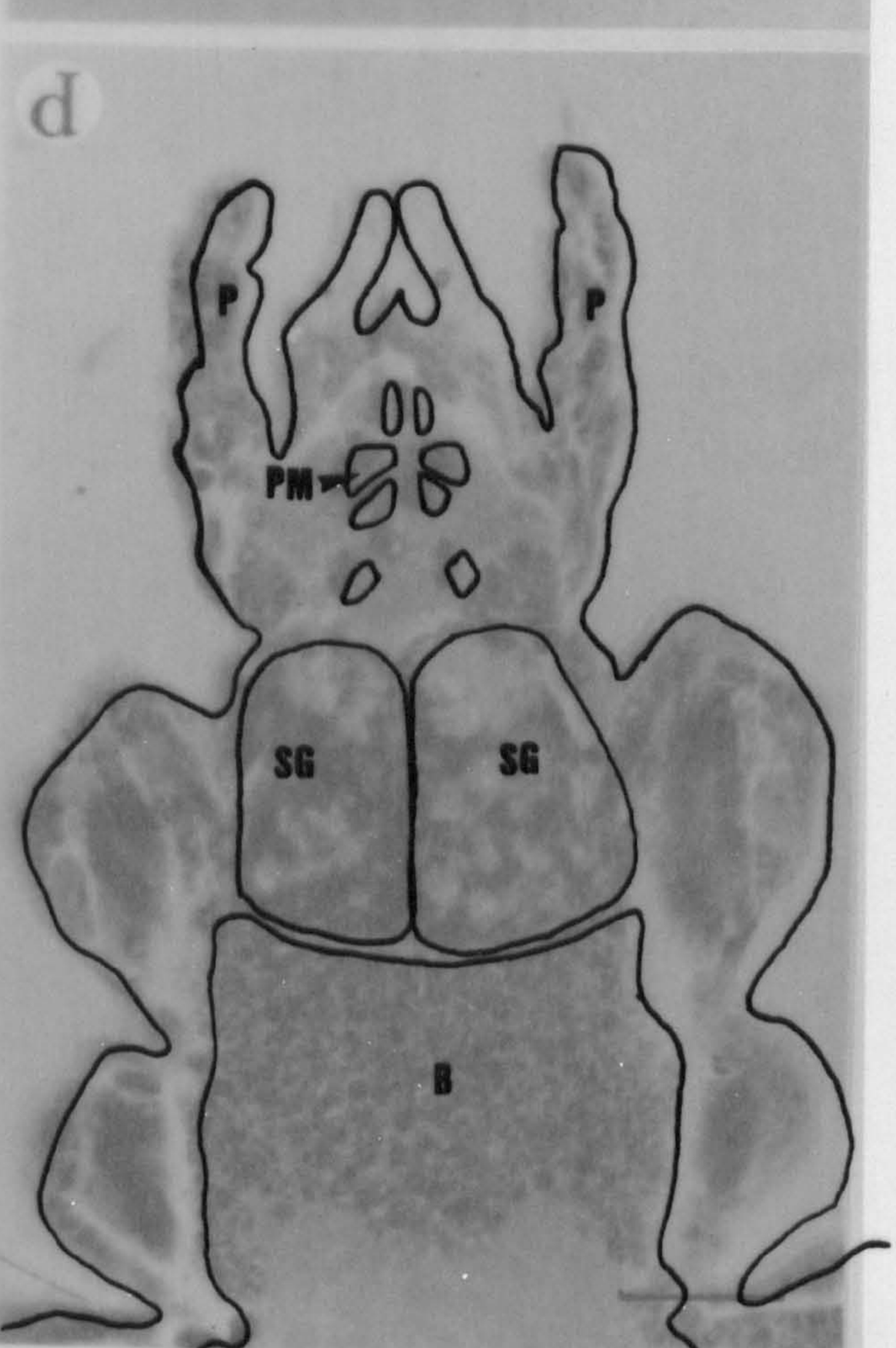
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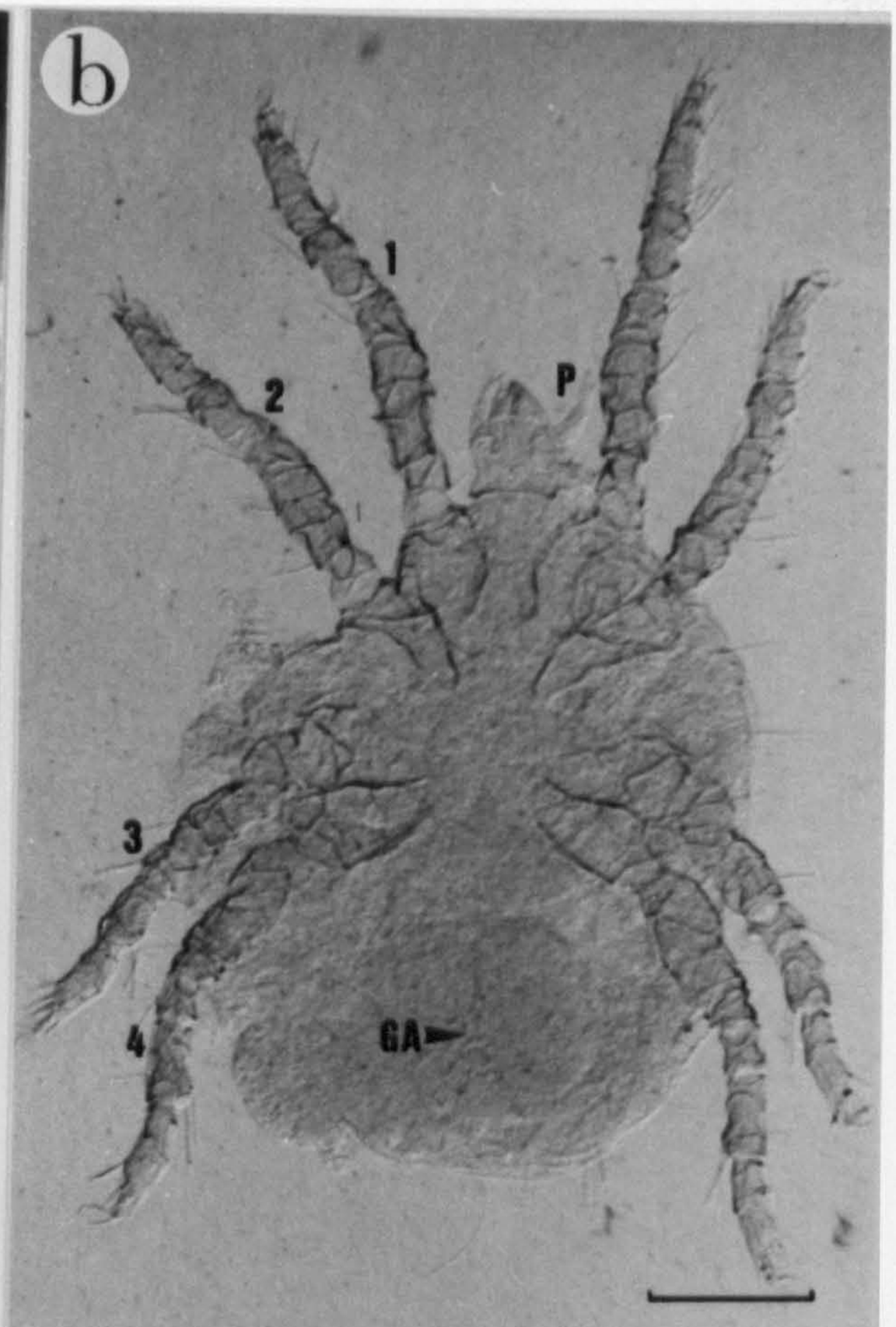
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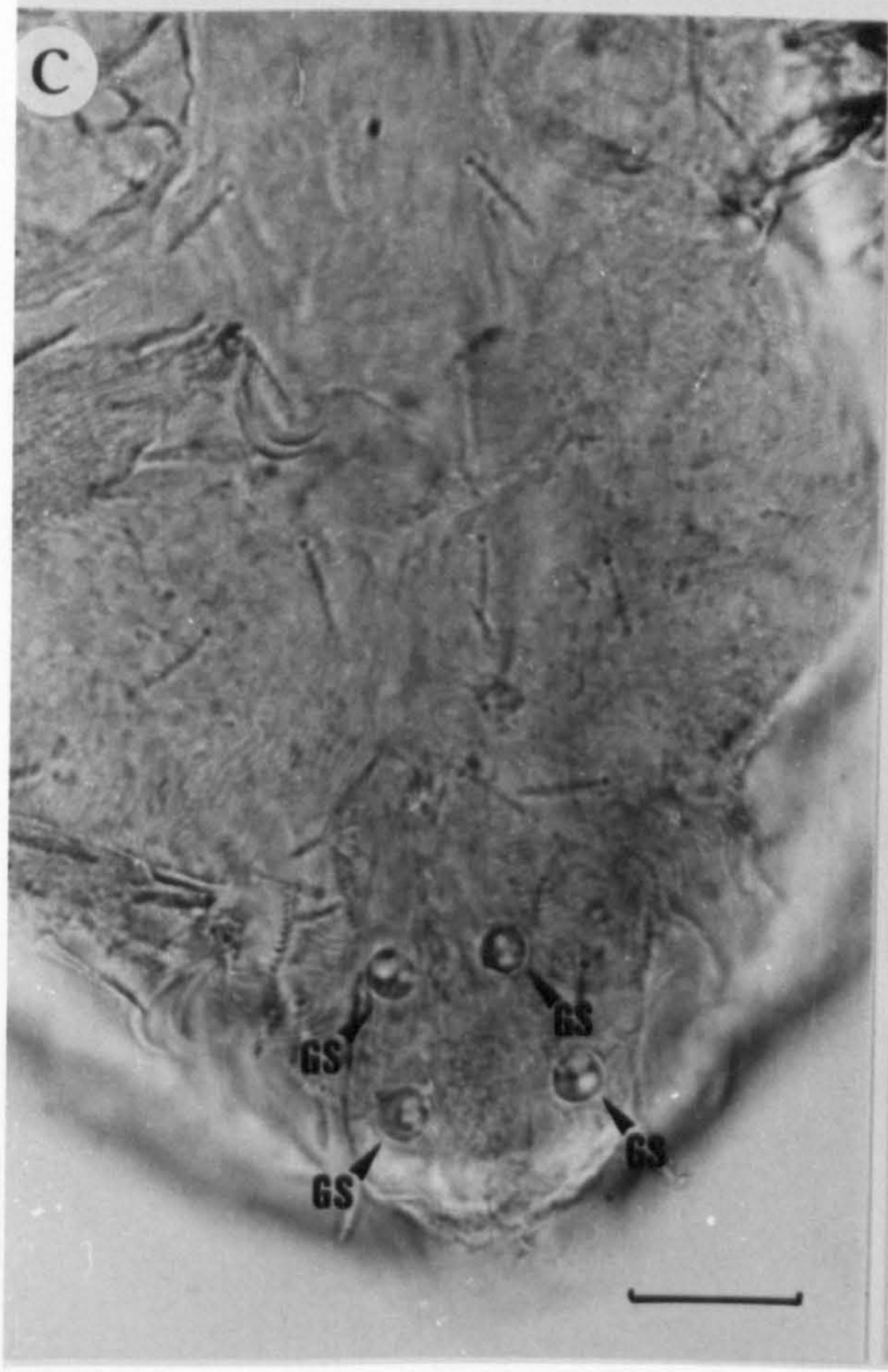
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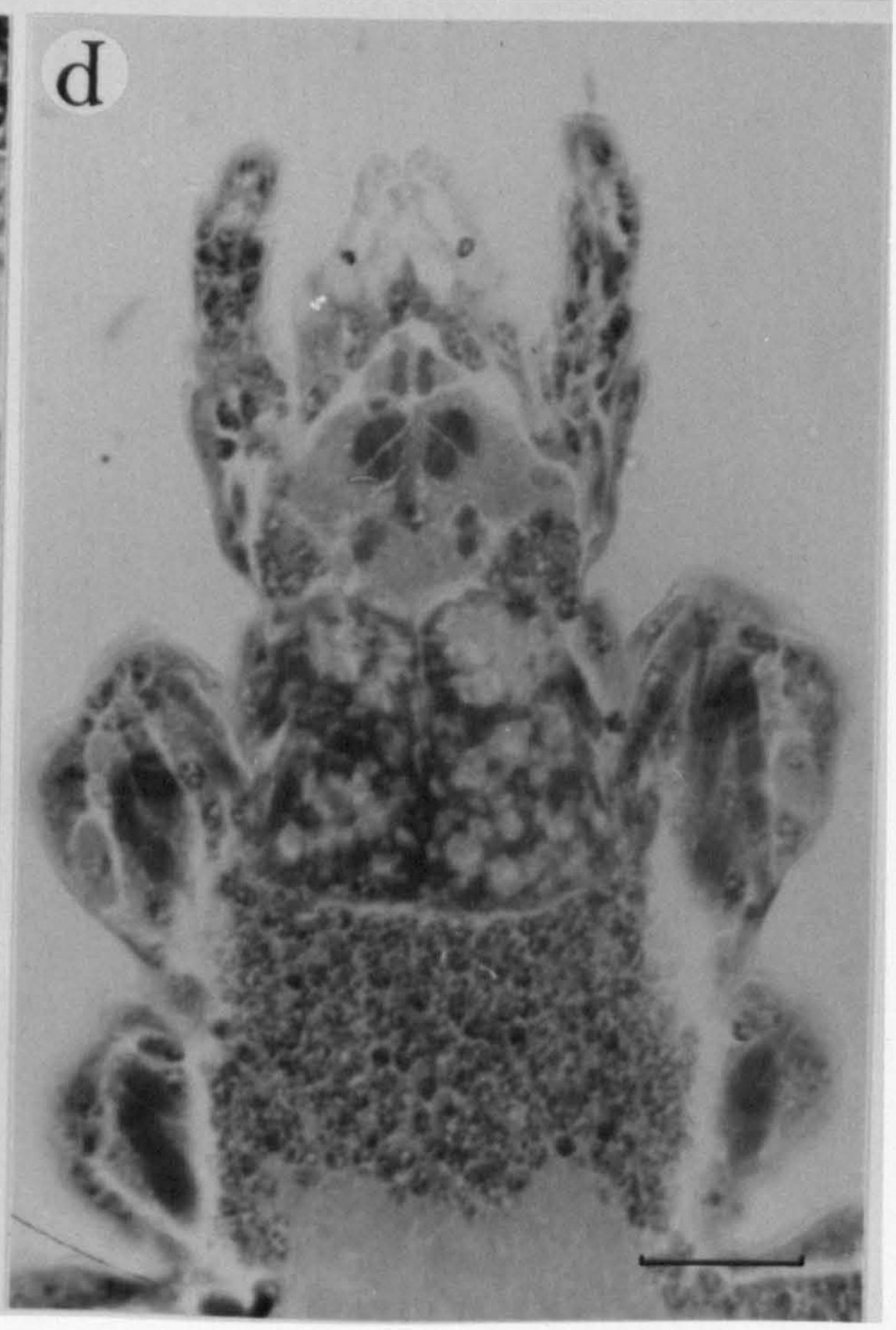

d

PLATE 7

a). Vertical, longitudinal LM section (3μm) of *R. limacum* showing main organs of the body; gnathosoma (GN), salivary gland (SG), brain (B), ventriculus (V), digestive caeca (DC), rectum (R), ovary (OV), oviduct (OD), oocytes (OO). Scale bar = 56.6μm.

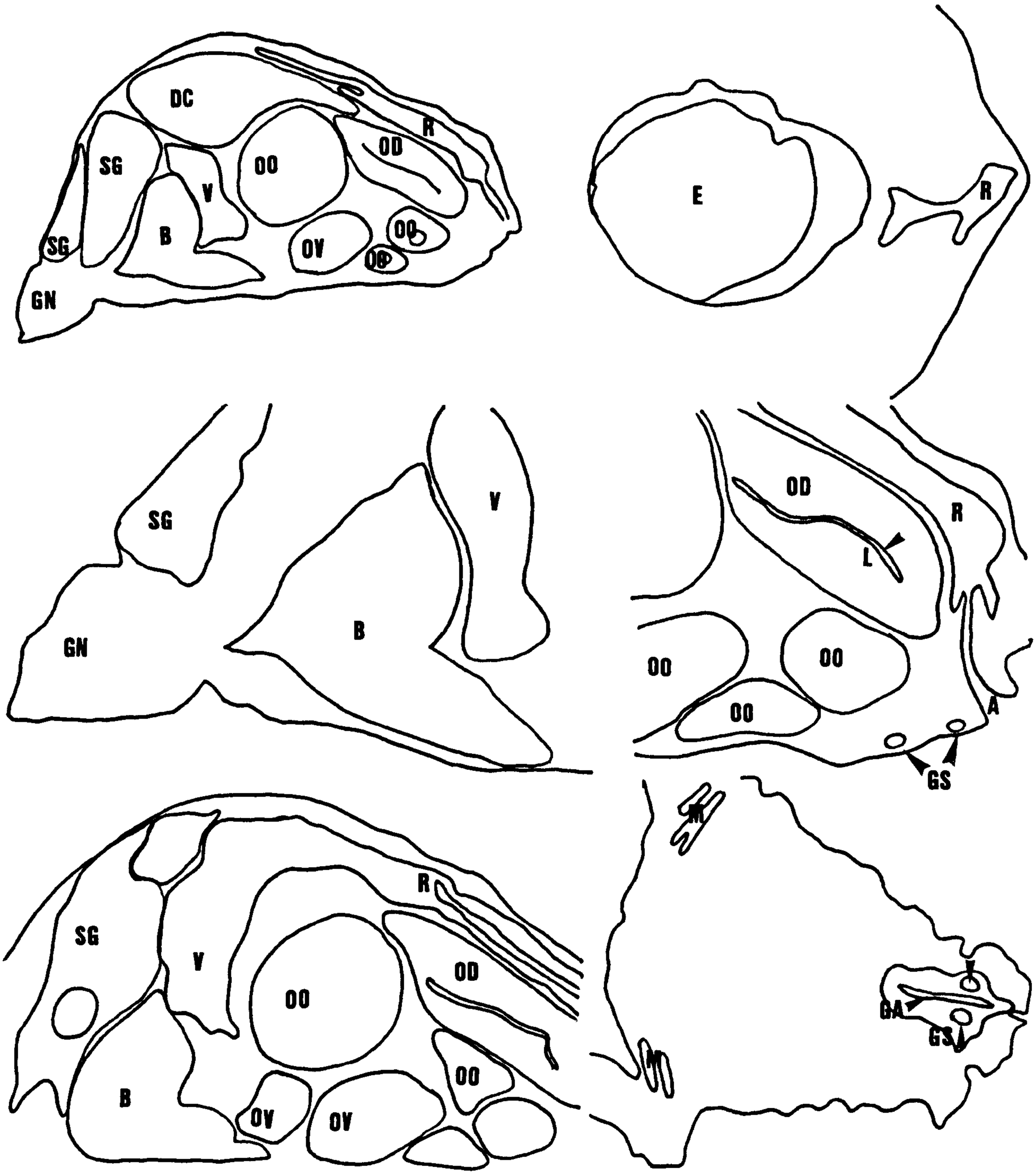
b). High power view of anterior portion of above section, abbreviations are the same. Scale bar 18.2μm.

c). High power view of mid-portion of mite, abbreviations are the same as above. Scale bar = 27.1μm.

d). Horizontal, longitudinal LM section (3μm) of posterior of *R. limacum* showing descending part of the rectum (R) and mature oviducal egg (E). Scale bar = 16μm.

e). High power view of posterior portion of (a) showing highly expandable rectum (R), anus (A), oviduct (OD), lumen of oviduct (L), oocytes (OO) and genital suckers (GS). Scale bar = 25.3μm.

f). Horizontal, longitudinal LM section (3μm) of posterior portion of *R. limacum* in the region of the genital aperture (GA), with genital suckers) (GS) and muscles to the legs (M). Scale bar = 25.6μm.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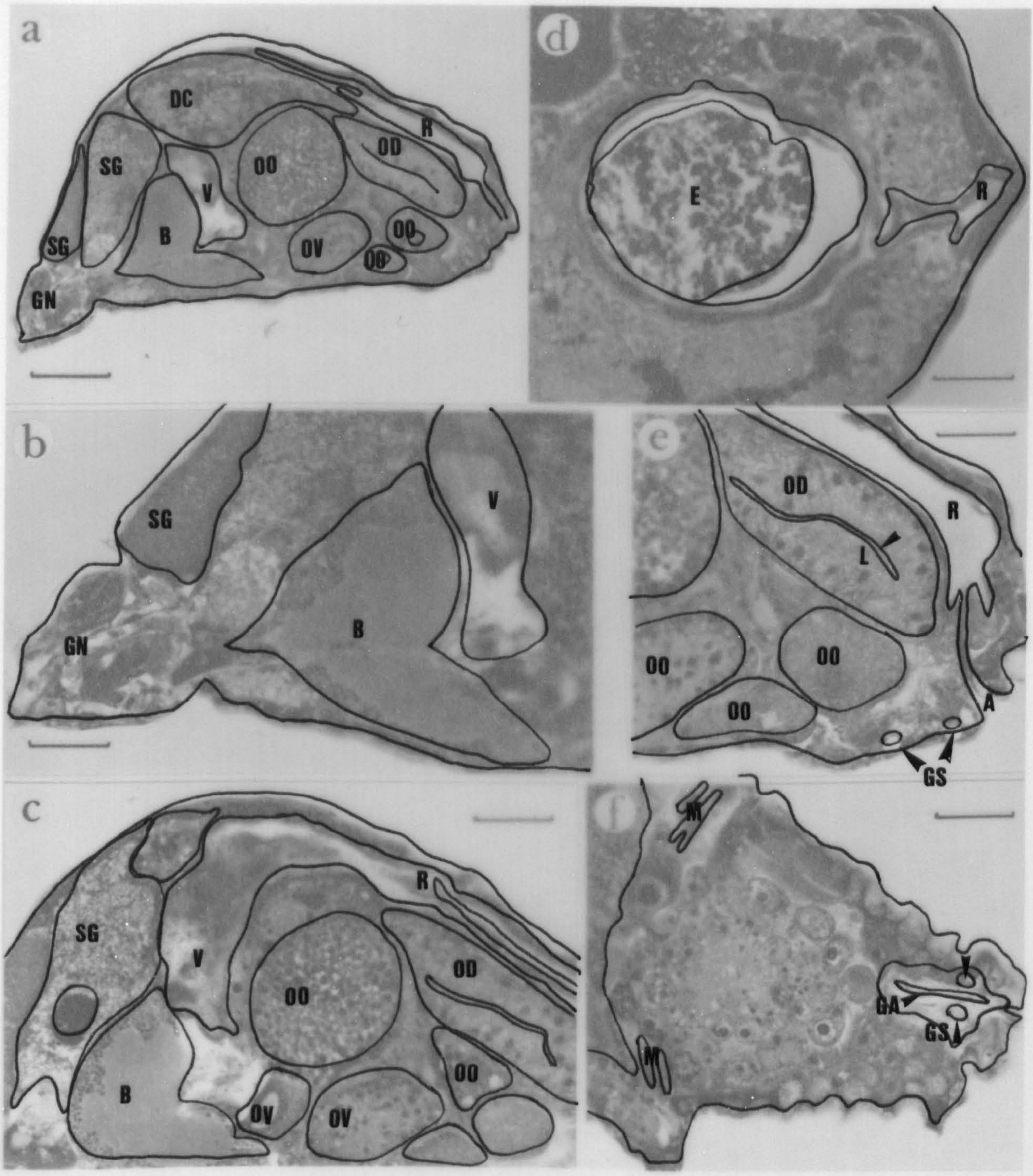
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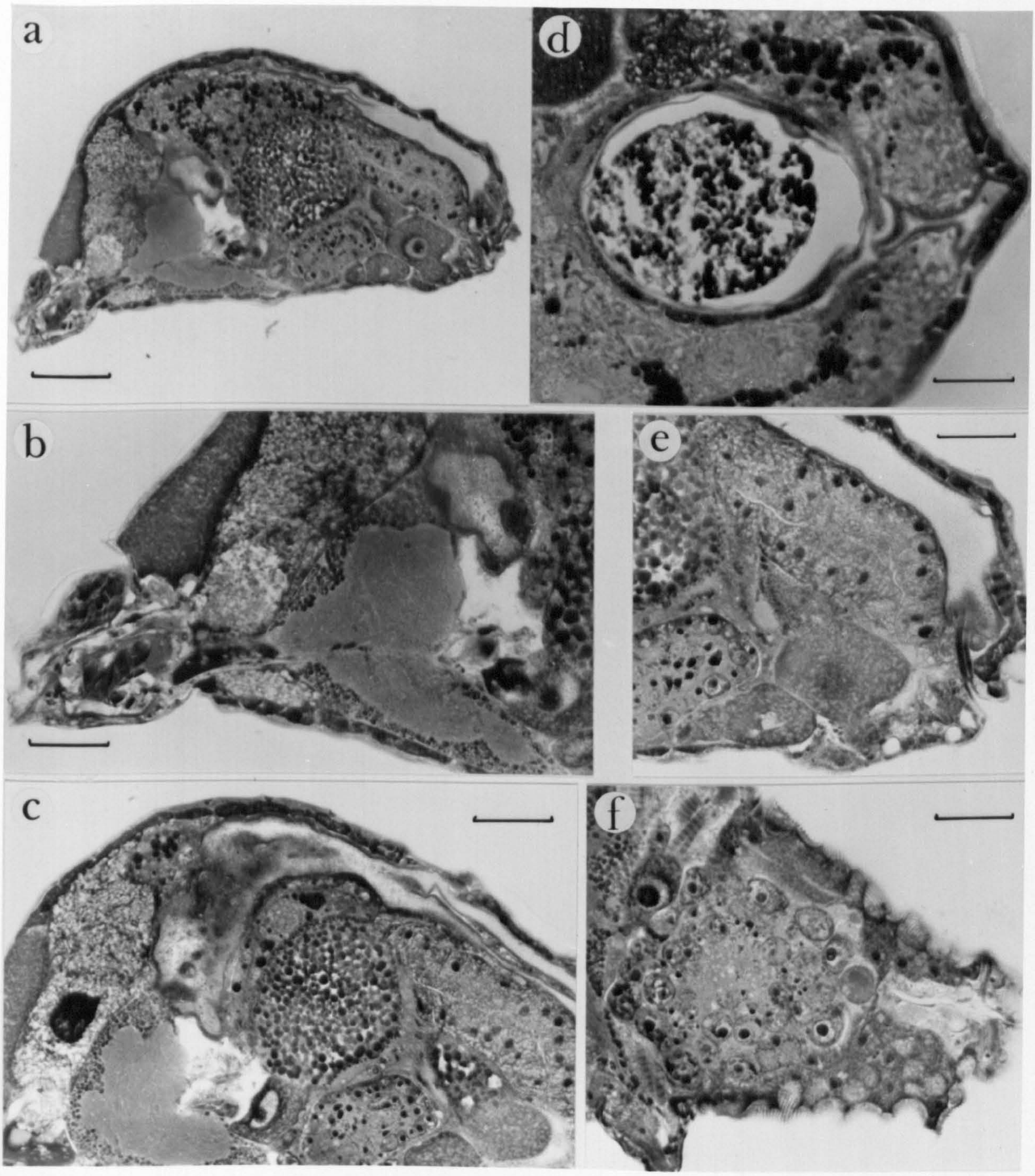
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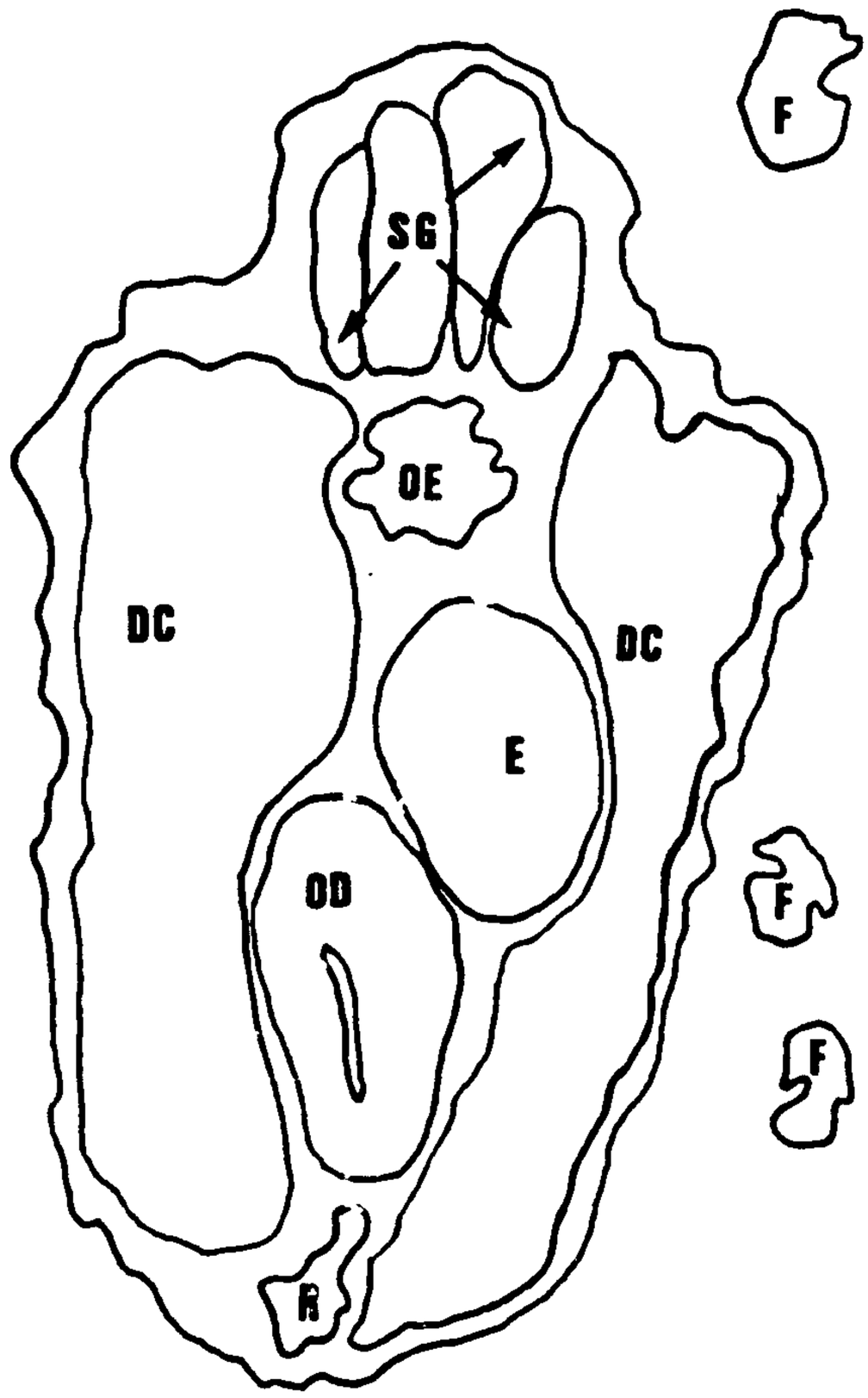
PLATE 8

a). Horizontal, longitudinal LM section (3μm) of *Riccardoella limacum* with oesophagus (OE), rectum (R), salivary glands (SG), mature egg (E), oviduct (OD), digestive caeca (DC) and feet (F). Scale bar = 55.2μm.

b). Section of the same mite taken in a more dorsal position showing ventriculus (V) and brain (B), all other abbreviation are the same as above. Scale bar = 54.3μm.

c). Horizontal, longitudinal LM section (3μm) of *R. limacum* showing ovaries (OV), branching oviduct (OD) and brain (B). Scale bar = 46.3μm).

d). High power view of posterior portion of mite showing developing oocytes (OO) and obliquely sectioned oviduct (OD). Scale bar = 35.2μm.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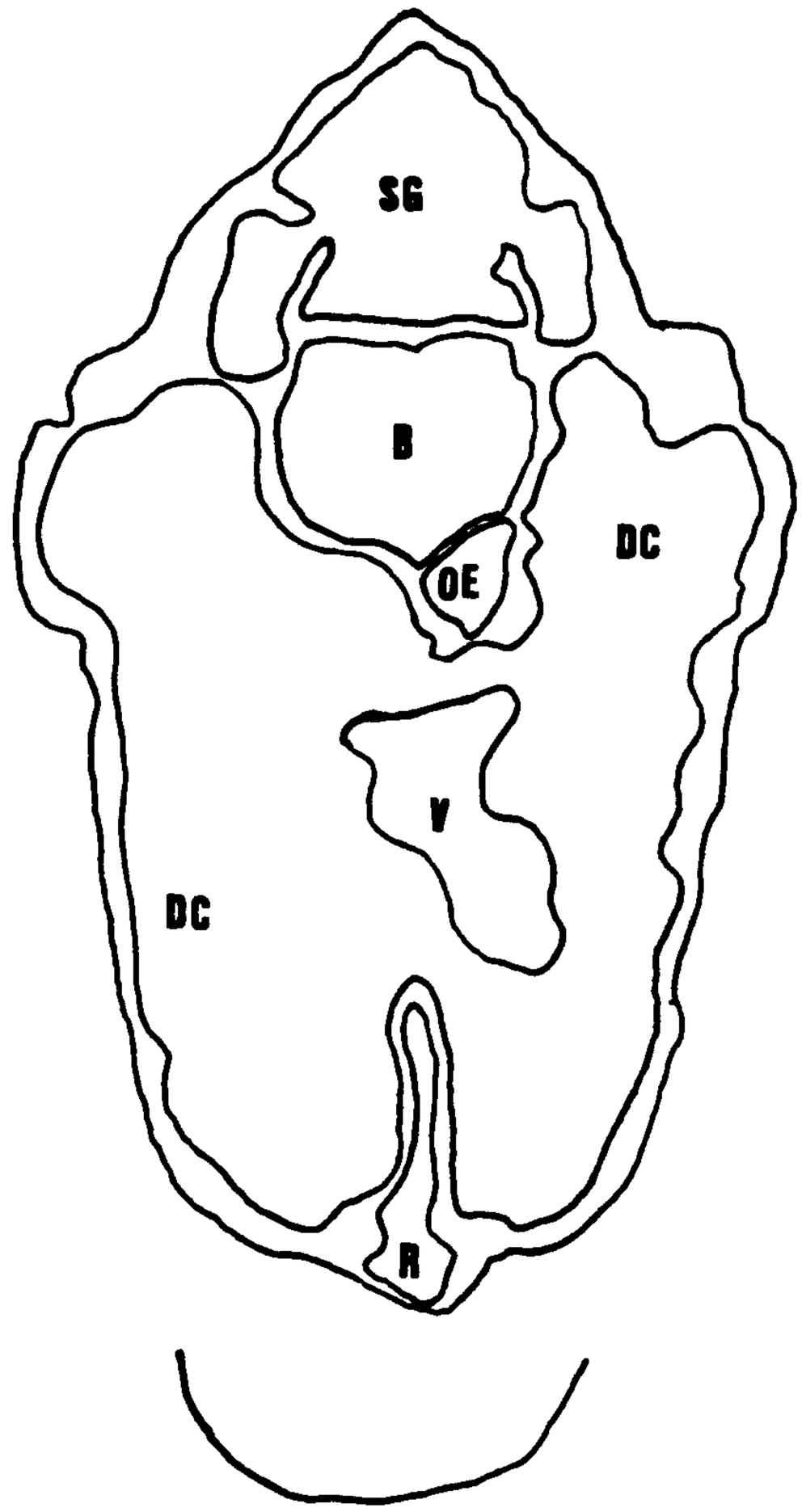
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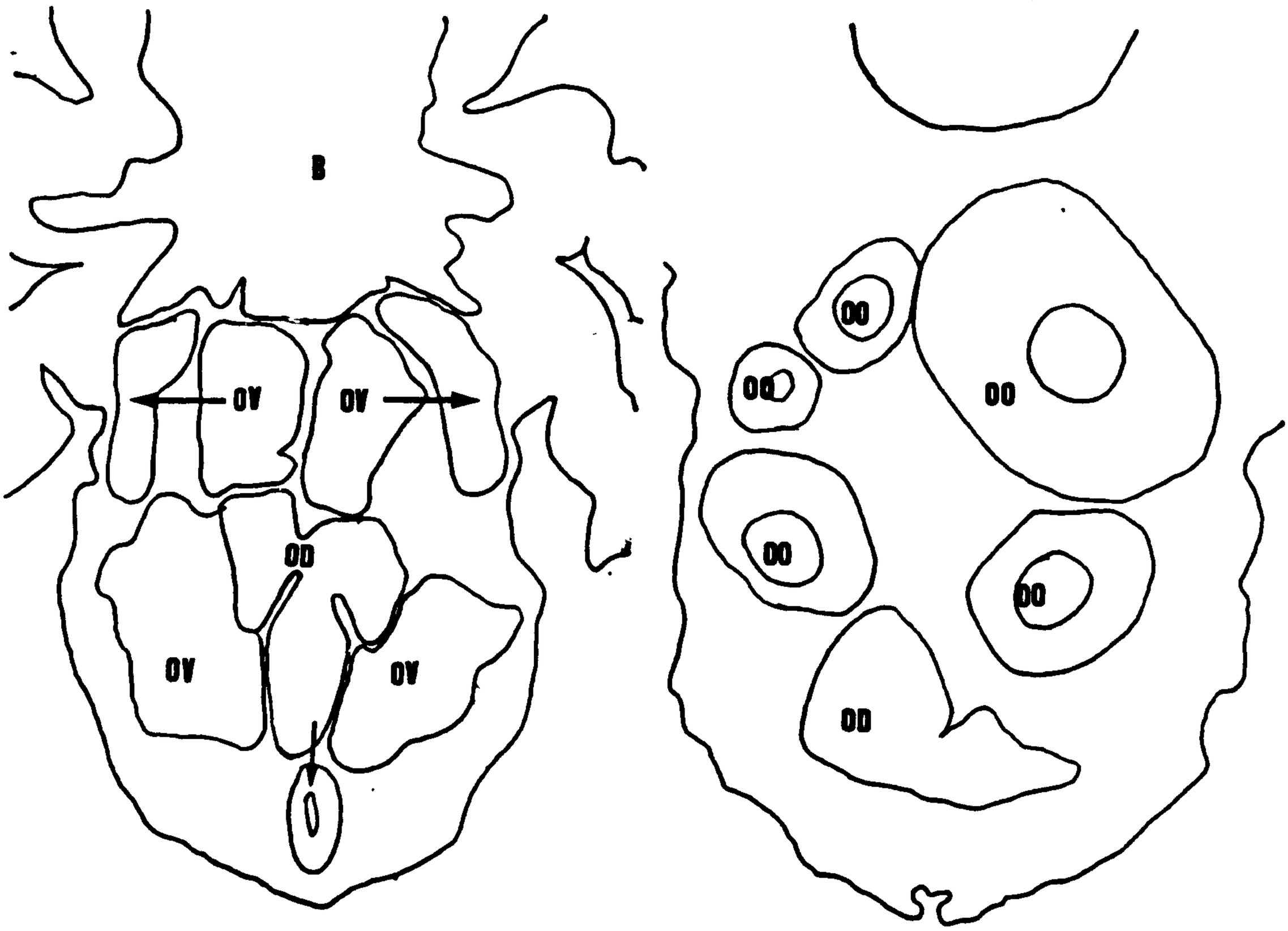
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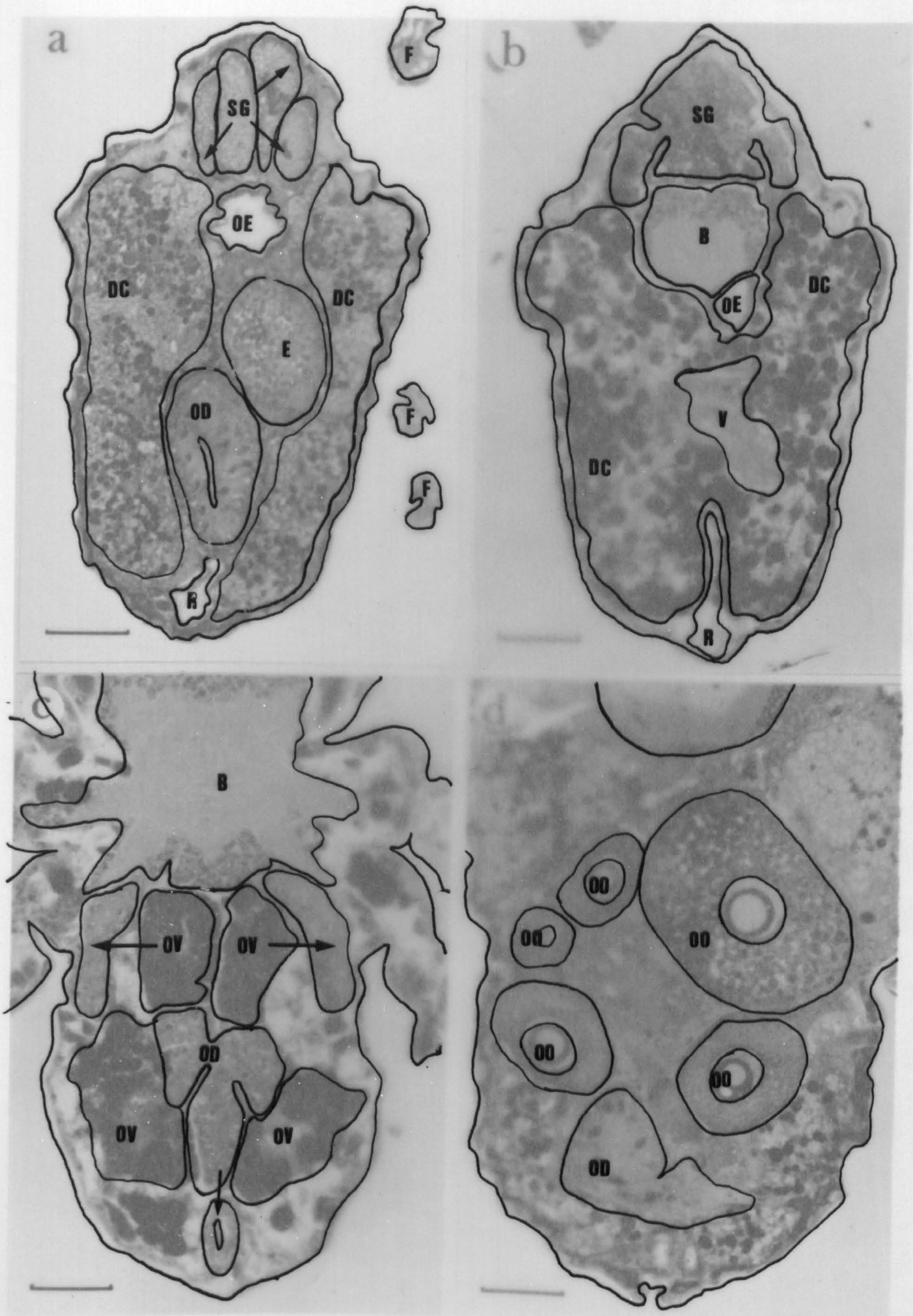
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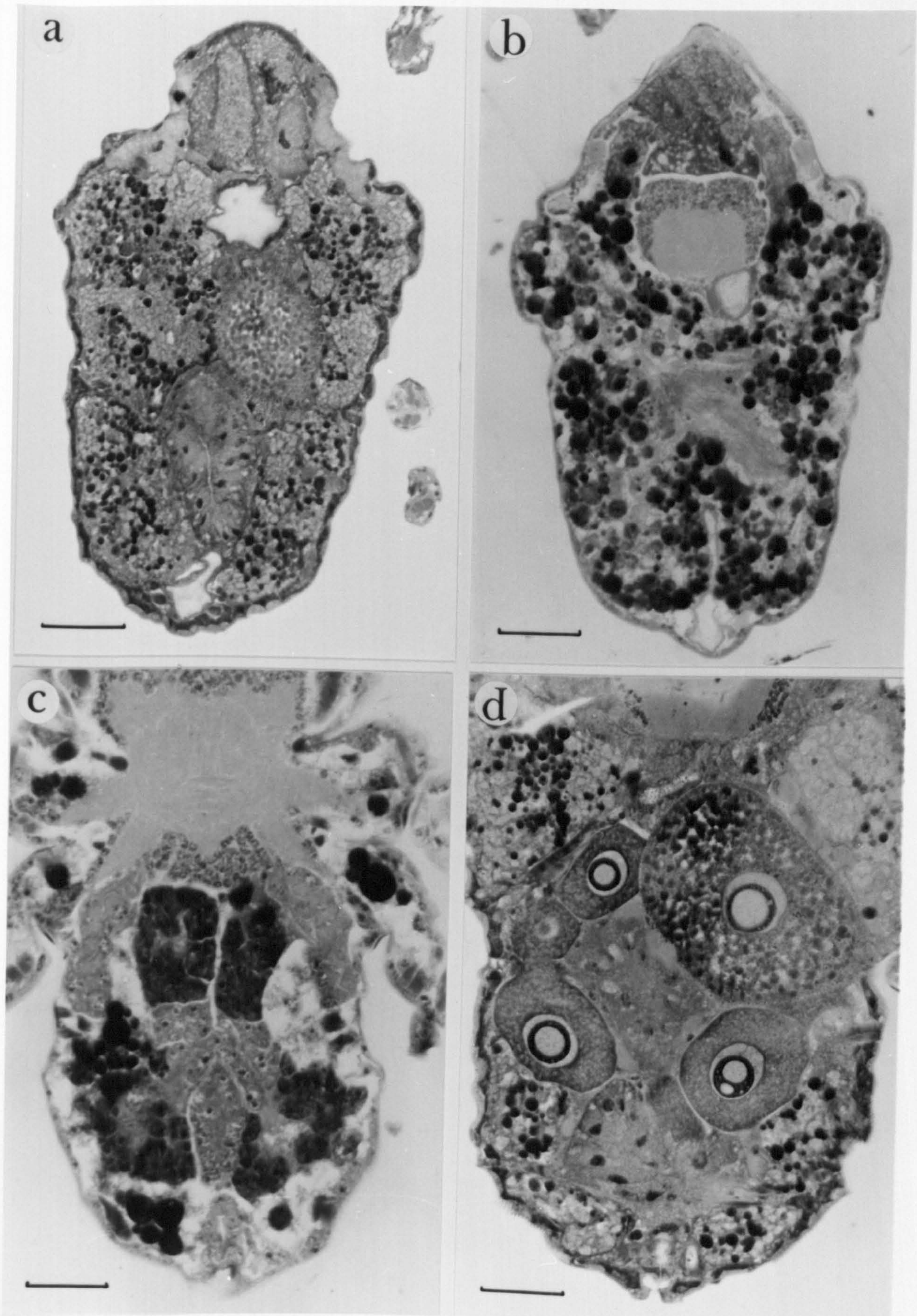
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PLATE 9

a). Transverse LM section (3μm) of *Riccardoella limacum* showing digestive caeca (DC) and oviduct (OD). Scale bar = 34.3μm.

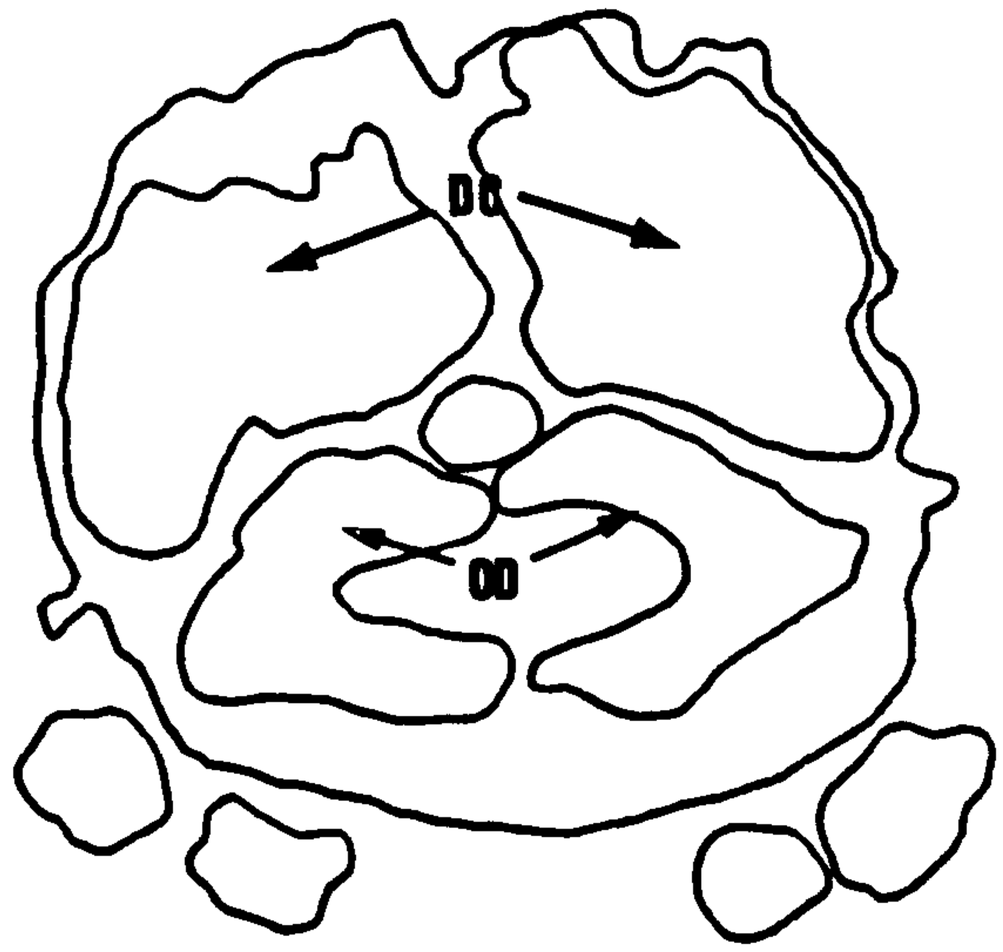
b). Transverse section at the anterior end of a mite showing dorsally situated oesophagus (OE) and ventral brain divided in to three branches which form nerves posterior to this section (B). Scale bar = 26.9μm.

c). Section taken through the same mite as in (a) in a more anterior position showing the oviduct forming into separate ducts (OD). Scale bar = 35.5μm.

d). Transverse section of same mite as in (b) taken in a more anterior position showing the salivary glands (SG) and brain not divided into nerves in this more anterior position. Scale bar = 29.4μm.

e). Section taken in a more anterior position in the same mite as in (a) & (c) showing the oviduct as two separate branches (OD). Scale bar = 26.6μm.

f). Transverse section of same mite as in (b) & (d) through region of reproductive organs showing cell divisions taking place (CD). Scale bar = 30.5μm.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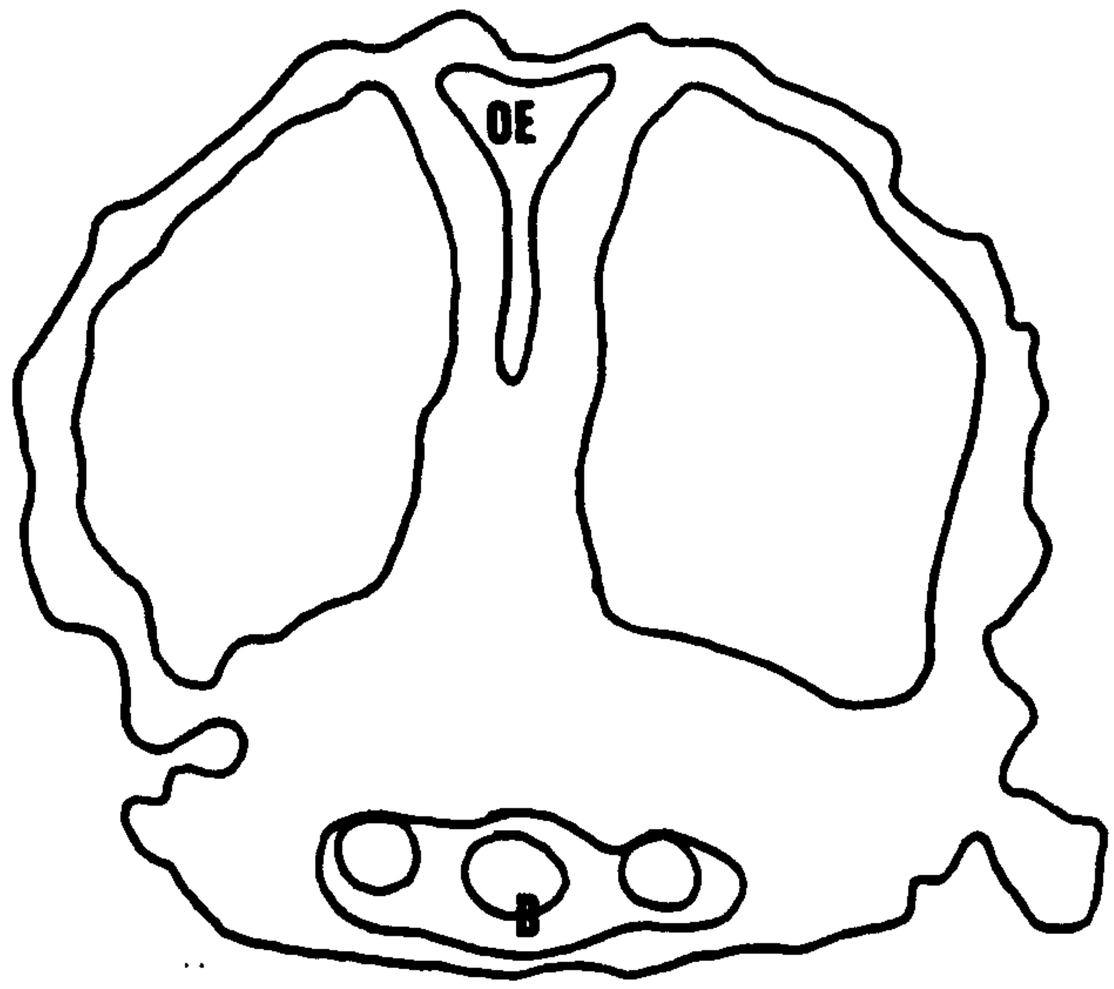
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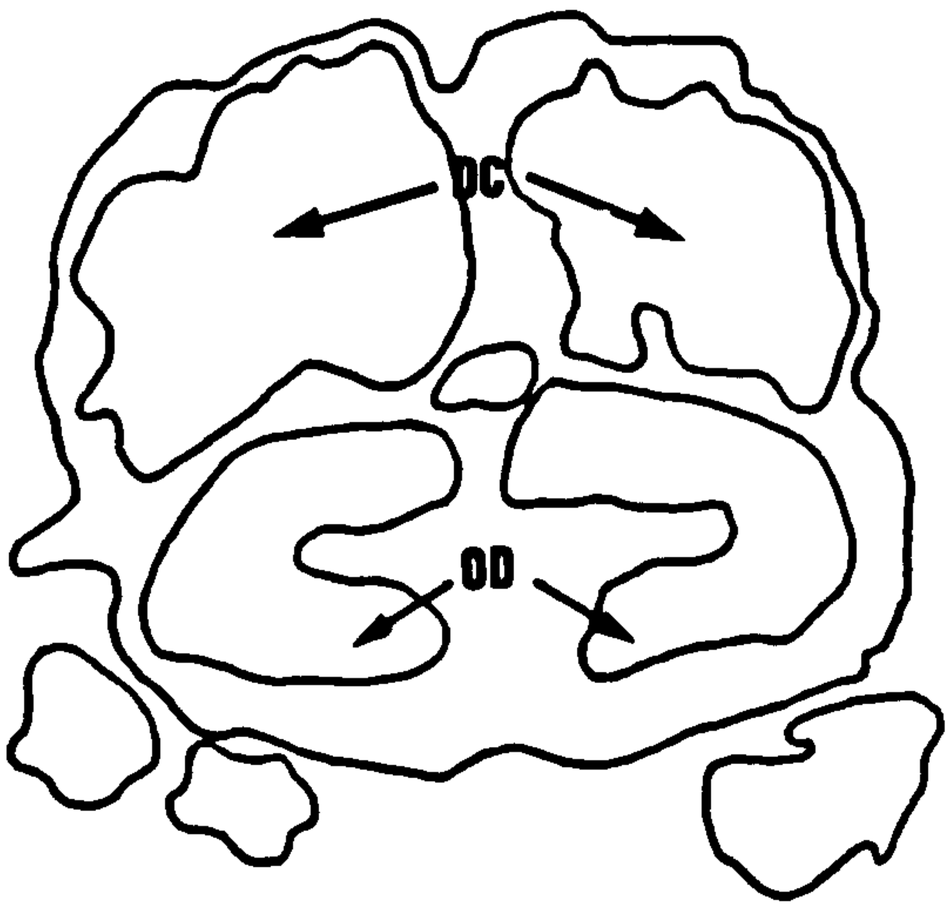
B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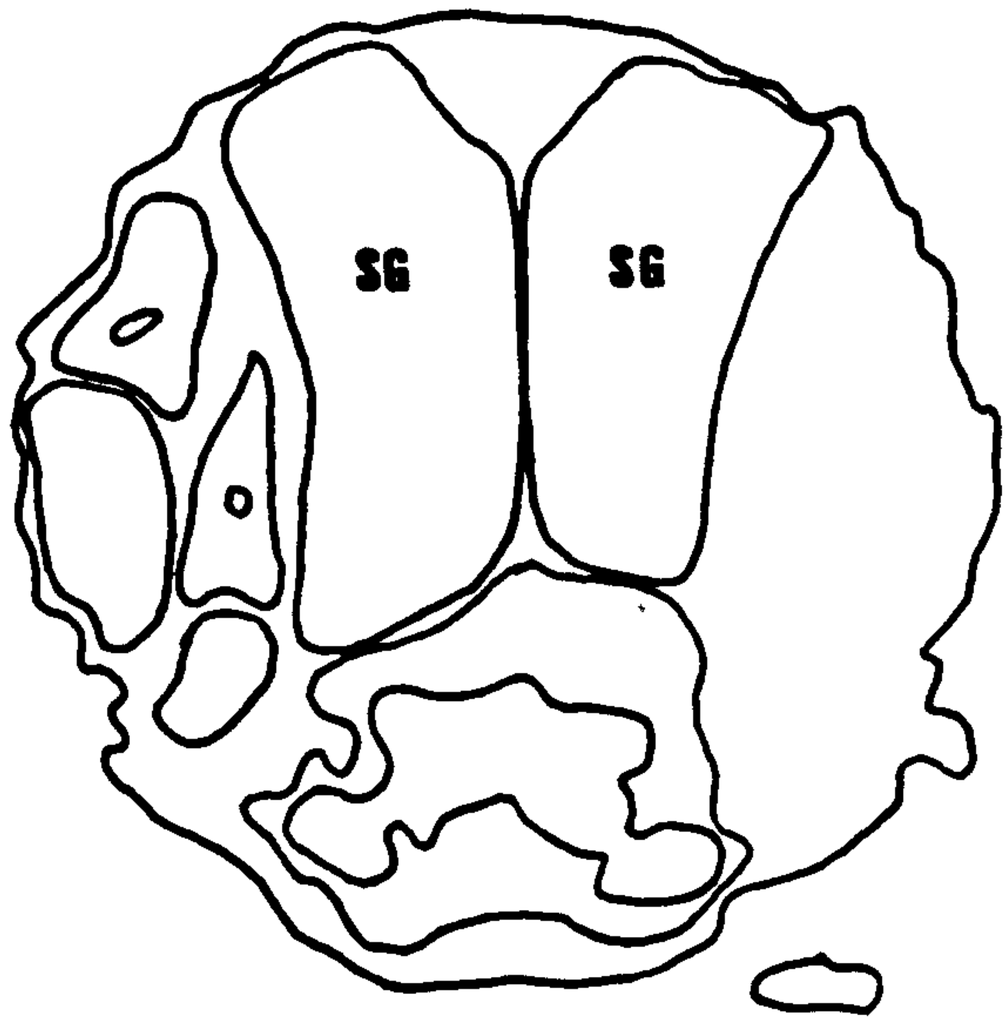
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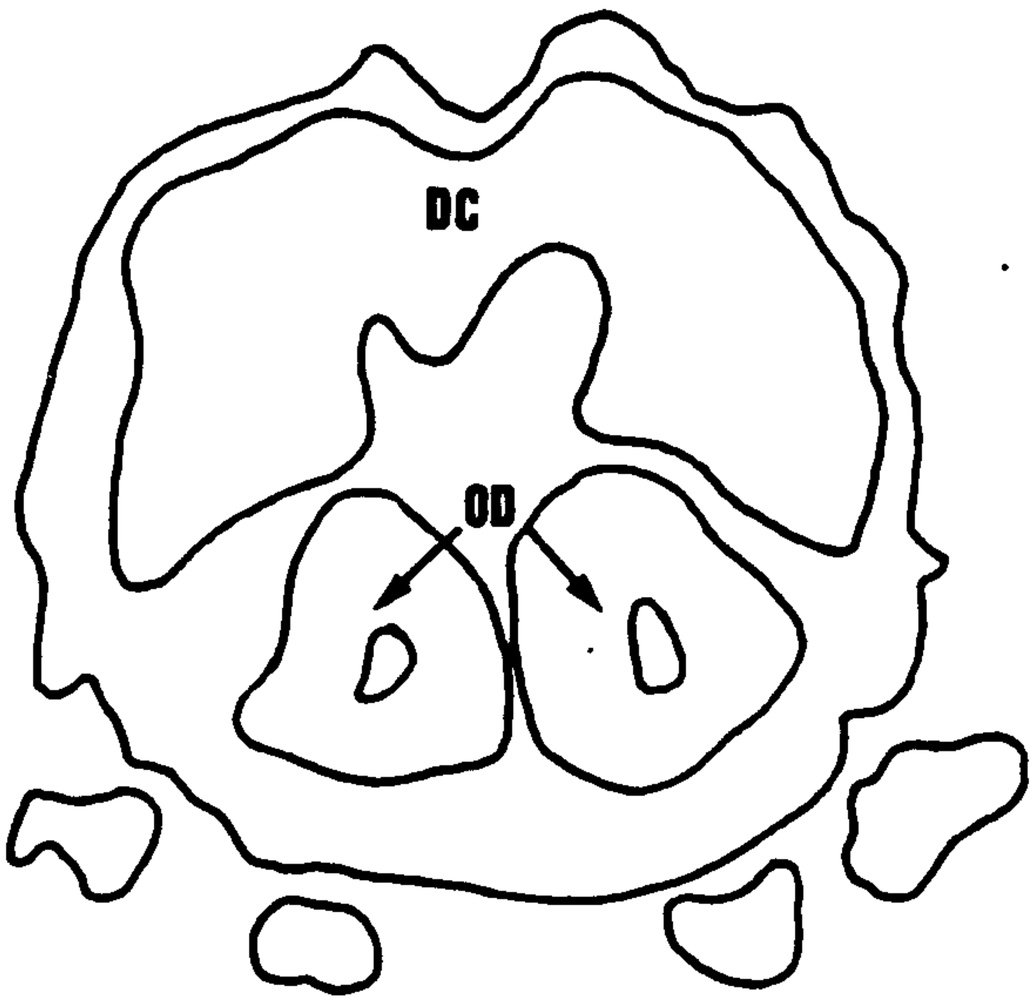
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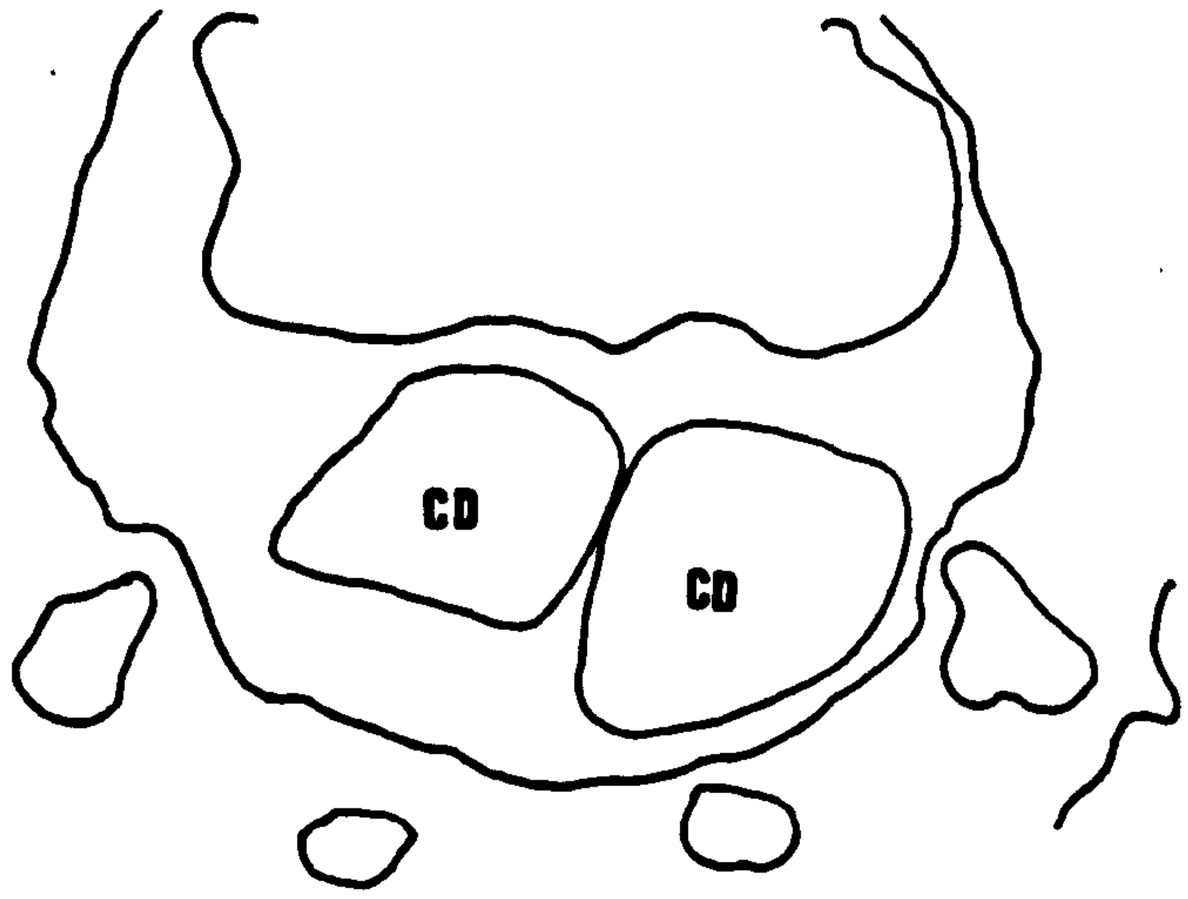
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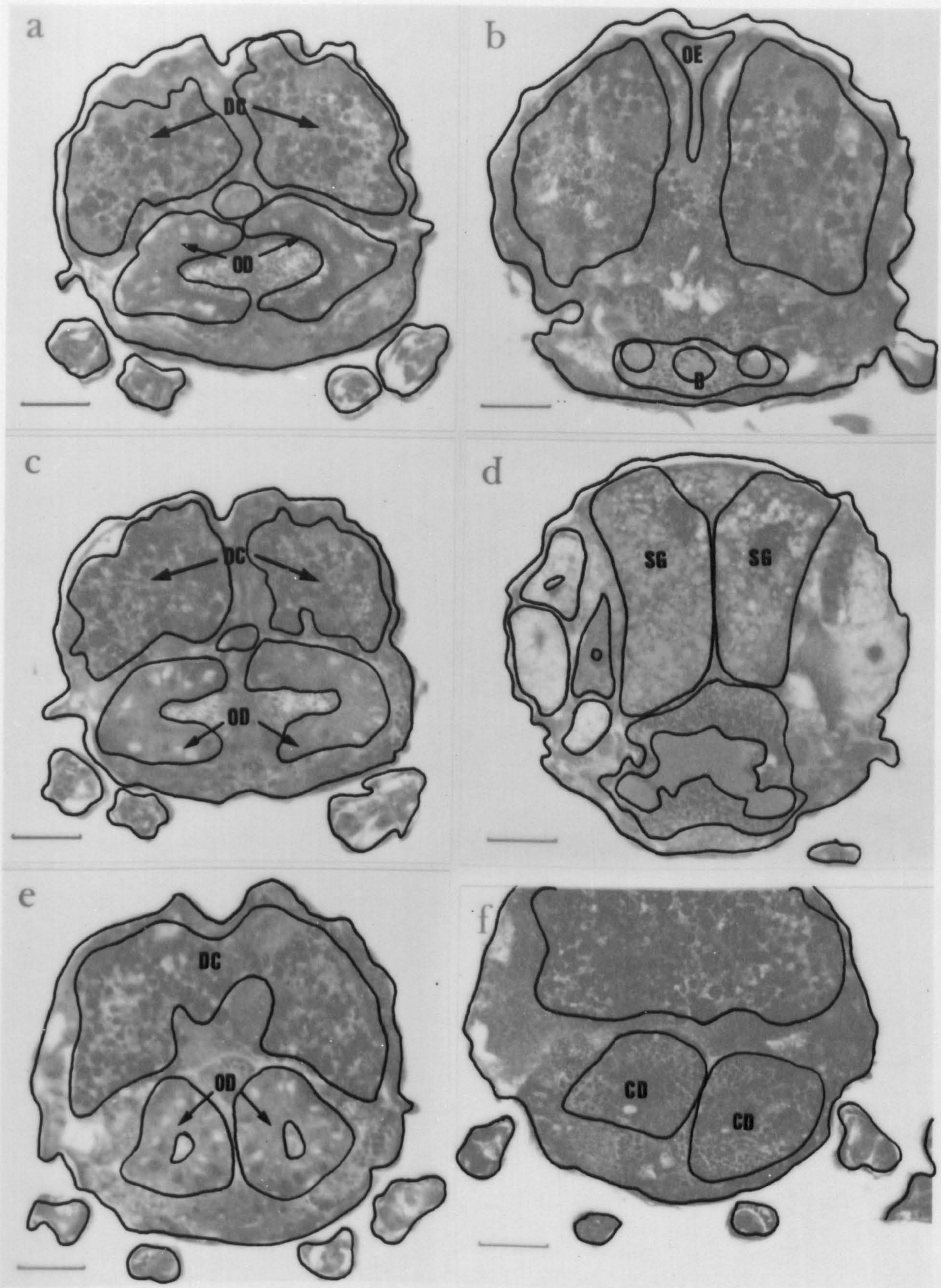
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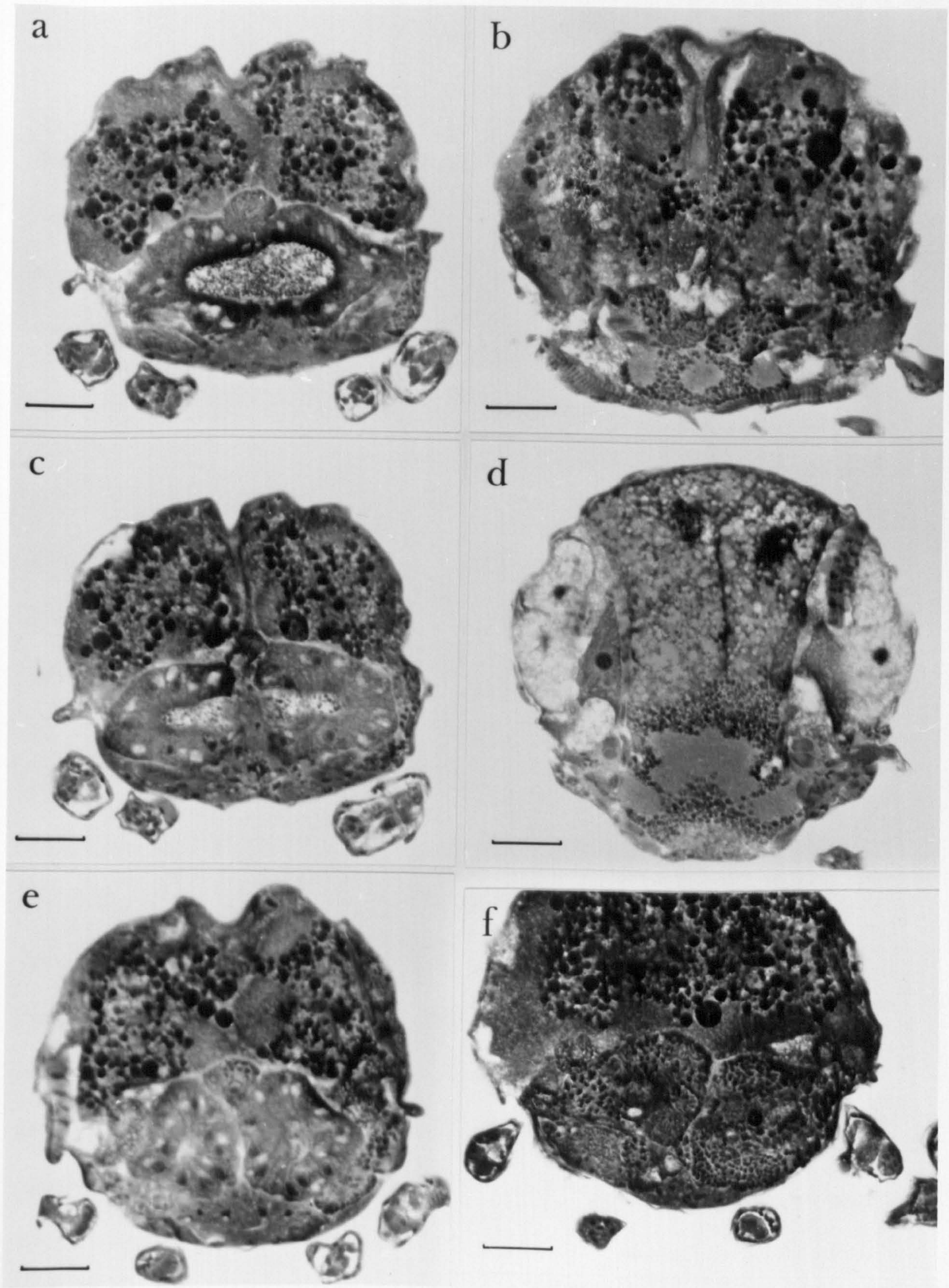
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Situated above the ovary in a posterior position there are a pair of what could be described as recepticula seminalis though no sperm were observed inside them. It was possible to discern the genital suckers in these sections (Plates 7e & 7f).

4.3.1.2 The digestive system.

This is somewhat more complex than the reproductive system as there are many component parts and numerous accessory glands. There is also a great deal of confusion as to the correct names for certain parts, with different authors using separate conventions.

The horizontal sections show a short but muscular pharynx which leads on to a very narrow non-muscularised oesophagus (Plate 6d). This almost immediately bends sharply upwards and ascends vertically to join the ventriculus which could be likened to a midgut. This in turn is in close association with paired blind ending caeca which increases the surface area of the ventriculus for the digestion of food (Plates 7c, 8b & 9a). The digestive caeca are rather extensive and occupy a dorsal position taking up most of the available body space. The contents appear as a mass of darkly staining spheres of varying sizes and densities, they obviously correspond to food material in different stages of digestion. Another region closely associated with the digestive caeca is the rectum which joins the caeca in the region of the ventriculus. The rectum runs along the dorsal margin of the body, in sections it usually appears empty, though sometimes a lightly staining mass can be seen within (Plates 71, 7c & 7e). The rectum expands into a sack-like structure which then opens to the outside through what is known in the prostigmata as the uropore (Evans, 1992)(Plates 7e, 8a & 8b). Throughout the rest of the body there is an extensive array of what have previously been labelled salivary glands (Plates 6d, 7a, 7b, 7c, 9a

& 9b). From examining the sections available it was not possible to say what the exact function of so many glands might be. Despite the semi-specific nature of the stains used, they all exhibited darkly staining glandular type tissue. The most extensive of the glands lie anteriorly, just behind the gnathosoma (Plate 9e). It can be seen from the accompanying photographs that they fit the contours of the body wall extremely well and are fairly closely packed. There are also a pair of glands present in a more central, posterior position in the body and three pairs situated near the coxae of the last three pairs of legs, these have a somewhat tubular appearance.

4.3.1.3 The muscular system.

At the anterior end there are several well defined muscle blocks in the gnathosomic region which are associated with the pharynx and chelicerae, in fact these muscles almost fill the entire gnathosoma (Plate 6d). The other even more obvious muscles of the body occur in the centre of the mite where a large chitinous mass known as the endosternite gives rise to blocks of muscle leading to the legs. Posteriorly there are a number of smaller groups which seem to be associated with the ano-genital region as well as the rear legs (Plate 7f).

4.3.1.4 The nervous system.

This is basically a mass of ganglia fused to form a large "brain" in the anterior, ventral part of the body. It is very easy to locate on the slides as at some points it occupies nearly half the available body space, it is divisible into the inner neuropile region and the outer cortex (Plates 7b, 7c, 8b, 8c, 9d & 9e). The oesophagus runs through the ganglion dividing it into two halves, the front of which gives rise to two large palpal nerves which run anteriorly. From the rear half of the ganglion it is

possible to trace four branches of nerves leading to the legs and also a thinner paired branch to the genital region.

4.3.2 Whole Mount Observations.

Although these were useful in the initial identification and separation of the two species, *R. limacum* and *R. oudemansi,* they were of only limited use in gaining information concerning the internal anatomy (Plate 6b). It was possible to identify certain structures such as the genital suckers found internally in the region of the genetal slit (Plate 6c). These were also reported by Baker (1970) though it was not possible to say what their function was. The chelicerae of the mouthparts are fairly clear and many of the leg and body hairs show up well. It was not possible to see clearly any of the major organ systems as had previously been reported as possible (Turk & Phillips, 1946) although a slightly denser region running dorsally down the back of the mite could have been the excretory canal.

4.3.3 Computer-aided Three-dimensional Reconstruction.

The results of this were very satisfactory and can be seen in the accompanying colour print-outs. Good reconstructions were obtained for the reproductive system (again only the female mite was available for examination), the pharynx, oesophagus, digestive caeca, excretory organ and anal atrium/rectum, several muscle blocks and the brain. The results served to back up information already gathered during the light microscope study and in some cases expanded it into more detail. For example, the true position of the reproductive structures can be appreciated and the extent to which the brain occupies the anterior region understood. The salivary glands provide a particularly good example of how the reconstruction program puts

the organs in their natural position and the true extent of these glands can be seen from the plots. It is also possible to see how large a volume is occupied by the caeca and if all the organs are plotted on the same picture the caeca will obliterate almost everything else. The ventriculus is seen to be in close association with the digestive caeca and then joins the rectum which expands into a much larger sac like structure before opening at the anus.

Figure 1

Three-dimensional computer-aided reconstruction of *Riccardoella limacum*. Red = body outline, dark blue & light blue = glandular tissue, the most anterior portions of which are salivary glands.

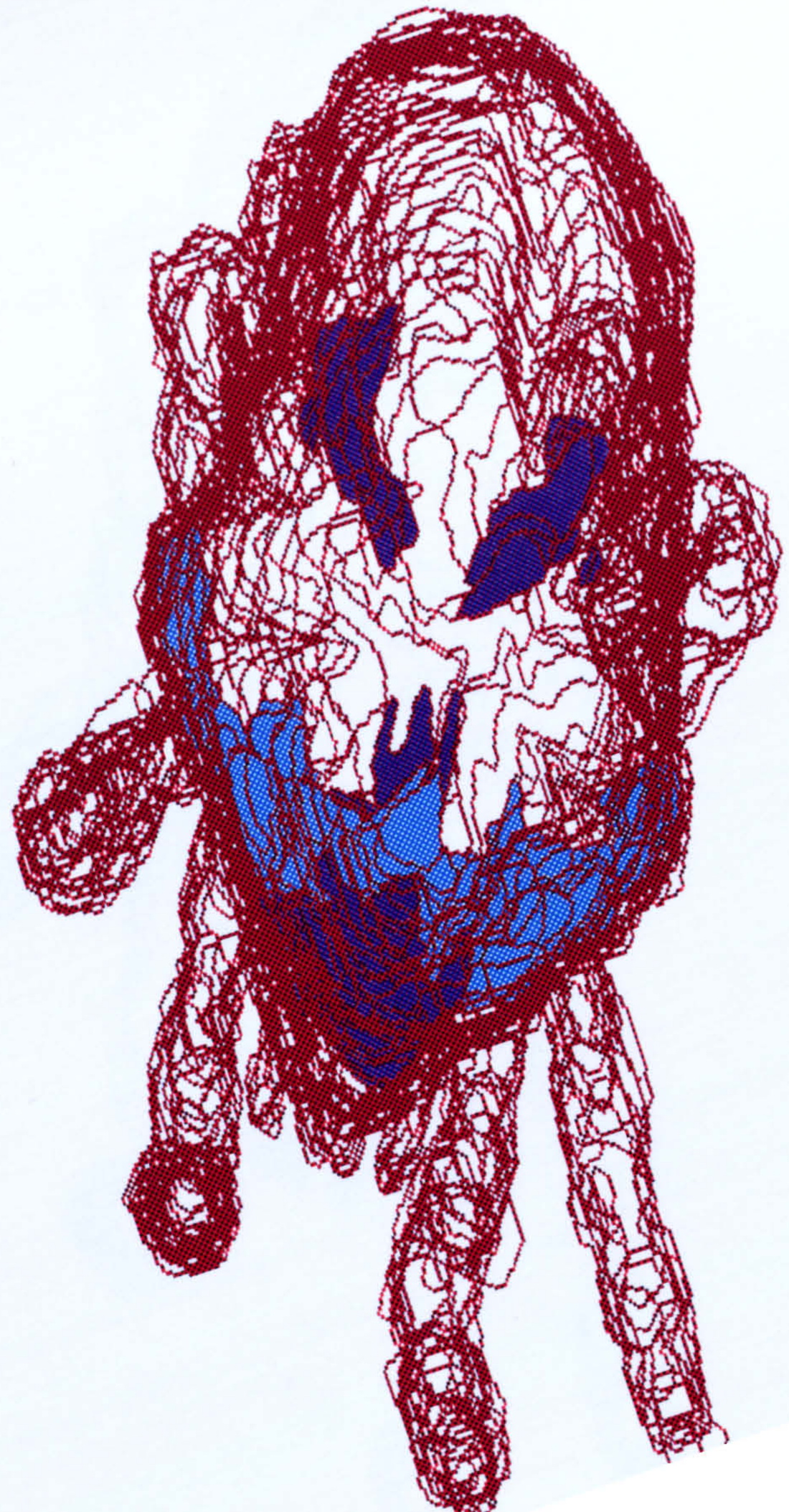


Figure 2

Three-dimensional computer-aided reconstruction of *Riccardoella limacum* showing the lobes of the ovaries (yellow) and the oviduct (turquoise), as before the outline of the body is in red.

Figure 3

Three-dimensional computer-aided reconstruction of *Riccardoella limacum* showing the brain (magenta), the oesophagus (turquoise) running through the brain and the ventriculus (dark blue).

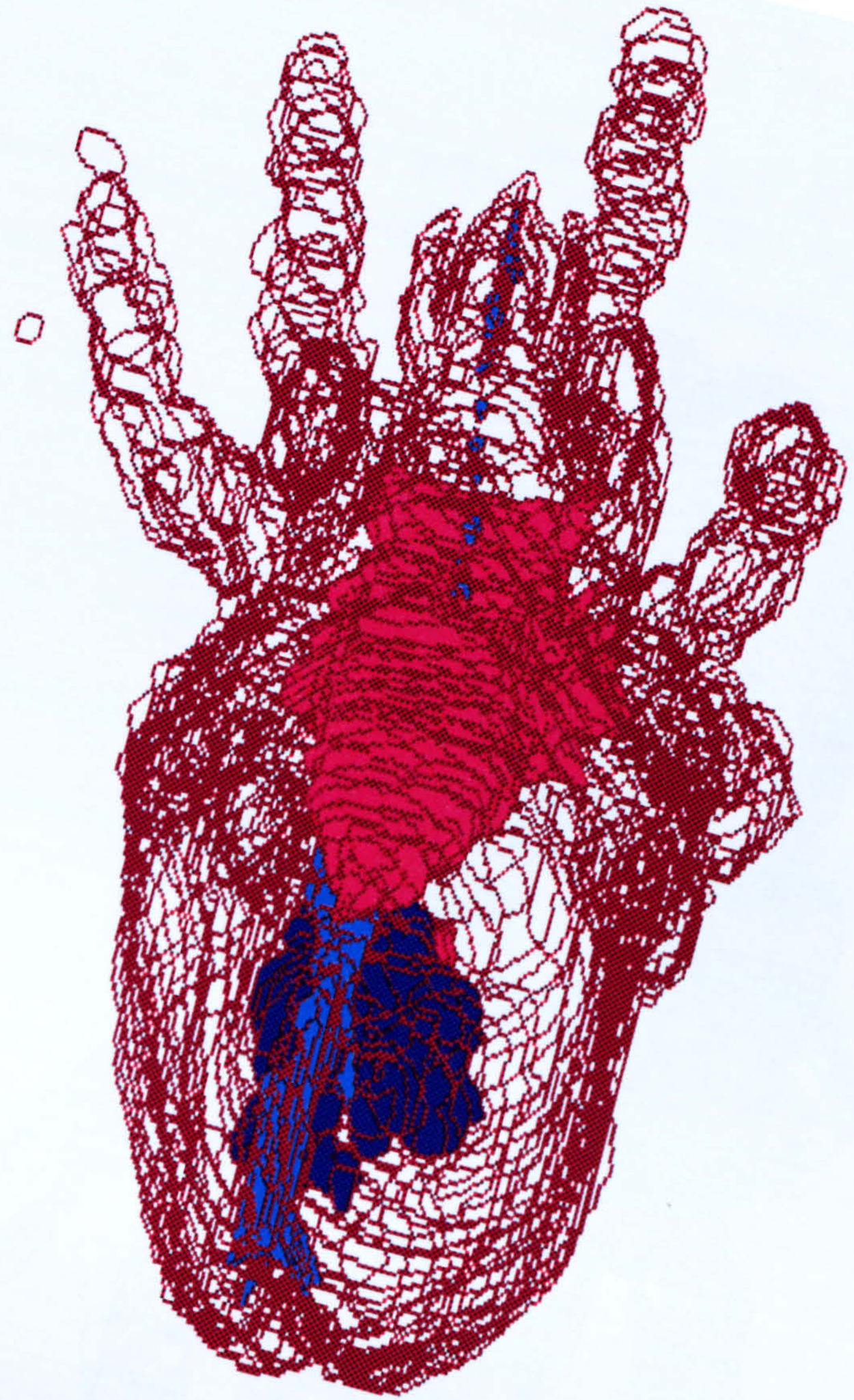


Figure 4

Three-dimensional computer-aided reconstruction showing the main muscles of the body and legs (purple/blue), the oesophagus (pink) and the ventriculus (magenta) of *Riccardoella limacum.*

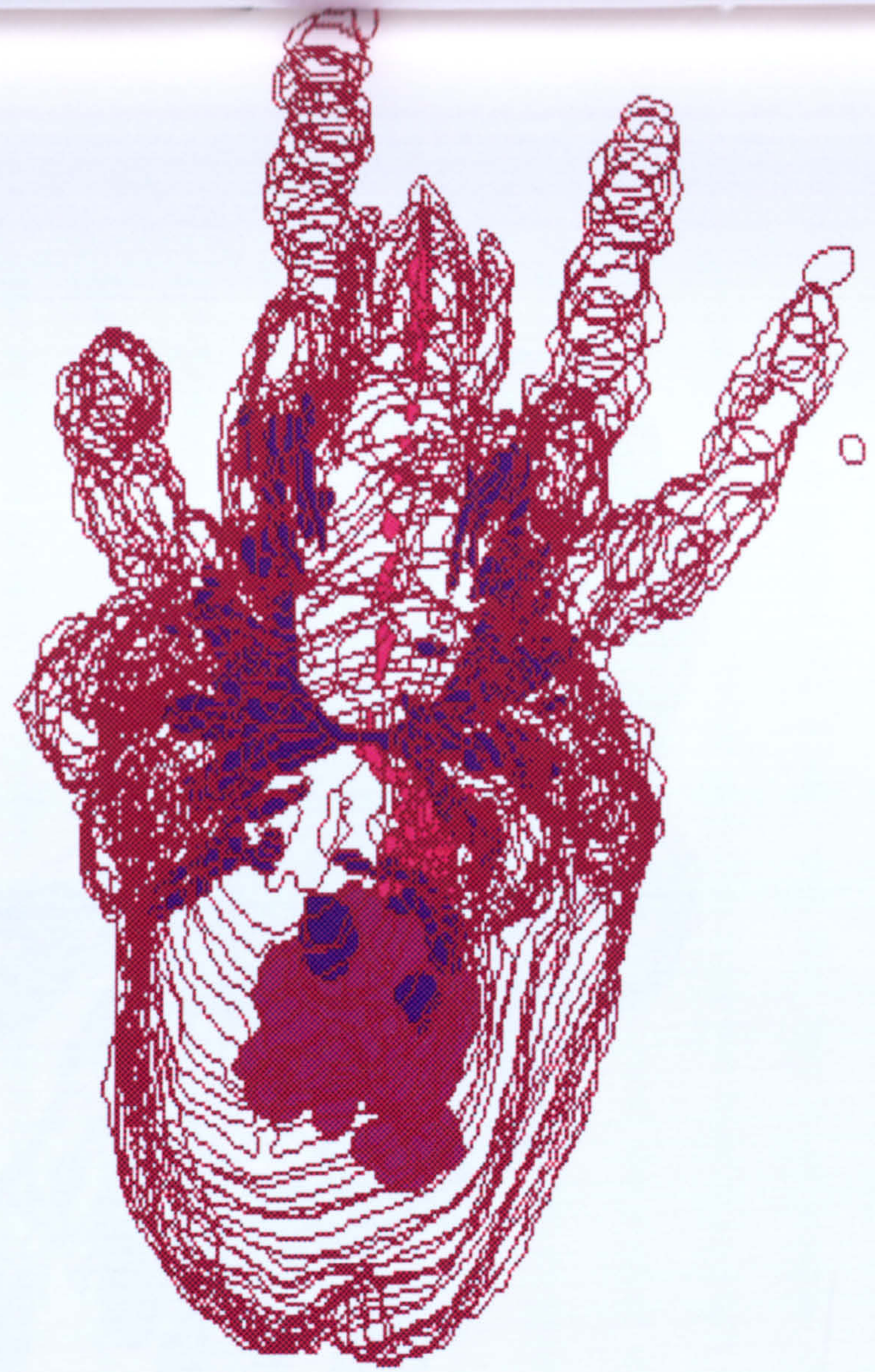
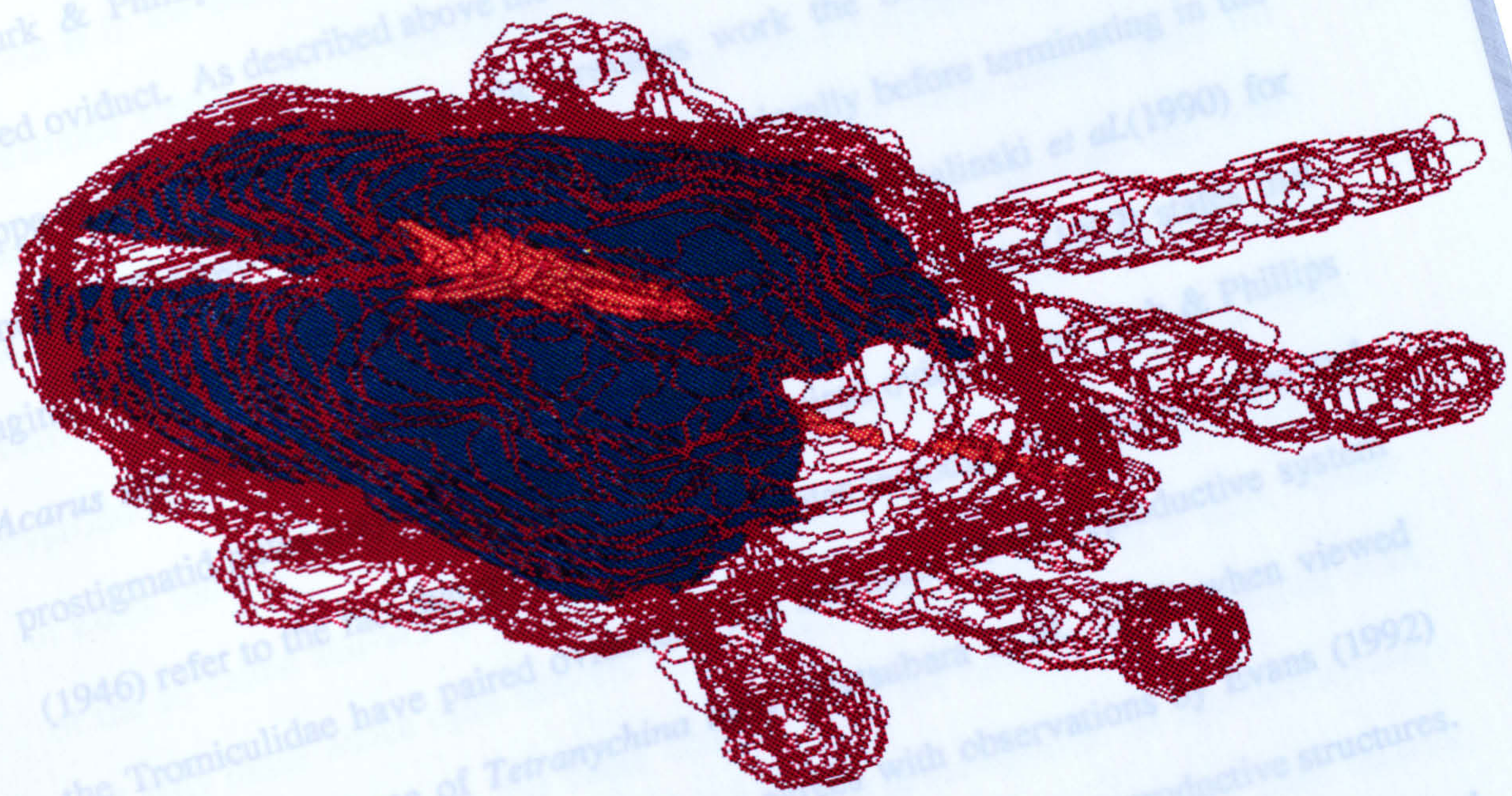


Figure 5

Three-dimensional computer-aided reconstruction of Riccardoella limacum showing the digestive caeca (Jade green) and the oesophagus (orange) running through it in close association.



4.4 DISCUSSION

From this study it has been possible to clarify many of the aspects of the major systems in the body, which had previously been dealt with only briefly or not at all. Like Turk & Phillips (1946) it was found that there was a distinct shortage of males to examine, though Baker (1967, 1973) subsequently managed to section specimens of *R. oudemansi.* The details of the female reproductive system agree with those published by previous workers (Turk & Phillips, 1946; Baker, 1967 & 1973) for the most part. However there are one or two differences that were noted, for example both Turk & Phillips and Baker report that there is an unpaired ovary and an unpaired oviduct. As described above the ovary was found to be single though lobed in appearance whilst contrary to the previous work the oviduct seemed to be branched. The two arms of the oviduct fuse posteriorally before terminating in the vagina, this arrangement is similar to that described by Witalinski *et al.*(1990) for *Acarus siro* where the fused oviduct is called the uterus. Evans (1992) states that prostigmatid mites may have either paired or unpaired oviducts and Turk & Phillips (1946) refer to the fact that most prostigmatid mites including the Hydrachnidae and the Tromiculidae have paired oviducts. The structures of the reproductive system also resemble those of *Tetranychina haiti* (Matsubara *et al.*, 1992) when viewed from a lateral point of view which also agrees with observations by Evans (1992) who stated that Tetranychidae are also known to have paired reproductive structures.

Baker (1967) described the presence of oviducal eggs in the adult female and records the presence of a shell and yolk prior to oviposition and this has certainly been the case in this study. There also appeared to be only one pair of accessory glands in the female which agree with other workers, as opposed to the male which has been reported to have many.

The digestive system has been described in detail many times previously but the combination of light microscopy and three dimensional reconstruction has allowed a much better understanding than before. It was possible to observe the pharynx as a tube with powerful pharyngeal muscles surrounding it, this arrangement was also seen by Baker (1971) who, when working on the related mite *X. africanus*, stated that this produced the sucking action required for this mite to feed. He goes on to describe the oesophagus running through the nerve ganglion and into a ventricular region. This is similar to that reported by Turk & Phillips (1946) though they refer to the ventriculus as the stomach, they also say that the main site for digestion and absorbtion is in the oesophagus due to the fact that it has thin walls and that there are peristaltic waves from the ventriculus forwards. This seems unlikely and other workers have since said that the caeca are the main sites for digestion and absorption (Baker, 1967 & 1973), Baker (1971) noted six pairs of caeca arising from the ventriculus in *X. Africanus* and they were full of haematin granules and other byproducts of digestion. Evans (1992) writes that in describing the internal anatomy of mites there is often some confusion in the terminology used. He says that some of the so called salivary glands described previously are not in fact salivary glands and only those which open into the buccal region should be named as such. Other glands previously given this name could well be the coxal glands associated with osmoregulation (Evans, 1992) which were noted at the bases of the legs in this study. However using the 3-D images it was possible to see clearly that the most anterior of the glands were in fact true salivary glands being in close association with the gnathosomic region. The function of so many salivary glands is not really known though Baker (1973) states that it is likely that they play a part in penetrating the host tissue. The most posterior portion of the alimentary tract has also been subject to many differing names, with the hind gut being likened to the excretory canal by Baker (1967) though he later calls this into question. It seems that the ventriculus

is a large chamber for holding the food until it passes into the caeca for digestion and absorption. The rectum, in close association with the caeca, leaves the ventriculus and is highly expandable, it has been seen to fill up with excretory products (Turk & Phillips, 1946) before they are discharged through the posterior anus to the exterior. The ability of the rectum to expand can be seen fully in the reconstruction plots. The nervous system and the musculature are very similar to those which have been previously described.

CHAPTER 5

SOME ASPECTS OF THE LIFE CYCLE AND DISPERSAL OF *RICCARDOELLA LIMACUM*

5.1 INTRODUCTION.

The life history of the family Ereynetidae was probably first described by Thor (1933) who noted the presence of a hexapod larva and an octopod nymph. He believed there to be no other stages preceding the adult and the female was said to lay eggs. Development in the Ereynetidae was further studied and the life cycle modified by Grandjean (1938 & 1939) who established that as a rule development in this family included a larval and three nymphal stages.

Some of the earliest and more detailed work carried out specifically on the life cycle of *Riccardoella limacum (= R. oudemansi)* was by Turk & Phillips (1946), they described all stages of the life history; egg, larva, protonymph, deutonymph and adult male and female. They did however state that the form they identified as the male, had previously been described by other authors as *R. jenynsi*. They also believed that the occurrence of the adult male was seasonal and possibly regressing from the life cycle as very few specimens were collected and those that were, appeared in poor physical health. The rarity of the male form was also observed by André & Lamy (1930) who reported greater numbers of females than any other stage. Turk & Phillips (1946) believed that the female form was also in the process of dying out from the life cycle, its role being filled by the deutonymph which was capable of

laying eggs or indeed being viviparous. No mention is made in their work of a third nymphal stage and they were only able to separate the two stages that they found on the strength of differences in body length. Baker (1973) when reviewing the Ereynetidae as a whole noted that viviparity occurred in the related mite *Xenopacarus africanus,* but not apparently in *R. limacum* and therefore Turk & Phillips must have been mistaken. Unusual development was also seen in the family Speleognathidae by Fain (1972), who found that some members have no free nymphal stage but undergo all development from larva to adult within the larval skin. The residual membranes containing sclerotized pharynxes of all the previous stages can be seen in the ecdysed skin, it is possible to see from this example how a false impression of viviparity could be given.

Baker (1967) set out to reveal the life cycle of *R. limacum (=R. oudemansi)* in full without basing his observations totally on size as had been the case before. He followed work already carried out by Grandjean (1938) amongst others, on the tydeid mites, in which the life cycle stages were separated on the basis of the genital and agenital setae. Baker confirmed that there were three nymphal stages in *R. limacum (=R. oudemansi)* thus following the pattern for ereynetids in general. This third nymphal stage was known as the tritonymph and was found only very occasionally which could explain it's omission in previous reports. He also expressed doubts that the female stage was disappearing from the life cycle as he could find no nymphs containing eggs, while most of the females he examined contained fully developed ova and there was no evidence for viviparity.

There is some difficulty in studying accurately the entire life cycle of this mite, which cannot be kept for any great length of time away from the host snail on an artificial food medium (Baker, 1967, 1970b). Fain *et al.* (1969) succeeded in keeping

specimens of *X. africanus* alive once off their hosts for several days and Baker (1967) transferred mites at different stages of development into culture cells (see introduction) and found it was possible to observe the life cycle through from egg to adult, though specific times for each stage were not given. Baker (1967) also sampled the mites of the wild population by collecting slugs and examining any parasites thus obtained under a microscope for information concerning the seasonality and percentages occupied by the different stages. He repeated this study later (1970b) but he did however note that this was not an accurate method of estimating the population as some specimens were often lost in transit. As far as the literature is concerned there are no further reports of *R. limacum* and the entire life cycle has not been established nor factors affecting the duration of it considered.

There has been a considerable amount of research on the life cycles and factors affecting their duration in other mites of the order Prostigmata, particularly the family Tetranychidae that contains the spider mites. Nickel (1960), working on *Tetranychus desertium* and *T. telarius (urticae)* found that the life cycles were affected quite dramatically by temperature. The egg to adult development time decreased as the temperature rose from 16-36 °C and he also found that the time was less if the humidity was higher. It also appears from this work that there is a threshold temperature of approximately 10 °C, below which no development can take place in these species. This threshold temperature of about 10 °C was also found by Herbert (1981) working with *Panonychus ulmi*, who also noticed that twice as many eggs hatched at 21 °C as at 15 °C. Tanigoshi *et al.* (1975) established an optimum temperature of about 35 °C for the growth and reproduction of *T. mcdanieli* and discovered that the whole life cycle took up to 1.98 days longer to complete when the temperature was altered by ±5 °C. Shih *et al.* (1975) showed that *T. urticae* required temperatures in the range 23-29 °C for their optimal development and a rise

in temperature would prolong the entire life cycle, thus increasing the reproductive potential and have considerable consequences in causing a population increase. In some cases the temperature affects different stages, for example Hazan *et al.* (1973) found that in *T. cinnabarinus* the temperature for optimal egg deposition was 24 °C and 38% relative humidity while the fastest development time for the entire life cycle was recorded at 35 °C and 38% relative humidity. Overall they found that in this species very high humidities retard development and high temperatures increase mortality.

The effects of temperature and humidity have also been seen to affect the life cycles of trombiculid mites in significant ways. Keay (1937) working on *Trombicula autumnalis* found that the larva took longer to develop in spring time when the temperatures were lower and that placing them in an incubator at 23 ^{0}C speeded up the process. Jones (1950a) working on the same species, found that at 100% relative humidity the life cycle could be shortened from 36 days at room temperature to 28 days at 25 ^{0}C. Melvin (1946) found that if he heated up his culture to 35 ^{0}C for two or three days then egg laying was increased. The effect of photoperiod on the behaviour and life cycle of mites is also well known, with a condition of diapause being induced primarily by low daylight hours but also by temperature and modified by host or environmental quality. According to Van Houten *et al.* (1992) a photoperiod of less than fourteen hours will induce a diapausing state, this is reduced to 6-13 hours in *Panonychus ulmi* (Evans, 1992). In this mite a special overwintering egg is produced by the females at the end of the summer, these eggs are less likely to hatch before the winter than those laid by females during the rest of the year (Garcia-Marí *et al.*, 1992). This special diapausing egg usually only hatches after a certain length of time has passed at low temperatures, (Evans, 1992). It is also very common in the Tetranychidae to undergo a period of diapause as an

adult.

From previous work on the Ereynetidae and other prostigmatid mites it is therefore evident that while a basic knowledge of the life cycle of *R. limacum* has already been gained, there is still much that is unknown particularly regarding the environmental influences on the duration of its life cycle.

5.2 MATERIALS AND METHODS

To discover more about the life cycle of the mite in general information was gathered from both wild and laboratory cultures in the following ways.

5.2.1 Collections From wild Populations.

Each month, at approximately the same time, 30 individual snails were gathered from a private garden in Menai Bridge where large aggregations of snails were known to exist. They were immediately taken back to the laboratory and the incidence of mites on the exterior of the snails noted and the specimens collected in a test tube. They were then given a lethal injection of magnesium chloride into the body cavity while in an extended position and the shells removed with a stout pair of scissors once all activity had ceased. The lung was then dissected out under a binocular microscope by inserting a fine pair of scissors into the pneumostome and cutting along the line formed by the rectum and ureter as far as the digestive gland of the snail. Another incision was made along the front edge of the mantle and the lung could then be folded back into a triangular shape exposing the inner respiratory surface. The pulmonary cavity was then examined for any signs of mites; eggs, larvae, nymphs, adults or moulted skins. The overall condition of the lung was noted.

This study was undertaken for a period of two years so that any trends in the population in the first year could be verified. At times of the year when the snails were hibernating due to the cold weather, they lay down a series of mucous layers which dry to a thin papery consistency and are collectively known as the epipraghm. It was necessary to carefully remove these layers before the snail could be dissected

and any incidence of mites occurring in these layers was also noted. Twice during the course of the study it was possible to obtain samples of snails from Cardiff and also once from Devon, in order to compare results from different areas. In the first year of the study it was also decided to collect slugs from the same local site to see what fluctuations were undergone by their mite population. They were prepared in the same way as the snails and the samples included specimens of *Arion ater, Arion hortensis, Limax sowerbii, Limax maculata, Limax maximus* and *Milax budapestensis.*

Most of the mites collected during this investigation were used for either SEM or light microscopy work, the mites from the slugs enabling the comparison with snail mites (Chapter 3) to be made. Occasionally mites were used to top up existing mite populations in culture or for other ongoing work.

5.2.2 Effects of Temperature, Humidity and Snail Physiology on Mite Populations in Wild Snails.

5.2.2.1 Effect of influencing snail activity by manipulating humidity.

Groups of snails hibernating on pieces of vertical slate were collected from the Menai Bridge site during the winter months of 1991. These were divided into four groups of approximately 15 snails each. Two groups were placed, still attached to their slate, into plastic bags which were sprayed daily with water and these formed the high humidity group. The other two groups were left as they were, these were the low humidity group. All the snails were then placed in an incubator at 20°C and left for ten days. It was thought that the snails kept at a higher humidity would wake up from their hibernation and that this might bring about a physiological change that would induce mite development. After ten days one of each of the high and low

humidity groups was removed from the incubator and dissected as previously described, incidence of mites being noted. The other two groups were examined a week later. It was thought that there might be a noticeable difference between the groups of mites on snails kept at high and low humidity, if not after the first time period, then at least after the second. This experiment was only repeated three times over the course of the study due to the limited availability of snails hibernating on substrate that could easily be removed to the laboratory without disturbing the snails.

The reverse of this experiment was carried out by collecting active snails during the summer months of the same year and forcing them into aestivation by dropping both the temperature and humidity in the incubator using silica gel crystals. If the life cycle of the mite is dependant on the activity or dormancy of the snail, this should have some effect on the mite population.

5.2.2.2 Effect of temperature.

As the mites appear to spend a greater part of their time inside the cavity of the lung where the humidity remains at a fairly high, constant level, it was thought that the effects of temperature on winter mite development would be interesting. If temperature was a controlling factor of development, then by raising it, the activity of the mites should increase and any eggs present should hatch.

Snails were collected from the site at Menai Bridge during the winter months of 1992 and divided into six groups of about fifteen in each. These were then placed into incubators set at 14-16 °C, 19-21 °C and 25-29 °C; the humidity was kept low to ensure that the snails remained in hibernation and that their physiology had no influence on the experiment. One group of snails from each temperature range was dissected after 10 days and the other after 20 days to see what stages in the life cycle

of the mite were present. This experiment was also repeated only three times due to the above reasons.

5.2.3 Life Cycle Observations in the Laboratory.

Previous work on the life cycle in the laboratory has resulted in proof of the existence of the various stages but not of their duration. It was hoped with this part of the study to be able to calculate the duration of at least some of the stages in the life history:

5.2.3.1 Addition of larvae.

Snails were taken from the "clean culture" and infected with approximately five larvae each by transferring the mites with a paint brush. Not all stages of mites were available at any given time and so the experiments were not all carried out simultaneously. Snails in each batch of experiments were kept separately in small plastic plant propagators (10cm x 7cm x 7cm) to prevent the mites from moving away from the new host snail. The snails were maintained at 25 °C and dissected each day in order to observe the stages reached by the mites. When the duration of each stage had been determined the experiment was repeated several times to confirm the result.

5.2.3.2 Addition of adults.

An experiment similar to the one above was carried out using adult mites to determine the duration of young stages. As the adult♀ were the most abundant stage it was possible to transfer more mites per snail for the experiments (approximately 10 per host), and to set up more of them simultaneously. Again, several replications were made to confirm the findings.

5.2.3.3 Use of culture cells.

When any eggs were found inside one of the dissected animals, they were transferred to a culture cell similar to those used by Baker (1970). The cells were made by drilling a 1cm diameter hole to a depth of 0.5cm in a piece of perspex (four cells to a block), with a small circle of high absorbency blotting paper (moistened with distilled water) placed in the bottom. The eggs were placed into these cells and a coverslip placed over the top with a drop of water on the underside. These were kept at 25 °C to see if they would hatch and if so, to determine hatching times and mortalities.

5.2.4 Dispersal of Mites.

It was not known which stages of the life cycle are more likely to leave the original host and colonise a new one. This was investigated by collecting both larvae and adults and transferring about ten of each to two "clean snails" which were marked on their shells with "L" (Larvae) and "A" (Adults). These two snails were kept separately for 24 hours to allow the mites to settle down and then transferred to an aquarium which contained a further 20 "clean snails" (marked 1 - 20). The snails were kept at 25 ^{0}C, and after three days were examined to locate the mites and dissected to discover whether any of the mites were inside the lung. This experiment was repeated using only adult mites to colonise the new hosts and all the snails where examined after 24 hours.

5.2.5 Proportion of Life Stages in the Population.

Throughout two years of the study large samples of mites were collected three times a year; in the spring as the snails were coming out of hibernation (March), in the

middle of the summer (July) and in the autumn, just before the snails began to go into their winter hibernation (October). These mites were prepared for SEM study as already described (Chapter 3) and mounted so as to reveal their genital region and therefore count the numbers of each stage present, this being more accurate than using a light microscope.

5.2.6 Observations on Internal:External Proportions.

As the study progressed it became evident that it would be useful to determine whether a snail was infested with mites without having to kill it. Since the majority of the mites live inside the lung cavity, the possible relationship between the numbers counted externally and those found internally was investigated.

5.3 RESULTS

5.3.1 Monthly Population Sample.

The results of the two year study of the wild population of snails are summarised in Table 5.2, including the observations made on the snails collected from Cardiff and Devon. The numbers of immature and adult snails that were collected throughout the year can be found in Table 5.1 while the results of the accompanying slug sampling are in Table 5.3. The percentages of snails collected which contained either mites or eggs in their lungs at any time of the year can be seen in Graph 5.1.

The snails begin their hibernation at the end of September as the weather worsens before winter and they break hibernation around March. However younger snails, i.e. those of that particular year class, delay hibernation for longer than fully mature individuals and may even avoid it altogether. They also wake up earlier if they do hibernate and are often mite free on examination. Over this period it became increasingly difficult to find snails for the samples and they were often found hibernating in old plant pots, under plastic bags, in dry stone walls and on vertical surfaces such as old roofing slates. The mite population dies back partially over the winter months leaving behind sometimes vast quantities of eggs that begin to hatch after any warm weather in March. The eggs are found as small pearly white oval structures, usually in groups near the pneumostome, but sometimes further into the lung itself. As the hatching time approaches their slightly translucent appearance becomes more solid white; at this stage they usually hatch within 24 hours. Those mites that did not die over the winter were often found living between the layers of the epiphragm when the snails were dissected. Most snails secrete between two and six layers of this mucus based covering and when it was removed layer by layer

Table 5.1 : Comparison of Mature and Immature Snails with and without an epiphragm.

	Numbers of Snails											
	1990				1991				1992			
	Mature		Immature		Mature		Immature		Mature		Immature	
Month	*With*	*With out*	*With*	*With out*	*With*	*With out*	*With*	*With out*	*With*	*With out*	*With*	*With out*
Jan.	xxx	xxx	xxx	xxx	40	0	1	11	28	0	2	8
Feb.	xxx	xxx	xxx	xxx	33	2	1	9	32	0	1	11
Mar.	xxx	xxx	xxx	xxx	25	7	0	5	29	2	0	12
Apr.	xxx	xxx	xxx	xxx	7	23	0	3	3	31	0	9
May	xxx	xxx	xxx	xxx	0	30	xxx	xxx	0	35	0	8
Jun.	xxx	xxx	xxx	xxx	0	30	xxx	xxx	0	37	0	10
Jul.	xxx	xxx	xxx	xxx	0	32	xxx	xxx	0	30	0	11
Aug.	xxx	xxx	xxx	xxx	0	35	xxx	xxx	0	30	xxx	xxx
Sept.	xxx	xxx	xxx	xxx	0	30	0	11	0	30	xxx	xxx
Oct.	xxx	xxx	xxx	xxx	3	28	0	10	2	25	0	9
Nov.	35	0	0	7	30	0	0	9	38	0	0	10
Dec.	31	4	0	10	31	0	1	10	32	0	2	7

Table 5.2 : Results of monthly snail samples.

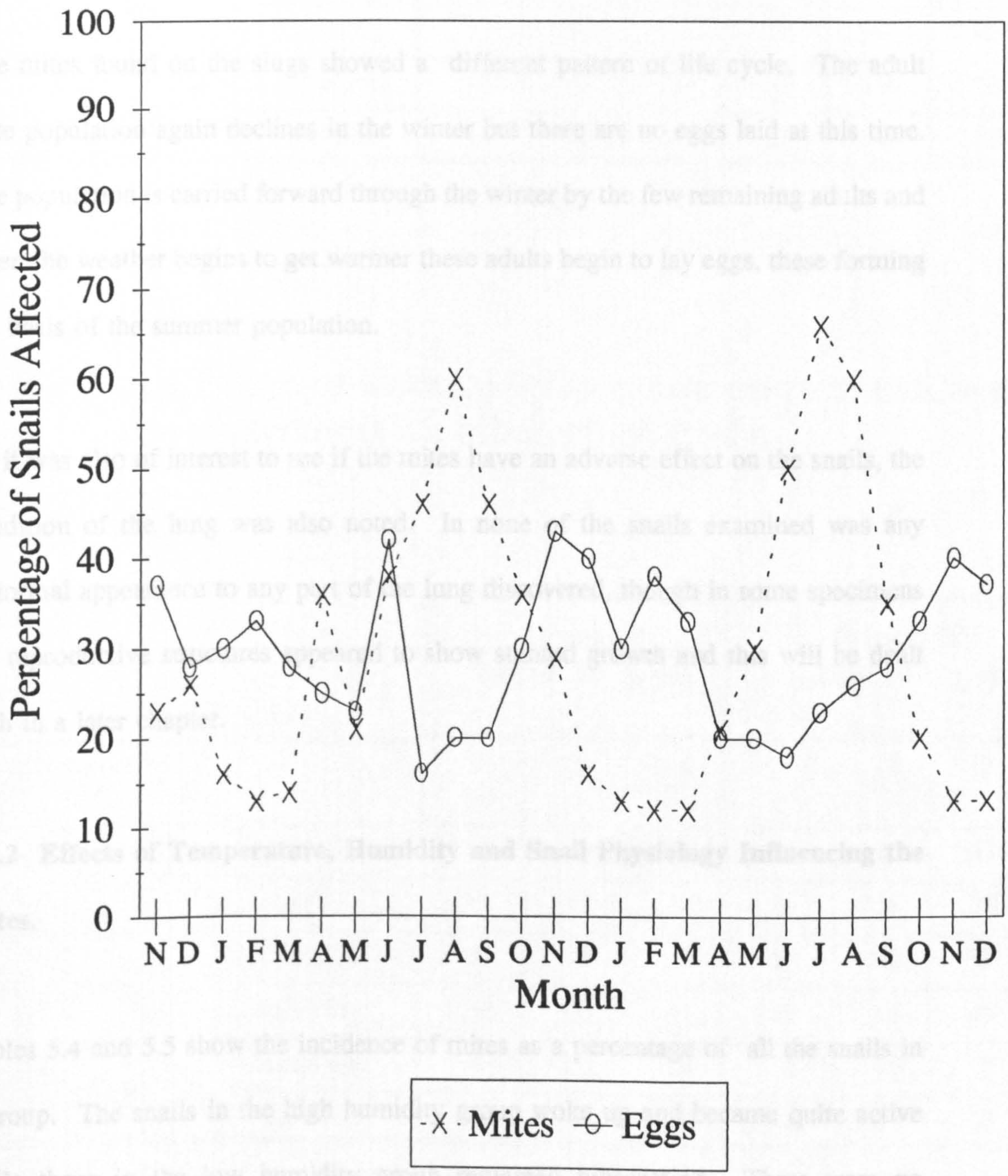
	Percentage of snails infested with mites or eggs					
	1990		1991		1992	
Month	Mites (%)	Eggs (%)	Mites (%)	Eggs (%)	Mites (%)	Eggs (%)
January	xxx	xxx	16	30	13	30
February	xxx	xxx	13	33	12 *8,**17	38 *33,**52
March	xxx	xxx	14	28	12	33
April	xxx	xxx	36	25	21	20
May	xxx	xxx	21	23	30	20
June	xxx	xxx	38	42	50	18
July	xxx	xxx	46	16	66	23
August	xxx	xxx	60	20	60	26
September	xxx	xxx	46	20	35	28
October	xxx	xxx	36	30	20	33
November	23	37	29 *25	43 *45	13	40
December	26	28	16	40	13	37

xxx No data for that month
* Cardiff sample
** Devon sample

Table 5.3 : Results of monthly slug sample.

	Percentage of slugs infested with mites or eggs	
Month	Mites (%)	Eggs(%)
November 1990	35	20
December 1990	28	0
January 1991	40	0
February 1991	36	0
March 1991	10	0
April 1991	28	10
May 1991	33	23
June 1991	28	34
July 1991	23	45
August 1991	37	21
September 1991	47	13
October 1991	59	6
November 1991	33	0
December 1991	23	0

Graph 5.1- Percentage of Snails with Mites or Eggs.



mites were revealed, those trapped in the outer layers were always dead but those in the innermost ones, near to the snails and within reach of the pneumostome, were often still alive. In these inner layers mite eggs were quite often discovered adhering to the small area of shell exposed between layers. As only two samples from Cardiff and one from Devon could be obtained it was not possible to gain enough information to discover if there was a significant difference between the timing of life cycles, proportions of stages or numbers of mites within the three populations.

The mites found on the slugs showed a different pattern of life cycle. The adult mite population again declines in the winter but there are no eggs laid at this time. The population is carried forward through the winter by the few remaining adults and when the weather begins to get warmer these adults begin to lay eggs, these forming the basis of the summer population.

As it was also of interest to see if the mites have an adverse effect on the snails, the condition of the lung was also noted. In none of the snails examined was any abnormal appearance to any part of the lung discovered, though in some specimens the reproductive structures appeared to show stunted growth and this will be dealt with in a later chapter.

5.3.2 Effects of Temperature, Humidity and Snail Physiology Influencing the Mites.

Tables 5.4 and 5.5 show the incidence of mites as a percentage of all the snails in a group. The snails in the high humidity group woke up and became quite active while those in the low humidity group remained hibernating. There were no significant differences when a chi-squared test was carried out between the numbers

Table 5.4 : Effect of humidity on mite and egg percentages on hibernating snails.

		Group 1 (after 10 days)			Group 2 (after 20 days)		
		1	2	3	1	2	3
High Humidity	Mites (%)	15	16	20	13	10	19
	Eggs (%)	33	31	45	25	31	46
Low Humidity	Mites (%)	16	10	20	10	9	20
	Eggs(%)	41	51	60	37	42	53

Table 5.5 : Effect of external temperature on mite and egg percentages on hibernating snails.

		Group 1 (after 10 days)			Group 2 (after 20 days)		
Temperature (°C)		1	2	3	1	2	3
14-16	Mites(%)	12	10	9	7	10	11
	Eggs(%)	35	41	47	36	39	28
19-21	Mites(%)	10	13	19	12	10	9
	Eggs(%)	29	36	41	39	43	45
25-29	Mites(%)	8	10	19	11	10	8
	Eggs(%)	37	41	49	41	36	37

of mites found in the snails that had been deliberately woken up and those that were dormant. Similarly there is no difference between the numbers of mites found inside snails in the groups set up at different temperatures. A second group of snails, examined either a week later (1st experiment) and ten days later (2nd experiment) also showed no significant differences when a chi-squared test was carried out on the data.

In both of the experiments the percentage of mite incidence recorded was mainly formed from the occurrence of eggs with few adult mites. All of the eggs where in the translucent state with none apparently about to hatch.

In the reverse experiment carried out in the summer causing the snails to go into artificial aestivation, there was no difference in the mite population to that observed in the wild. Mites did not lay overwintering eggs and the adult population did not go into a decline.

5.3.3 Life Cycle Observations in the Laboratory.

5.3.3.1 Addition of larvae.

The results of this experiment are summarised in Table 5.6. Mortality of the transferred larvae was high presumably as a result of handling, however, enough survived to estimate of the length of time for the development of nymphs, adults and eggs.

3.3.3.2 Addition of adults.

The results of this experiment are summarised in Table 5.7. Adults seem to be more resilient to handling than the larval stage and these experiments have met with

Table 5.6 : Occurrence of mite life cycle stages (incubation at 25°C)

Days after transfer of 10 larvae	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Mean number of eggs												1.6	4	7	6.3	12.6	26			
Mean number of nymphs		2.5	2	3.3	2.5	1.3	1.3	1.5		1.5										
Mean number of adults									2	3.3	4.3	4.5	16	20.4						

Table 5.7 : Occurrence of mite life cycle stages (incubation at 25°C)

Days after transfer of 20 adults	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Mean number of eggs					43	12	16	43.5	25.8	32.2	37.5	15	10	10.6	6	7.7	29	3	5	6
Mean number of larvae								1	1	3.3	10.6	3.3	6	3.8	3.7	4	6	5.5	6.5	5
Mean number of nymphs													5	1	1.5		16	15	19	20

Table 5.8 : Duration of stages in life cycle.

	Mite life stage (first appearance)			
	Eggs	Larvae	Nymphs	Adults
Larval mites added	9 days	-	3 days	9 days
Adult mites added	5 days	8 days	13 days	-

Note: Experiment carried out at 25°C for approximately 25 days.

greater success. A summary of the duration of time taken for the first specimens of any stage to appear after the initial introduction to snail can be found in Table 5.8. The number of stages present were confirmed as egg > larva > protonymph > deutonymph > tritonymph > adult and the approximate durations were as follows:

i. Eggs take approximately five days to hatch from when they are laid.

ii. The larval stage lasts for approximately three days.

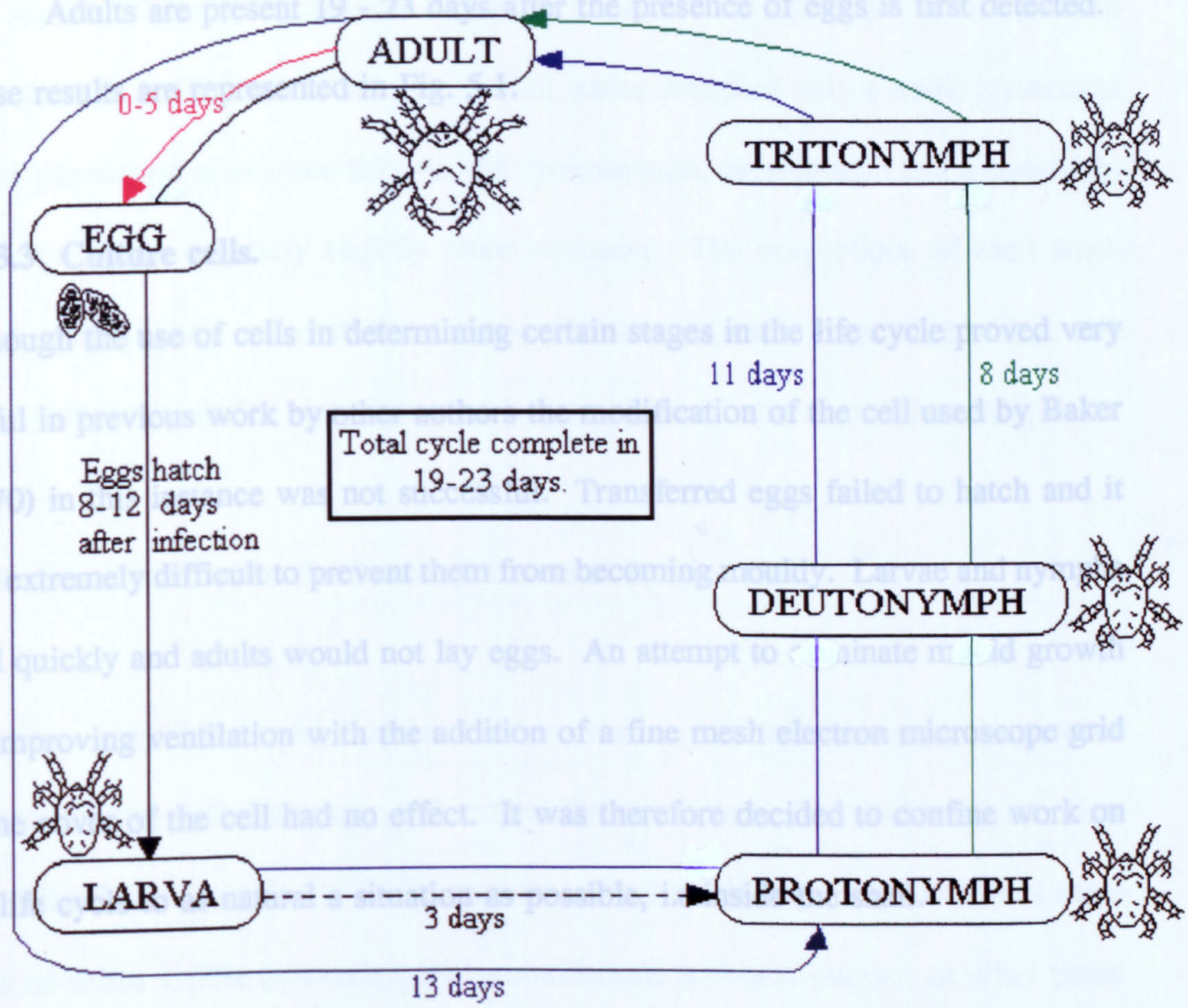
iii. The protonymph exists for approximately two days.

iv. The deutonymph and tritonymph last a further six days altogether, it was not possible to separate them further.

v. Adults are present 19 - 23 days after the presence of eggs is first detected.

These results are represented in Fig. 5.1.

Figure 5.1 : Life cycle of *Riccardoella limacum* at 25°C.



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These results are represented in Fig. 5.1.

5.3.3.3 Culture cells.

Although the use of cells in determining certain stages in the life cycle proved very useful in previous work by other authors the modification of the cell used by Baker (1970) in this instance was not successful. Transferred eggs failed to hatch and it was extremely difficult to prevent them from becoming mouldy. Larvae and nymphs died quickly and adults would not lay eggs. An attempt to eliminate mould growth by improving ventilation with the addition of a fine mesh electron microscope grid in the cover of the cell had no effect. It was therefore decided to confine work on the life cycle to as natural a situation as possible, i.e.inside the snail.

5.3.4 The Dispersal of Mites.

The mites disperse actively and after three days with an infected snail, most of the snail population had some sign of mites, the lowest percentage found in any of the

experiments being 45%. Table 5.9 summarises the results and indicates the incidence of each stage. Transference of live larvae met with limited success but some did survive and spread to other members of the snail population as can be seen from the table. Adult mites were much better subjects for transfer and fewer dead adults were recovered. Both stages were found running around on the body of the snail as well as inside the lung and where adults had been added, it was common to find snails without mites, but with many eggs laid inside the pulmonary cavity.

5.3.5 Proportion of Life Stages in the Population.

The results (Table 5.10) indicate that adult females were by far the most common stage present at all times of the year. Adult males occupied only a small percentage of the population at any one time, as did protonymph, deutonymph and tritonymph; the larval stage was only slightly more common. The proportions of each stage noted, only slightly reflected the relative durations of each stage observed in this study and as few males were observed it is not known what their relationship is.

5.3.6 Observations on the Internal:External Proportions.

Whenever a snail had to be examined for any reason, and then dissected, the number of mites found both internally and externally was noted. The incidence of mites found inside and outside the snails appears to be virtually random; sometimes there were no mites visible externally, with considerable numbers inside. At other times the entire mite population of a snail was present externally with no evidence of their presence internally. Certain trends were noted however:

1. Larger, older snails tend to be more likely to have mites than smaller,

Table 5.9a : Extent of mite dispersal after 3 days (snails 1-10 inclusive)

				Snail Number										
Trial Number	Stage found	Adult Source	Larvae Source	1	2	3	4	5	6	7	8	9	10	Spread of mites or eggs (%)
1	Larvae		O					O					O	**65**
	Adult	O*		O*		O*			O*	O*	O			
2	Larvae	O	O		O				O			O	O	**75**
	Adult	O*	O				O		O*		O*			
3	Larvae	O	O	O	O*	O		O			O		O	**80**
	Adult	O*	O	O*	*	O*	O	O	O	O	O		O*	
4	Larvae	O	O	O			O		O				O	**80**
	Adult	O*	O		O*	O*		O*	O*			O*		
5	Larvae	O	O		O	O				O			O	**90**
	Adult	O*	O	O	O		O*	O*	O*		O	O*	O	

O - Mite present * - Egg present O* - Mite and egg present

Table 5.9b : Extent of mite dispersal after 3 days (snails 11-20 inclusive)

				Snail Number										
Trial Number	Stage Found	Adult Source	Larvae Source	11	12	13	14	15	16	17	18	19	20	Spread of mites or eggs(%)
1	Larvae		O						O	O				**65**
	Adult	O*			O		O*		O*		O*	O*		
2	Larvae	O	O		O									**75**
	Adult	O*	O			O	O	O*		O*	O*	O*	O*	
3	Larvae	O	O			O			O	O				**80**
	Adult	O*	O					O	O*		O	O*	O	
4	Larvae	O	O		O			O			O			**80**
	Adult	O*	O	O	O			O	O	O*		O*	O*	
5	Larvae	O	O		O				O				O	**90**
	Adult	O*	O			O*	O*	O	O	O*	O*			

O - Mite present * - Egg present O*- Mite and egg present

Table 5.9c : Dispersal of mites after 3 days: adults only (snails 1-10 inclusive)

				Snail Number										
Trial Number	Stage Found	Adult Source	Larvae Source	1	2	3	4	5	6	7	8	9	10	Spread of mites or eggs (%)
1	Adult	O*	xxx	O*	O*	O*	O*		O*		O*	O	O*	60
2	Adult	O*	xxx	O		O*		*		O*	O*	O*		50
3	Adult	O*	xxx		O	O	O*	O*	O*		O*		O	70
4	Adult	O*	xxx	O*		O	O	O*	O		O	O	O	70
5	Adult	O*	xxx	O*		O*	O		O		*		O*	45
6	Adult	O*	xxx	O*		O			O*		O	O	O*	60
7	Adult	O*	xxx			O			O*	O*				50

O -Mite present *- Egg present O*- Mite and egg present xxx- Larvae not used

Table 5.9d : Dispersal of mites after 3 days: adults only (snails 11-20 inclusive)

				Snail Number										
Trial Number	**Stage Found**	**Adult Source**	**Larvae Source**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	Spread of mites or eggs (%)
1	Adult	O*	xxx		O		O*		O*	O				**60**
2	Adult	O*	xxx	O	O*				O*	O				**50**
3	Adult	O*	xxx	O*	O*		O	O			O*	O*	O*	**70**
4	Adult	O*	xxx	O				O		O*	O*	O*	O*	**70**
5	Adult	O*	xxx	O*			O	O						**45**
6	Adult	O*	xxx		O*	O*			O*		O	O	O	**60**
7	Adult	O*	xxx	O		O*		O	O*	O*	O		O	**50**

O -Mite present *- Egg present O*- Mite and egg present xxx- Larvae not used

Table 5.10 : Proportion of different life stages present in wild population throughout the year

Month	Adult females (%)	Adult males (%)	Nymphs (%)	Larvae (%)
March 1991	92	1	3	4
July 1991	94	2	3	3
October 1991	96	1	1	2
March 1992	93	1	1	5
July 1992	96	1	1	2
October 1992	94	2	2	2

younger individuals.

2. If mites are present they are more likely to be seen on a snail that has adhered to the side of its enclosure and is withdrawn inside the shell. The mites will be running around on the exposed surface of the snail near to the pneumostome occasionally entering it.

3. If no mites are found on the surface of a snail in the resting position, then it is likely that individual is not infected.

5.4 DISCUSSION

The stages in the life cycle of *R. limacum* are as follows; egg > larva > protonymph > deutonymph > tritonymph > adult. Other workers have found only two nymphal stages (Turk & Phillips, 1946) with no record of a tritonymph, and have separated nymphal stages by size difference only. In this study specimens were examined with an SEM and the different stages were separated by their genital regions (Plates 2c, 2d, 2e, 3a, & 3b). The protonymph had only one pair of genital setae and one patterned area, the deutonymph had two pairs of genital setae and two patterned areas and the tritonymph had four pairs of genital setae and two patterned areas (Plate 2e). These observations agree with Baker (1970a) although it is likely that he was working on *R. oudemansi*. Previous workers (Turk & Phillips, 1946; Baker 1970a) used mites obtained solely from outside the host animal and mites for the purpose of this study were regularly obtained from inside, this may have influenced the proportions observed. These workers also suggest that the rarity of nymphs may be due to the fact that they are very short-lived stages and indeed in the present work it was observed that all three nymphal stages took only six days to complete. In general adults laid eggs within about five days of being transferred to the snails, these hatched after a further three days, in agreement with Baker (1970a) who noted the same hatching time. It is hoped that these hatching times are as close to the natural occurrence as possible and it was noted by Lebrun *et al.* (1992) in all of the observations he made, that fecundity of mites in the laboratory was always very close to that observed under natural conditions.

The larval stage persisted for about five days before moulting into a protonymph, Baker (1970a) noted that his larvae died after five days in the culture cell and it is possible that they could not moult because they had not fed, Turk & Phillips (1946)

also say that the larval stage is no longer than a week. As previously stated, all of the nymphal stages occur within about six days and then the adult appears. It is not known whether the male and female have the same development times, Herbert (1981) found that in *Panonychus ulmi* they were both the same, while Laing (1969) noted that in *Tetranychus urticae* the male developed faster by approximately nineteen hours and waited by the female so that he could mate with her as soon as she moulted. The complete cycle of *R. limacum* takes about ninteen days at 25 °C which is similar to other trombidiform mites such as *Tetranychus cinnabarinus* which is sixteen days (Wrensch & Young 1978) and *T. urticae* which is between sixteen and seventeen days depending on the sex of the mite and temperature (Laing 1969).

In this study the proportions of different stages of mite present on snails that were sampled at three times over the year were on average;

Adult females = 94.2%

Adult males = 1.3%

Nymphs = 1.8%

Larvae = 2.7%

compared to the findings of Baker (1970);

Total adults = 85%

Nymphs = 4%

Larvae = 11%

These differences are due in part to difference in sampling techniques but mainly the fact that Baker was actually working on the mite *R. oudemansi* which has been shown to have a different annual life cycle anyway. Baker also noted that the number of males found was high, comparable to that of females, while the results of

this study confirm André & Lamy (1930) and Turk & Phillips (1946) conclusion; that there are very few males relative to females. There is no evidence that the male is disappearing from the life cycle and possibly the male is very short lived and undertakes to mate as soon as the females moults as in *T. urticae* (Laing, 1969), and the female then stores the sperm. It is also possible that facultative parthenogenesis plays a part since it is fairly widespread in other prostigmatid mites (Evans, 1992).

In the wild snail population, it was observed that younger individuals went into hibernation much later than larger mature individuals, presumably because they require time to store enough food reserves to see them through the winter months. This observation was also made by Staikou *et al.* (1988) working on *H. lucorum.* Adults of this species hibernate at the end of September while the young delay the onset of hibernation until November, snails emerged in March, young first and adults a few weeks later. In the present study it was also found that these younger snails were usually mite free which indicates a certain amount of selectiveness on the part of the mite, in not wanting to damage the host so much that it dies. It is therefore advantageous for the mite to colonise larger individuals that are better able to support them. It may also be that the size of the pneumostome is a limiting factor, with mites being unable to gain access to the pulmonary cavity of the smaller snails. During the winter large numbers of eggs were found in the snails examined from the wild and very few adults. None of these eggs ever seemed about to hatch as characterised by the whitening of the egg membranes. It was decided that these must be overwintering eggs and this was confirmed by the fact that the eggs from these snails did not hatch with the change in snail physiology brought about by bringing the snails out of hibernation. Neither did they hatch when the temperature was raised for a short while, indicating that they are diapausing eggs, laid when light and temperature fall below acceptable limits in the autumn (Van Houten *et al.*, 1992).

These eggs are less likely to hatch; in *Panonychus ulmi* hatching is 65% (Garcia-Marí *et al.*, 1992) and they will also only hatch after a certain length of time at a low temperature. In *P. ulmi* this is 120 - 150 days at 1-9 °C (Evans, 1992). It is possible that this condition also exists in *R. limacum* though the temperatures and "chilling time" involved are not known. As this happens to be one of the warmer regions of the United Kingdom, it is likely that the critical temperature for laying diapausing eggs will be close to the upper end of this range. Examination of the mites obtained at different times of the year from slugs indicates that the closely related mite *R.oudemansi* has a completely different life cycle with no overwintering eggs laid at all. It is not known how the mite perpetuates it's population through the winter when the majority of slugs die off, though it is thought that they may survive by changing host to colonise species such as *Derocerus reticulatum* which may have several generations a year and *Milax spp.* which are among the few that live longer than a year.

As far as dispersal in these mites is concerned, they are very quick to spread to available host snails. When clusters of snails were examined from the wild, the incidence of mites among them was usually very high and if mobile stages were not present then eggs were. There is no evidence that these mites lay eggs singly as suggested by Baker (1970a) working on *R. oudemansi* as vast numbers have been found on occasion grouped together. This is supported by the work carried out on life cycles and dispersal which showed large numbers of eggs present after only three days after the initial infestation. Both larvae and adult mites are able to leave the lung of their host snail to colonise a new one. Hall (1959) frequently found the young stages of *Histiostoma laboratorium* jumping from the top of *Drosophila* culture jars in an attempt to find a new host fly. The delicate nature of the larval form in *R. limacum* might act as a limiting factor in the dispersal of the mites.

Once the mite is established on a new host, it can quickly lay numerous eggs that give rise to a new generation in about two and a half weeks. They leave the original animal to lay more eggs and spread rapidly to a high percentage of the population of snails. If the conditions become unfavourable they sustain their population by producing diapausing eggs that hatch when conditions have improved. It was clear then that this particular mite could build up a substantial population in a snail farm once introduced and it is necessary to find out if they actually harm the snails.

CHAPTER 6

OBSERVATIONS ON THE FOOD AND NUTRITION OF *RICCARDOELLA LIMACUM*

6.1 INTRODUCTION

The study of nutrition in the Acari has been mainly carried out using ticks, because of their economic importance, large size and their ready availability. The saliva produced by a tick either contains or causes to be produced, a complex mixture of anticoagulants, anti-capillary dilation and haemorrhaging agents and factors that prevent total tissue destruction (Evans, 1992). Such information is not available for parasitic mites and indeed in many cases the nature of their food is by no means certain. The only indication of the preferred food is gained from looking at the mouthparts and assessing what food stuffs it would be possible for the mite to eat. Studies in the laboratory on behaviour and examination of gut contents have provided some information.

The greatest variety of adaptations is found in the Prostigmata and although most predacious forms feed on small arthropods (Evans, 1992), it is known that others, particularly members of the family Trombiculidae, will attack vertebrates. Some types of trombiculid are responsible for the spread of human diseases such as Tsutsugamushi (mite borne typhus). The larva of *Trombicula autumnalis* (harvest mite) is able to pierce human skin and must therefore have mouthparts capable of

such activity. Jones (1950a) states that once this mite has penetrated the host tissue it does not feed directly on solid matter. The solid tissue is liquefied by the injection of saliva which facilitates extra-intestinal digestion and allows the products to be sucked back into the mite. To enable these activities to take place in the first place the mite must have suitable mouthparts. Jones described a buccal cone or rostrum similar to that described by Nuzzaci & De Lillo (1992) for another species of mite, *Cenopalpus pulcher*, and dorsal to this are the chelicerae which, as in all prostigmatid mites, are protracted by special muscles rather than by hydrostatic pressure (Nuzzaci & De Lillo, 1992). The chelicerae that are hinged to the gnathosoma are able to pierce the skin with the aid of a vigorous pushing motion and, once inside, they rotate slightly on their hinges to widen the hole. Saliva is pumped into the opening, causing the breakdown of host tissues and the formation of a tissue canal that can be used both for the influx of saliva and the withdrawal of liquefied tissue. The arrangement of pharyngeal muscles is such that a powerful pump action is created which causes a flow of liquids in either direction along the canal (Jones, 1950a). In other species such as *C. pulcher* (Nuzzaci & De Lillo, 1992) the same basic principal applies but instead of only one tissue canal there are two; one formed from interlocked cheliceral stylets for the passage of saliva and another formed from the preoral groove and the labrum for liquid food. By the time food enters the alimentary tract it has been partially broken down already and further digestion occurs quickly.

According to Baker (1967), this arrangement forms the basis of the feeding mechanism in the mite *R. limacum (=R. oudemansi)* despite the fact that previous workers have thought that the mouthparts of this species are far too short and blunt to pierce anything (Turk & Phillips, 1946). This has led to much speculation in the literature as to what the mite actually feeds on. Whilst Thor (1933) simply states

that the mite sucks in unspecified fluids, Turk & Phillips were convinced that it could not pierce the skin and therefore must have fed on mucus. The mucus was drawn up the channel made by the mouthparts and their suggestion was supported by the fact that the survival of mites was greatest in cells provided with a supply of snail mucus, on which they were presumably feeding. Another theory they put forward for the nutrition of this mite was that it obtained a certain amount of B complex vitamins from a haplosporidian sporozoan, *Rhagidiasporidium*, acting as an intracellular symbiont.

The view that *R. limacum* feeds on mucus was partially accepted by Lawrence (1951) working on the closely related species; *R. eweri* which parasitises the South African toad, *Bufo regularis*. He believed that this species obtained nourishment from the mucus in the nasal passages of the host toad. When he compared the mouthparts to those of *R. limacum* he found them remarkably similar and as Turk & Phillips (1946) had already pointed out that piercing was out of the question, he took this as confirmation of mucus feeding in *R. eweri*. However Lawrence must have had some doubts as he noted that the alimentary tract of *R. eweri* not only closely resembled blood feeding mites, but was full of a dark crystalline mass similar to that seen in recognised blood feeders.

This question of feeding in *R. limacum* remained unsolved satisfactorily and Baker (1967) decided to test some substances in feeding experiments, to see if he could finally determine what the mite *(R. limacum = R. oudemansi)* was feeding on. Some of the substances tried were as follows:

1. Fresh yeast.
2. Mosquito eggs.

3. Macerated slug.

4. Slug haemolymph.

5. Slug homogenate

6. Slug mucus.

Unfortunately the mites would not feed artificially on any of these substances and so he concentrated his efforts on the gut contents of the mite. He found that there were a large number of nucleated cells in the gut which slowly disappeared over a few days if the mites were starved and these closely resembled the cells found in the heart and connective tissue of the slug (Baker, 1970b). These cells were ingested whole and passed quickly into the caeca for digestion. Although Turk & Phillips (1946) stated that the mouthparts are wholly unsuitable for piercing and blood sucking, Baker insisted that they are far from ideal for dealing with large quantities of highly sticky mucus which appears to be the alternative. Baker therefore suggested that in this mite the use of a tissue canal formed in the same way as already described by Jones (1950b) in *T. autumnalis*, for obtaining liquefied tissue, is the method employed for feeding. Another member of the Ereynetidae, *X.africanus* which lives in the respiratory tract of the toad *Xenopus laevis* (Baker, 1971) has also been shown to exhibit large quantities of haematin in the gut after feeding. This is a breakdown product of blood digestion and these granules were observed to disappear over a period of a few days when the mite was prevented from further feeding (Fain *et al.*, 1969). The alimentary canal when examined closely is very similar to *R. limacum (=R. oudemansi)* with the exception that the salivary glands in *R. limacum (=R. oudemansi)* are better developed (Baker 1971), this seems to indicate that *R. limacum* is suitably equipped at least for digesting a blood meal once it has obtained it.

Modern immunological techniques can be very useful in feeding studies by determining the presence or absence of specific ingested proteins. The blood of *Helix aspersa* contains haemocyanin, a high molecular weight, copper containing glyco-protein, which functions as an oxygen transport molecule dissolved in the haemolymph (Van Kuik *et al.*, 1986). It occurs in many pulmonate snails (Markl *et al.*, 1991) and by testing for the presence of this protein in the mites it is possible to see if they have been feeding on snail haemolymph. This can be a very time consuming process as it is first necessary to prepare antisera to snail haemocyanin (antigen), (Yoshino & Bayne, 1983; Rivera-Marrero & Hillyer, 1985) or to use a commercially available alternative, in which case the cross reactivity must be tested (Ouchterlony, 1958; Rivera-Marrero & Hillyer, 1985). Then by carrying out gel electrophoresis with the correct concentration of gel (Studier, 1973; Rasmussen *et al.*, 1985) followed by a technique called "Western Blotting" (Towbin *et al.*, 1979; Rivera-Marrero & Hillyer, 1985) it is possible to detect the presence of antigens by using the previously prepared antisera and then staining the antigen:antibody complex with a substance which makes it visible. This technique, although fairly lengthy, provides accurate results and was used to determine whether or not *R. limacum* feeds on snail haemolymph.

6.2 MATERIALS AND METHODS

6.2.1 Location of Mites Feeding *in situ.*

Sections of snail lung, approximately 0.5cm X 0.5cm with mites attached were collected during the dissection of monthly snail samples described in Chapter 5 (see also examination of mite infested lungs in Chapter 7). The pieces of tissue were prepared using the same methods as for the preparation of mites for light microscopy in Chapter 4 but there was no need to remove the cuticle with xylene, stain with lignin pink or embed in agar. This tissue was made into blocks and sectioned at 3μm as previously described. The sections were examined for any signs of mites present in the lung tissue.

6.2.2 Immunological Techniques.

6.2.2.1 Raising antisera.

Before mites could be tested for the presence of haemocyanin and therefore snail haemolymph, it was necessary to obtain anti-haemocyanin antibodies. This is done by injecting an antigenic solution into a host animal and allowing the immune response to raise antibodies to the antigen, in this case haemocyanin. The antigen can be obtained using the methods described by Yoshino & Bayne (1983) and Rivera-Marrero & Hillyer (1985) in which the haemolymph is obtained and ultracentrifuged before injection into the host in order to stimulate antibody production. Alternatively a similar, commercially obtainable antigen can be used to raise antibodies which must then be tested to see that they react favourably with the original test antigen. For the purposes of this study, the latter approach was adopted as considerable amounts of antigen were required in the first instance. Keyhole

limpet haemocyanin (KLH) is a powerful immune stimulant often used in research (Markl *et al.*, 1991) and this was used to raise the antibodies for this experiment. The following procedure was used:

1. 100μg KLH was mixed with complete Freund's adjuvant (to stimulate antibody production) and injected subcutaneously into multiple sites in a Sandy Half-lop rabbit.

2. After three weeks a booster was given consisting of 50μg of KLH mixed with incomplete Freund's adjuvant, this was again administered over multiple sites, subcutaneously. A further two boosters at two week intervals were given using the same mixture.

3. After all three boosters (over approximately six weeks) the blood serum was tested for the presence of anti-KLH antibodies. The serum was obtained as follows:

A. Up to 10ml of blood was removed from the ear veins of the rabbit using a syringe.

B. The blood was placed into cytology tubes, kept at 37 °C in an incubator for one hour and then stored overnight at 4 °C.

C. The cytology tubes were centrifuged at 1000g for ten minutes. This caused the separation of the blood cells from the serum which was removed and stored at -20 °C until it could be tested to see if it could be used in place of anti-*Helix spp.* haemocyanin serum.

6.2.2.2 Testing for cross reactivity with *Helix spp.* haemocyanin.

To test whether the antibodies raised against KLH would bind to *Helix spp.* haemocyanin, an immunodiffusion assay was performed. Approximately 12ml of molten agar was poured onto a dry glass plate and allowed to set. A stencil was

used to cut four channels with three wells between them, into the agar. Into the first well was placed a small quantity of snail haemolymph, into the second KLH in phosphate buffered saline (PBS) and in to the last one, KLH in barbitone buffer. An electric current was passed through the agar via filter paper soaked in barbitone buffer connected to the terminals. The current separates the test substances into constituent proteins, -ve proteins to the anode and +ve ones to the cathode, in this case the snail haemocyanin protein was separated from the haemolymph. After three hours the antisera containing anti-KLH antibodies was added to all of the channels and left for two hours, then overnight in a covered box to keep the humidity high and prevent drying out of the agar. The plate was examined the following day over a diffuse light source. Any reactions between antigen and antibody could be seen as small arcs in the gel, this is evidence for cross-reactivity and can provide an estimate of anti-serum strength. The next stage is to carry out polyacrylamide gel electrophoresis (PAGE) to assess how much protein is contained in a rough homogenate of the sample.

6.2.2.3 Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE).

This method utilizes a stacking gel with a low percentage of acrylamide to ensure that the proteins are concentrated which gives better resolution, and a resolving gel with a high percentage of acrylamide for the separation of the proteins. Sodium dodecyl sulphate (SDS) is added to coat all of the protein molecules which gives them an equal small negative charge. This ensures that the proteins are separated only on the basis of their molecular weight and not their surface charge. An electric current causes the proteins to move through the gel which can then be fixed and stained, or used to "Western blot", the proteins present can then be examined.

The ingredients used for the gels are as follows:

Stacking gel (3%):	Stacking buffer	2.5ml
	Acrylamide (Protogel)	0.5ml
	Distilled water	2.0ml
	SDS (10%)	50.0µl
	Temed	10.0µl
	*Ammonium persulphate	75.0µl
	Phenol red	2.5µl
Resolving gel (12%):	Resolving buffer	2.5ml
	Acrylamide (Protogel)	3.8ml
	Distilled water	3.7ml
	SDS (10%)	100µl
	Temed	10µl
	*Ammonium persulphate	100µl

*This has to be made freshly every day to a concentration of 50mg/ml distilled water.

Preparation of gels:

1. The resolving gel was made by mixing the buffer, acrylamide and distilled water in a flask and then degassing for ten minutes to remove air bubbles and then warming gently to aid setting. The SDS, ammonium persulphate and Temed were added - Temed last as this causes the gel to set.

2. The molten gel was poured between two glass plates clamped in a holder with a 0.75mm spacer between them and held in an upright position; a drop of butanol was then placed on the surface to help the gel to set level. Due to the nature of the

apparatus used to "run" the gels, two must be run simultaneously, therefore the molten gel was used to create two gel plates.

3. After polymerisation of the gel the butanol was washed off with distilled water and the gel carefully dried with filter paper.

4. The stacking gel was made in the same way using the relevant ingredients, the addition of phenol red dyed the gel slightly pink which made it easier to see the divide between the two, adding too much however can cause the gel not to set.

5. This gel was poured in a layer above the existing resolving gel and a plastic comb placed into it to create the wells necessary for loading samples. Again enough gel mixture was made for both of the resolving gels.

6. After polymerisation, the comb was removed and the wells washed out with distilled water and dried using filter paper. The gels were then ready to be placed in the electrophoresis equipment; in this case a Biorad "Mini Protean II" was used, and the samples loaded.

Preparation of samples:

The samples were prepared at the same time as the gels were polymerising.

1. About 50 mites were hand homogenised in 100µl of saline to produce a rough homogenate.

2. Different dilutions of this were made with saline and samples of 20µl of each were placed in Eppendorf tubes.

3. To half of these 2µl of iodoacetamide (freshly made daily to a concentration of 36mg/ml distilled water) was added. This strengthens the existing disulphide bonds between the protein chains thus preserving the quaternary protein structure. These samples are known as "native", the samples with no iodoacetamide are called "non-native" as they lose the disulphide bonds. All the samples were then placed in the

dark for half an hour.

4. Loading buffer was then added to all samples (5µl per 20µl sample), this contains glycine to make the sample sink to the bottom of the well and bromophenol blue so that the position in the gel can be known. The native samples had non-reducing loading buffer and the non-native one had reducing loading buffer.

5. All the samples were then boiled for two minutes to denature the protein; this straightens out the coiled protein chains allowing them to pass more easily through the gel.

6. Known molecular weight protein markers were prepared at the same time by diluting the stock solution 1:3, i.e. 20µl of marker and 60µl of distilled water. These also had 5µl of loading buffer added and were boiled for two minutes.

Samples used during this study:

1. *R. limacum* homogenised in saline.
2. *T. urticae* " " "
3. *H. aspersa* " " "
4. Mucus from *H. aspersa.*
5. Known molecular weight markers.

The samples and known weight markers were then loaded into the wells created by the comb templates in the gel; native samples were placed in one gel plate and non-native in the other. A Hamilton syringe was used to deliver approximately 10µl to each of the wells and was washed thoroughly with distilled water between each application to avoid carry over.

The "upper tank" of the electrophoresis apparatus used was formed by the two vertically positioned gels. A space between the gels was filled with "upper tank"

buffer and the gels placed into the "lower tank" which was partially filled with "lower tank" buffer. The equipment was connected to a Biorad 500 power supply and the gels electrophoresed at 200 volts until the samples, indicated by the blue line of bromophenol blue, were at the bottom of the gels (care must be taken not to run the samples off the bottom of the gels!) This normally took about 45 minutes, after which the glass plates containing the gels were removed and the gels silver stained as follows:

1. Gels were fixed in a solution of 40% ethanol and 10% glacial acetic acid for 45 min.
2. Washed in 10% ethanol for 2 X 5 min.
3. Washed in distilled water for 3 X 5 min.
4. Stained in 0.2% silver nitrate in the dark for 45 min.
5. Washed briefly in developing solution; 2.5g sodium carbonate and 100µl formaldehyde in 100 ml distilled water. Developed for 15-30 min.
6. The developing reaction was then stopped with 1% acetic acid for 10 min.
7. Washed in distilled water for 3 X 10 min.
8. Excessive background staining was removed by reducing in 0.6g sodium thiosulphate, 0.3g potassium ferricyanide and 0.1g sodium carbonate in 200ml distilled water until the desired strength staining was achieved.
9. Washed in distilled water for 2 X 10 min.

At this stage it was possible to see any proteins present and decide from the colour what level of dilution gave the best result. A more satisfactory method of doing this once suitable sample concentrations have been established is to perform a "Western Blot".

6.2.2.4 Western blotting of SDS PAGE.

This enables the proteins separated by gel electrophoresis to be transferred to a medium which accepts antibodies as a specific indicator of a particular protein antigen; in this case haemocyanin.

The method for this is identical to that for the electrophoresis of SDS PAGE described above with the exception that both the native and non-native samples were loaded into the same gel and replicated exactly in the duplicate gel on the other side of the apparatus. This was so that when the antibody probe was carried out, the gel from one side could be used as a control with normal rabbit serum (NRS) instead of KLH antisera. When the gels are removed from the Biorad apparatus the following techniques were followed, instead of silver staining, to produce a "Western Blot":

1. The gels were carefully removed from the glass plates and placed into a shallow bowl containing electroblotting buffer to allow them to equalise. The two sheets of Whatman filter paper, two absorbent pads and the hinged, perforated perspex support were also placed in the buffer to equalise.
2. Two sheets of nitrocellulose paper (NCP) were cut so that they were just bigger than the gels, this paper has a high affinity for proteins so it was necessary to handle it very carefully to avoid transferring protein from the fingers that would obscure the result.
3. A "sandwich" was made with one side of the perspex support, then an absorbent pad, followed by filter paper, then a piece of NCP. The gel was carefully laid on top of this and the rest of the layers placed over the top in reverse order, being careful not to trap any air bubbles. There was enough room for the two gels to be placed side by side in this manner.
4. The sides of the perspex support were brought together and the whole thing

placed into a blotting tank filled with electro-blotting buffer cooled to 6 °C. Extreme care must be taken to ensure that the position of the NCP and the gels is such that the paper is nearest to the anode of the tank as the proteins have a negative charge and flow towards the positive electrode and hence the paper.

5. A current of 0.45 amps was applied to the tank for 3-4 hours after which time it was assumed that the proteins had "blotted" onto the paper.

6. The NCP was carefully removed and the region with the samples placed in a solution of 2g Marvel milk powder in 200ml distilled water for two hours. This blocks all nonspecific protein binding sites left free on the NCP thus preventing them binding with the antibody later and masking the result.

7. The regions that had the molecular weight markers was treated as follows:

A. Wash in PBS for 3 X 5 min.

B. Leave in 0.3% Tween 20 in PBS at 37 ^{0}C for 30 min.

C. Wash in PBS for 3 X 5 min.

D. Develop overnight in reusable Protogold solution.

8. After two hours the samples were washed in Tween 20 transblotting solution (TTBS) for 3 X 5 mins and left overnight in the final wash.

9. The primary antibody was made up to a dilution of 1:100 with TTBS, i.e. 200µl serum : 20mls TTBS. One gel was treated with anti-KLH serum while the other was treated with normal rabbit serum (NRS) which acted as a control as the donor rabbit would not contain any antibodies to haemocyanin. The gels were incubated for four hours on an agitator.

10. Both gels were then washed in TTBS for 3 X 5min.

11. The secondary antibody was applied at a dilution of 1:2000, i.e. 10µl serum: 20mls TTBS and incubated for two hours. In this case the secondary antibody was

a general enzyme conjugated goat-anti-rabbit antibody (conjugated with horseradish peroxidase). This recognised any antibodies previously produced by a rabbit, bound to them and was detected by the ensuing enzyme-substrate reaction.

12. The gels were washed in TTBS for 3 X 5 min.

13. They were incubated in the enzyme substrate solution which allowed the visualization of any proteins present via the binding of the enzyme conjugated secondary antibody with its appropriate substrate.

Enzyme-substrate complex:

20mg of 1, 4 chloronaphthol dissolved in 4ml of methanol

20ml of warmed TBS.

10µl hydrogen peroxide (as the antibody was conjugated with horseradish peroxidase.)

14. These were mixed in a small dish and the NCP's allowed to incubate for 5-15 minutes in the dark to let the protein bands develop.

15. The NCP's were washed in distilled water and then photographed along with the known molecular weight markers, as soon as possible as the colour degenerates very quickly.

N.B. See appendix for list of buffers used.

6.3 RESULTS

6.3.1 Location of Mites Feeding *in situ*.

Despite repeated sectioning of highly infested snail lung, this part of the study was unsuccessful in obtaining evidence for the mite feeding in the pulmonary cavity. Many sections were obtained of the lung and although they demonstrated the structure of the lung no mites were found feeding there. No evidence for the presence of feeding tubes (stylostomes) as reported by Jones (1950b) and Baker (1967, 1970b) was obtained. Some structural changes were however observed, these will be dealt with in Chapter 7.

6.3.2.1 & 6.3.2.2 Raising anti-sera and testing for cross-reactivity with *Helix spp.*

The anti-sera were successfully raised and then used in an adaptation of the method described by Ouchterlony (1958) for an immunodiffusion assay. When the agar plate was left overnight and examined the next day with a diffused light source it was possible to see that arcs had developed in the area where the KLH antibodies had reacted with the snail haemolymph and the KLH antigens. The KLH antigen in barbitone buffer gave a stronger result than the one in PBS. The presence of these arcs indicated that despite being haemocyanin from a different species there was a reaction between the antibodies raised to it and the haemocyanin of *H. aspersa*, and therefore the antiserum was suitable for use in further study.

6.3.2.3 Sodium dodecyl sulphate (SDS) gel electrophoresis (PAGE).

This was carried out using an adaptation of the methods used by Studier (1973). Gels were successfully run to assess all the test samples and these were all tested in

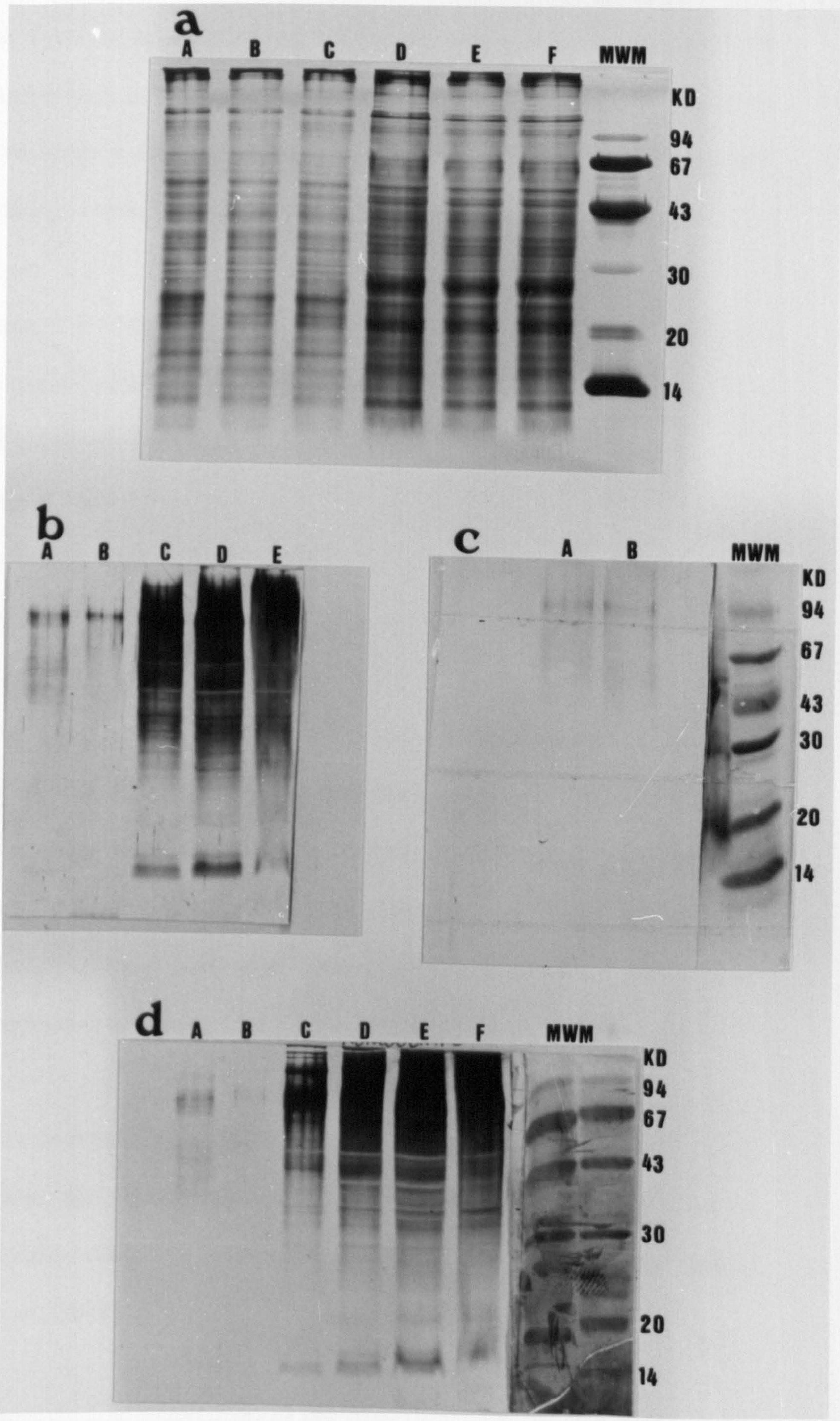
PLATE 10

a). Photograph of SDS PAGE dilution assay of *Helix aspersa* homogenate. A, B & C = 1/50 dilutions and D, E & F = 1/20 dilutions. MWM = high molecular weight markers. All gels and Nitrocellulose papers shown actual size.

b). Western Blot NCP, non-native samples, A & B = *Riccardoella limacum* homogenate 1/20 and 1/50 dilutions respectively and C, D & E = *H. aspersa* homogenate 1/10, 1/20 and 1/50 dilutions respectively.

c). Photograph of SDS PAGE dilution assay of *R. limacum* homogenate. A = 1/20 and B = 1/50 dilution.

d). Western Blot NCP, native samples (gel run in conjunction with (b) above), A = *R. limacum* homogenate 1/20 dilution, B = *R. limacum* homogenate 1/50 dilution, C = *H. aspersa* homogenate 1/100 dilution, D = *H. aspersa* homogenate 1/50 dilution, E = *H. aspersa* homogenate 1/20 and F = *H. aspersa* homogenate 1/10 dilution.


a
A
B
C
D
E
F
MWM
KD
94
67
43
30
20
14
b
A
B
C
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E
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MWM
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A
B
C
D
E
F
MWM
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94
67
43
30
20
14

the native and non-native forms. A slight brown staining of the gels indicated that there was a reaction taking place and that proteins were present but as at this stage no antibodies were used, it was not possible to say what those proteins were (Plate 10a). The bands in the channels containing the known molecular weight markers stained darkly brown. Throughout this study the markers used were as follows:

Cytochrome C = 14kD

Soybean trypsin inhibitor = 20kD

Carbonic anhydrase = 30kD

Ovalbumin = 43kD

Bovine serum albumin = 67kD

Phosphorylase b = 94kD

The results from samples in the native and non-native forms were identical, the degree of staining was the same as was the position of the proteins in the gel. The dilutions of all the samples run appeared to be adequate except for the whole snail homogenate, this one needed to be diluted somewhat. For future samples it was decided that dilutions of 1:20 and 1:50 would give better results and would not be obliterated by over staining.

6.3.2.4 Western blotting of SDS PAGE.

Once the basic SDS PAGE had succeeded for the test samples, and the best dilutions were established (Plate 10a & 10c), the gels were blotted using an adaptation of Towbin *et al.* (1979).

This method produces a record of the experiment on the NCP and can be photographed and studied at a later date. Care must be taken not to mix up the

molecular weight markers from one gel with another as slight variations in the consistency of the gels make them run at different rates, varying the distance between the markers. Again the native and non-native samples gave the same results and the halves treated with NRS, instead of anti-KLH sera, were all blank showing no reaction of "normally" produced rabbit antibodies with any of the proteins commonly found in snails, *R. limacum,* snail mucus or *T. urticae.* However in the gels that had been incubated with the anti-KLH sera definite darkly staining bands appeared in the regions corresponding to 94kD, when compared to the molecular weight markers, on all the samples of *R. limacum* homogenate and also snail homogenate (Plates 10b, 10c, & 11). The reaction in the snail gave large "smeared" bands which spanned a large downwards portion of the gel indicating that the *Helix spp.* haemocyanin had dissociated into different molecular weight constituents and had a strong reaction to the antibodies. The bands which developed for *R. limacum* were very small, clear and discrete, while the channels containing *T. urticae* homogenate were totally unaffected by the antibody reaction, as were the channels containing the snail mucus (Plates 10 & 11).

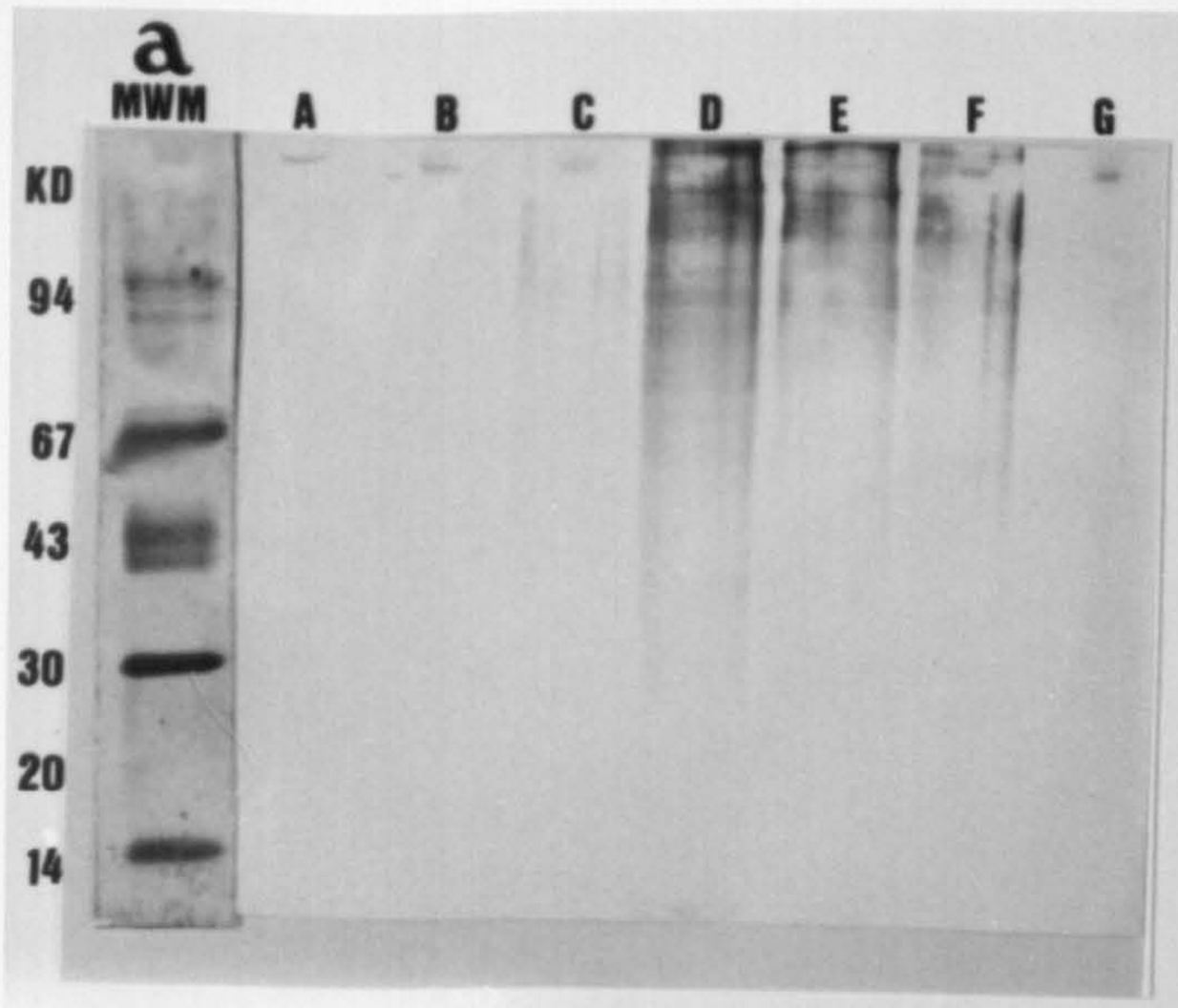
PLATE 11

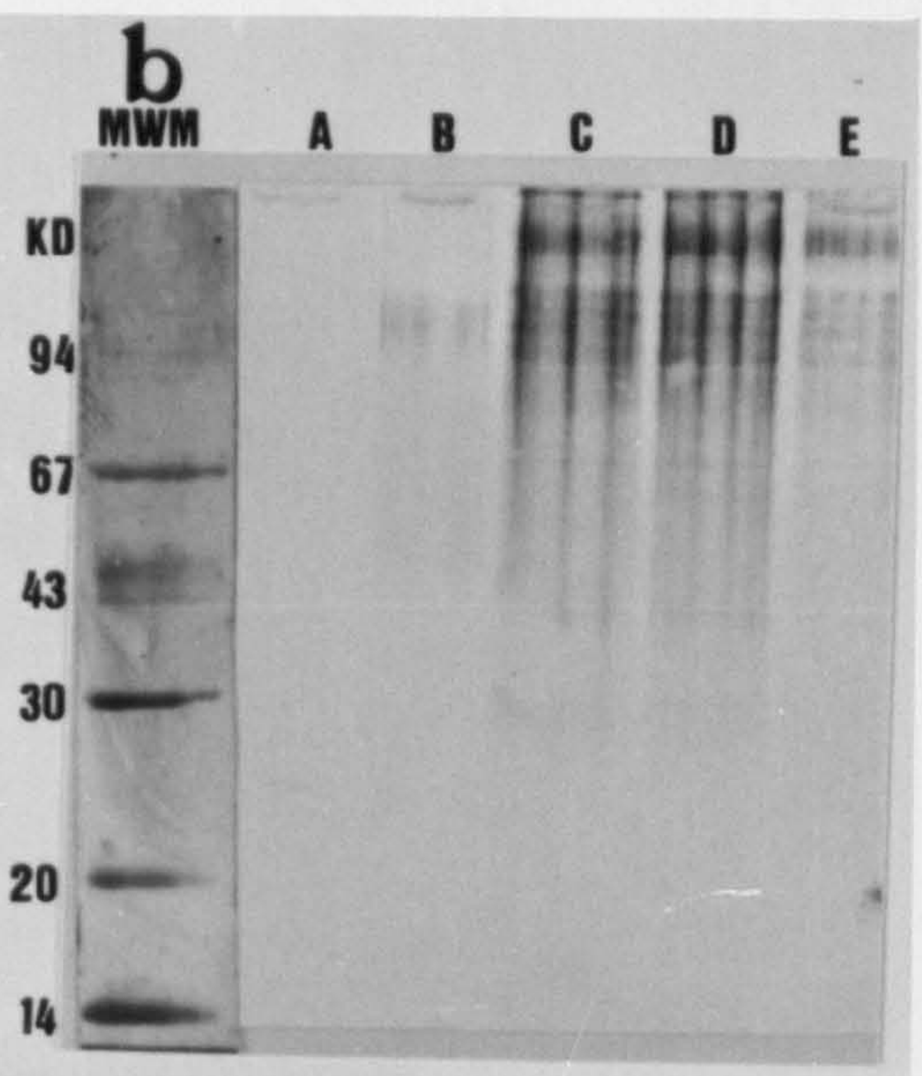
a). Western Blot NCP, native samples, A = *Tetranychus urticae* (control) homogenate 1/20 dilution, B = *T. urticae* (control) homogenate 1/50 dilution, C = *Riccardoella limacum* homogenate 1/20 dilution, D = *Helix aspersa* homogenate 1/20 dilution, E = *H. aspersa* homogenate 1/50 dilution, F = *H. aspersa* homogenate 1/100 dilution and G = blank. MWM = high molecular weight markers. All gels and NCP's shown actual size.

b). Western Blot NCP, non-native samples run in conjunction with (a) above, A= *T. urticae* homogenate (control) 1/20 dilution, B= *R. limacum* homogenate 1/20 dilution, C = *H. aspersa* homogenate 1/20 dilution, D = *H. aspersa* homogenate 1/50 dilution and E = *H. aspersa* homogenate 1/100 dilution.

c). Western Blot NCP, native samples, A = *T. urticae* homogenate (control) 1/20 dilution, B = *R. limacum* homogenate 1/20 dilution, C = *H. aspersa* homogenate 1/10 dilution, D = *H. aspersa* homogenate 1/20 dilution, E = *H. aspersa* homogenate 1/50 dilution, F = *H. aspersa* mucus undiluted and G = *R. limacum* homogenate 1/10 dilution..

d). Western Blot NCP, native samples, A = *R. limacum* homogenate 1/20, B = *R. limacum* homogenate 1/10, C = *T. urticae* homogenate (control) 1/10, D = *H. aspersa* mucus undiluted, E = *H. aspersa* homogenate 1/20, F = *H. aspersa* homogenate undiluted and G = *H. aspersa* homogenate 1/5.


a
MWM
A
B
C
D
E
F
G
KD
94
67
43
30
20
14


b
MWM
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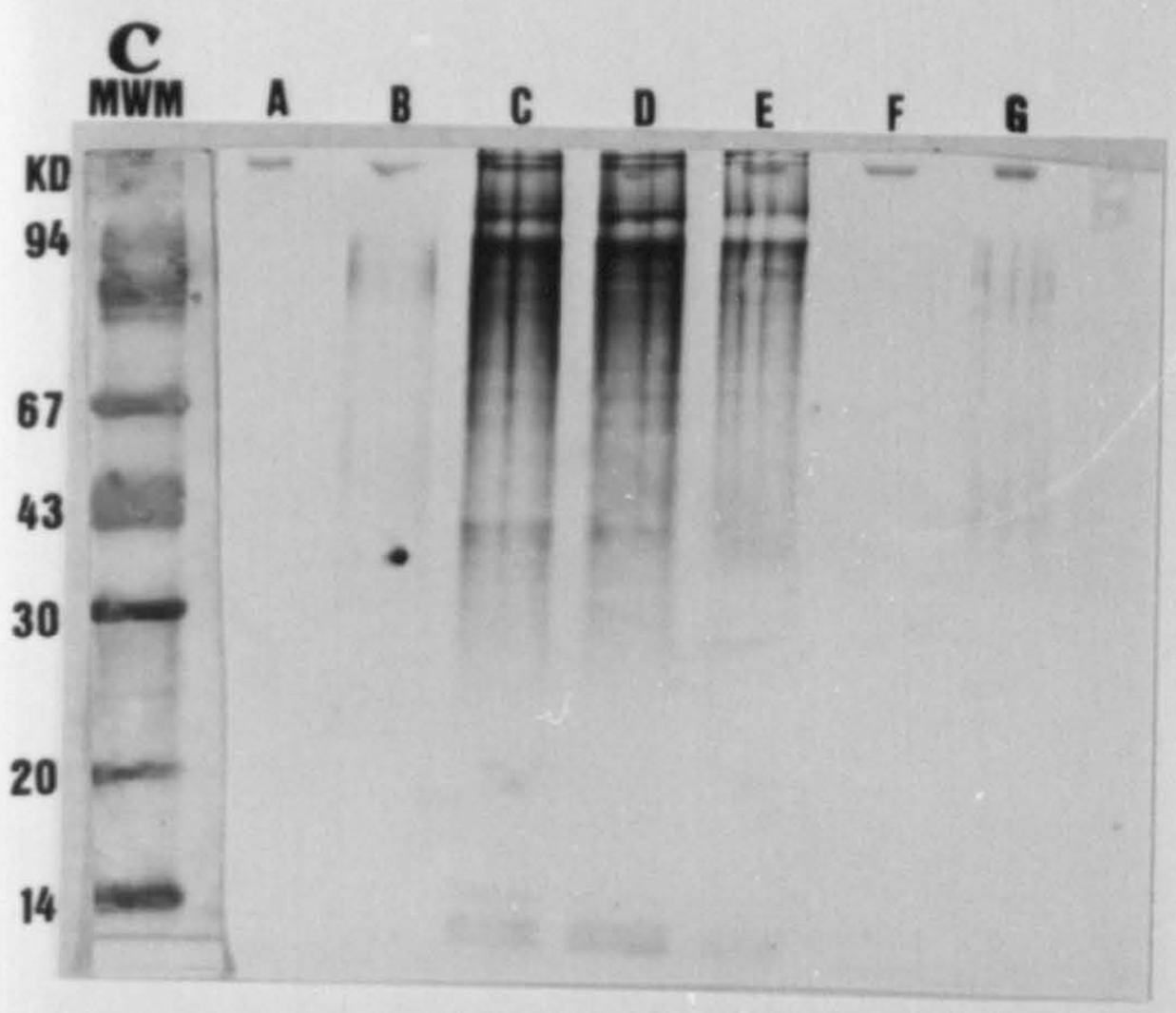
c
MWM
A
B
C
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KD
94
67
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d
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C
D
E
F
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MWM
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6.4 DISCUSSION

The results of the immunological survey into whether *R. limacum* feeds on the haemolymph of *H. aspersa* indicated that it probably does. The bands which regularly appear in every test homogenate of *R. limacum* in the high molecular weight, 94kD region show that a protein in the mite is reacting with an anti-KLH antibody produced by the rabbit. This antigenic substance is not necessarily KLH as this rabbit would also have produced any number of other antibodies to substances to which it had been exposed during the immunisation period. If any of these other antigenic substances were present in the mite then a reaction would occur but KLH is the most likely candidate.

A way of making the results of this experiment more conclusive would be to raise antibodies specifically for *H. aspersa* haemocyanin and purifying the antisera produced so that it contained only those antibodies raised against *H. aspersa* haemocyanin. This however is very time consuming and also very expensive, so in this instance the only way of eliminating the effects of some of the other nonspecific antibodies was by carrying out the control with NRS. This replaces the anti-KLH sera with the normal range of antibodies found in rabbits, when this is compared to the specific serum any differences in the appearances of bands on the NCP's is due solely to the fact that rabbit was stimulated with KLH antigen. The fact that both mucus and *T. urticae* homogenate also showed no reactions to any antibodies in the KLH anti-sera or the NRS indicated that the anti-serum raised for KLH was fairly specific in the first place, as neither mucus nor *T. urticae* share any antigenic substances with the rabbit. A compound found in one mite would also probably be found in the other species, especially if it was so common that even a rabbit not stimulated to produce antibodies against it nevertheless contained antibodies enabling

a reaction to take place. This is definitely not the case as none of the gels revealed any reactions at all in the channel with *T. urticae* homogenate. Gas transport in mites does not of course involve haemocyanin or any other respiratory pigments and so the mites contain none. Therefore, the only difference between the mites is in the food of both species; plant juices in *T. urticae* and apparently snail blood in *R.limacum*. The negative result for snail mucus confirms that it contains no haemocyanin and the snail is therefore not obtaining haemocyanin from the mucus. Mucus cannot be eliminated as a source of food on the strength of these results, to do that would require an antibody to mucus to test for its presence within the mites. Mucus is in part an ultrafiltrate of the blood with the addition of a complex carbohydrate (Runham & Hunter, 1970) and as such it would not be possible for it to contain any haemocyanin so the bands present on the gels are not due to mucus. Haemocyanin does however dissociate under certain conditions as already mentioned, one of these being a pH less than 9.8 (Markl *et al.*, 1991).

The natural molecular weight of haemocyanin is in the range of 900 to 20kD which is far too big to be shown up on the type of gel used. However the electrophoresis process causes the polymer to break down into its lighter constituent parts, and the band that appears at 94kD is most likely one of these parts. The streaking effect seen in the samples of snail homgenate was caused by the lighter elements of haemocyanin reacting with the antibodies. This did not occur in most of the *R.limacum* samples (despite the presence of the 94kD band) due probably to a concentration effect, i.e. the mite contains extremely small quantities of haemocyanin and the paler lower part of the streak does not show up.

When looked at in context with published work there does not seem to be any doubt at all that *R. limacum* feeds on blood. Gastropod molluscs have blood systems

comprising arteries, veins and haemocoelic spaces that are in close contact with the organs and the blood therefore contains many useful food substances. The lung contains a well developed network of veins thus providing a ready food supply for any mites living inside the pulmonary cavity. Several mite species are known to like living in similar conditions, for example *Acarapsis woodi* pierces the tracheal wall of the honey bee and feeds on the haemolymph (Evans, 1992). Not all the members of the Ereynetidae are blood feeders but it is clear that they do not really possess the equipment for dealing with mucus (Baker, 1973) which is most commonly mistaken as the food source in several species. The solution to this problem lies perhaps in the addition of saliva to the mucus to dilute it sufficiently to enable the mite to ingest it. Alternatively the mucus on the surface of the lung may be of a liquid enough consistency to make it possible for *R. limacum* to feed on that mucus in addition to blood. The internal anatomy of *R. limacum* is similar to that seen in other known blood feeders, particularly the musculature of the pharynx. This closely resembles that of *T. autumnalis* in which the muscles are arranged radially (Jones 1950b) and can cause a powerful sucking action. The "salivary glands" at the anterior of the mite are the most likely source of digestive juices that help in the operation of penetrating the cell walls lining the lung and also in the formation of a tissue canal (if one is formed) providing passage for both food and saliva (Baker, 1973). These glands are more extensive than those found in the related mite *X. africanus* that has previously been established as a blood feeder and also has a similar digestive system to *R. limacum* (Baker, 1971). The digestive system in *R. limacum* is well suited to a blood feeding way of life as like many other known blood feeders it consists of a large sack-like pair of caeca that can slowly expand when filling with blood. *X.africanus* has also been shown to have large quantities of haematin, which is a byproduct of the breakdown of haemoglobin, in the gut and this is most likely to be the identity of the black substance noted by Lawrence (1951) in the gut of *R. eweri.*

Baker (1967) also confirmed that the nucleated cells found in the gut of *R. limacum* (= *R. oudemansi*), were the same as those found in the heart and connective tissue of slugs. These were in fact amoebocytes and were the likely source of food for this mite. These cells disappeared after several days without food, suggesting that they had undergone digestion.

Although the mouthparts of *R. limacum* are not as highly modified for blood feeding as in other mite taxa, they are certainly good enough to achieve a degree of attachment which can then be enhanced by the addition of saliva from the accessory glands. The environment in which they live is well suited to blood feeding. The blood vessels of pulmonates do not have a liquid tight lining (Runham & Hunter, 1970) and therefore even relatively minor wounds on the surface would cause a leakage of blood from the vessel. The very thin epithelium of the lung surface (see Chapter 7) also provides easy access to the blood within the vessels and it is not really surprising that no permanent tissue canal was found during the study as there is very little room to accommodate one and certainly no apparent need. Once the blood vessel has been ruptured it would be relatively simple for the mite to ingest blood; the musculature of the pharynx is well suited for the task of withdrawing fluids from the snail and the gut is capable of holding a substantial amount of blood. The question then arises of what effect, if any, the regular removal of blood has on the snail.

CHAPTER 7

LONG-TERM EFFECTS OF *RICCARDOELLA LIMACUM* LIVING IN THE PULMONARY CAVITY OF *HELIX ASPERSA*

7.1 INTRODUCTION

Since *Riccardoella limacum* feeds on the haemolymph of the host, the question arises of what effect this has on the snail when relatively large populations of mites occur in confined conditions, such as would be found on a snail farm. It has already been shown in Chapter 5 that the mite is readily able to spread to a large proportion of the population of snails in a short space of time and lay considerable numbers of eggs. Even if they did not derive their nourishment from the snails, the actual presence of a large number of mites in the lung would surely cause a physical problem to the host.

As with the problem of whether the mite feeds on blood or not, there has been much discussion among workers as to whether there is a deleterious effect on the snail. Some believe that there are no ill effects, Turk & Phillips (1946) say that they have never observed any abnormalities in the slug that could be attributed to the presence or activities of the mites and therefore conclude that they do not affect the host. They also pointed out that the slug does in fact possess the capability of removing the mites by releasing copious quantities of sticky mucus in which the mites become trapped and then "sneezing" them out of the pneumostome. Mites have been found

by these workers embedded in the mucus secreted when the slugs are irritated. Lawrence (1951) working on the related species, *Riccardoella eweri* states that *R. limacum* lives on the host snails without appearing to produce any harmful effects. The workers André & Lamy (1930) were also certain that the snail derived no ill effects from the infestations of mites. Indeed they go as far as to say that the relationship is a purely commensal one and the snail does not suffer at all.

This scenario seems unlikely, as Baker (1970b) states "there is no doubt that in large numbers they cause considerable loss of blood in slugs", the loss of similar amounts of blood would be a serious drain on the resources of the snail and therefore reduce the efficiency of the snail farm. The act of simply attaching to the host tissue in order to obtain nourishment surely affects the snail. Although mites living in such a sheltered environment do not need to possess elaborate attachment organs, it is likely that the ambulacral claws or small chelicerae are used (Fain, 1969). If, as is suspected by Baker (1970b, 1973) and Jones (1950b), a feeding tube or stylostome is formed then considerable cell disturbance must occur within the host tissues and this structure is thought to persist in the host tissue even after the mite has finished feeding and detached itself (Evans, 1992). A feeding tube requires the injection of a considerable quantity of saliva which produces a localized reaction. The results of such irritations as piercing and injection of a foreign substance are likely to cause some lasting effects, especially if they are over the long term. The effect of mites on snails has been studied in the past by Oldham (1931) working on *Arianta arbustorum*. He discovered that mites living on these snails in nesting boxes caused total mortality in some of the boxes while in others growth was irregular, fecundity lessened and badly deformed shells a regular occurrence. This mite was not *R. limacum* as it was apparently introduced to the boxes while living freely in some soil, which means that it was probably a species of Ereynetes or Ospreynetes

that are nonetheless still closely related to *R. limacum.*

The adverse effects of the above mites on the shell formation of snails suggests that looking at the shell could be a good primary indicator of the snail's general well being. It is known in other species that in times of hardship, a permanent record is often left in the shell in the form of thickened bands. Ridges on the shell form after times of food shortage in *Nucella lapillus* when feeding activities return to normal (Crothers, 1985). In *Ampullarius glaucus* the shell thickness depends on the rate of calcium deposition which falls considerably in times of starvation, and after ten days with no food the deposition of calcium has fallen by 50% (Zischke *et al.*, 1970). Pollard *et al.* (1977) working on *Helix pomatia* note that the shells of damaged snails quickly repair themselves but again a permanent record remains of the event visible as thickened ridges. Shells may also show ridging from periods when the snail went into summer aestivation, possibly due to adverse weather conditions such as drought.

Helix aspersa are fairly easy to keep in the laboratory once a culture has been established. However it must be noted that the weight of individual snails is subject to considerable variation even between snails from the same clutch of eggs (Smith, 1966; Sampelayo *et al.*, 1990; Pollard *et al.*, 1977). It is also possible for the weight of a snail in the wild to vary considerably from day to day by as much as 50% of the body weight (Howes & Wells, 1933) and by 10% in farmed animals (Klein-Rollais & Dauzan, 1990) Snails are known to hibernate when their body weight is reduced and the water content is low possibly due to summer drought conditions, hence the reason many snails aestivate in the summer months. However Lucarz & Gomot (1985) state that weight measurements are reliable indicators of growth, even considering the natural variation within a population, although weight is not

indicative of age.

Besides weighing the animals to assess growth, maturation can be assessed by examination of the reproductive system. In general this is subject to less natural variation than the weight and is very similar in a number of species, being therefore readily comparable. Descriptions of the reproductive organs of other snails and slugs have been supplied by Duncan (1958) working on *P. gyrina,* Rigby (1963) for *Oxychilus cellarius*, Kugler (1965) working on the slug *Philomycus carolinarus,* Smith (1966) for the slug *Arion ater*, Runham & Laryea (1968) on *Agriolimax reticulatus* and also Breckenridge & Fallil (1973) working on the snail; *Achatina fulica.* Watts (1952) working on the slug *Arion subfuscus* remarks on the similarity of the reproductive system with *H. aspersa.* From the above work it is possible to build up a general picture of the main reproductive structures of *Helix spp.* The most important parts with which this study is concerned are the gonad and the albumen gland as these are easily found and changes in their structure are readily visible when examined histologically. The gonad consists of several creamish/white lobes made up of acini with a germinal epithelium, embedded in the digestive gland (Breckenridge & Fallil, 1973). Within all acini can normally be found both male and female gametes in all stages of development (Smith, 1966; Runham & Laryea, 1968). In young snails the gonad consists of a compact mass of undifferentiated cells which later develop into the characteristic acini. As sperm mature they fall into the lumen of the acinus and the ova remain attached to the acinar wall, (Rigby, 1963). The albumen gland is the last part of the reproductive system to develop (Rigby, 1963). It varies in colour, size and consistency from small and cream coloured in immature animals, to large, yellow and quite brittle when sexually mature, and small, brown and degenerate over the winter months after egg

laying (Duncan, 1958). The purpose of the albumen gland is to secrete a nutritive substance that coats each oocyte and nourishes the embryo until hatching. During these experiments, even if there is a large variation in the weights and body size of individual snails, the stage of maturation of the reproductive tracts of a similar aged group of animals should be comparable unless they have been affected by the experimental factors.

Another important part of the snails that would be expected to suffer and cause general ill health would be the lung surface. Although previously (Chapter 6) examination of the lung of mite infested snails revealed no obvious damage such as puncture marks, abrasions or the presence of tissue canals, a more thorough histological survey was thought to be necessary.

Several workers have looked at the structure of the lung, namely Pohunkova (1967) working on *Helix pomatia* and more recently Maina (1989) looking at the lung of the tropical terrestrial slug, *Trichotoxon copleyi*. Maina reports that the lung of *T. copleyi* is very similar to that in *H. aspersa*: it has all the features of an efficient gaseous exchange system, i.e. large surface area, a good blood supply and a thin blood/gas barrier. He describes the blood supply in this particular species as being like a sheet of blood with only the basement membrane separating cells from haemolymph in smaller vessels. The respiratory surface consists of a cuboidal or squamous epithelium covered by a layer of mucus to prevent drying out, which is secreted by numerous goblet cells which are present there. Underlying the respiratory epithelium and blood supply is a layer of connective tissue with collagen fibrils and pigment cells that form the roof of the lung cavity, (Pohunkova, 1967). If this is taken to be the normal condition of the lung in *H. aspersa*, then the presence of a number of mites ought to be detectable by some change away from the

normal. Therefore the body weight, development of the sexual characteristics and the state of the lung should provide a good indication, when taken together, of whether infestation by mites has any long-term lasting effects on snails.

7.2 MATERIALS AND METHODS

To assess the long term affects of different numbers of mites on snails the following experiments were carried out and the physical effects of mite infestation assessed on the strength of the following characteristics:

i. Final wet weight of the snail including the shell.

ii. Shell dimensions; height and diameter taken to be those represented in fig. 7.1.

iii. Shell condition; smoothness, colour and visible damage, also whether the characteristic rim of sexually mature animals is present.

iv. Wet weight of whole albumen glands; Experiment B only.

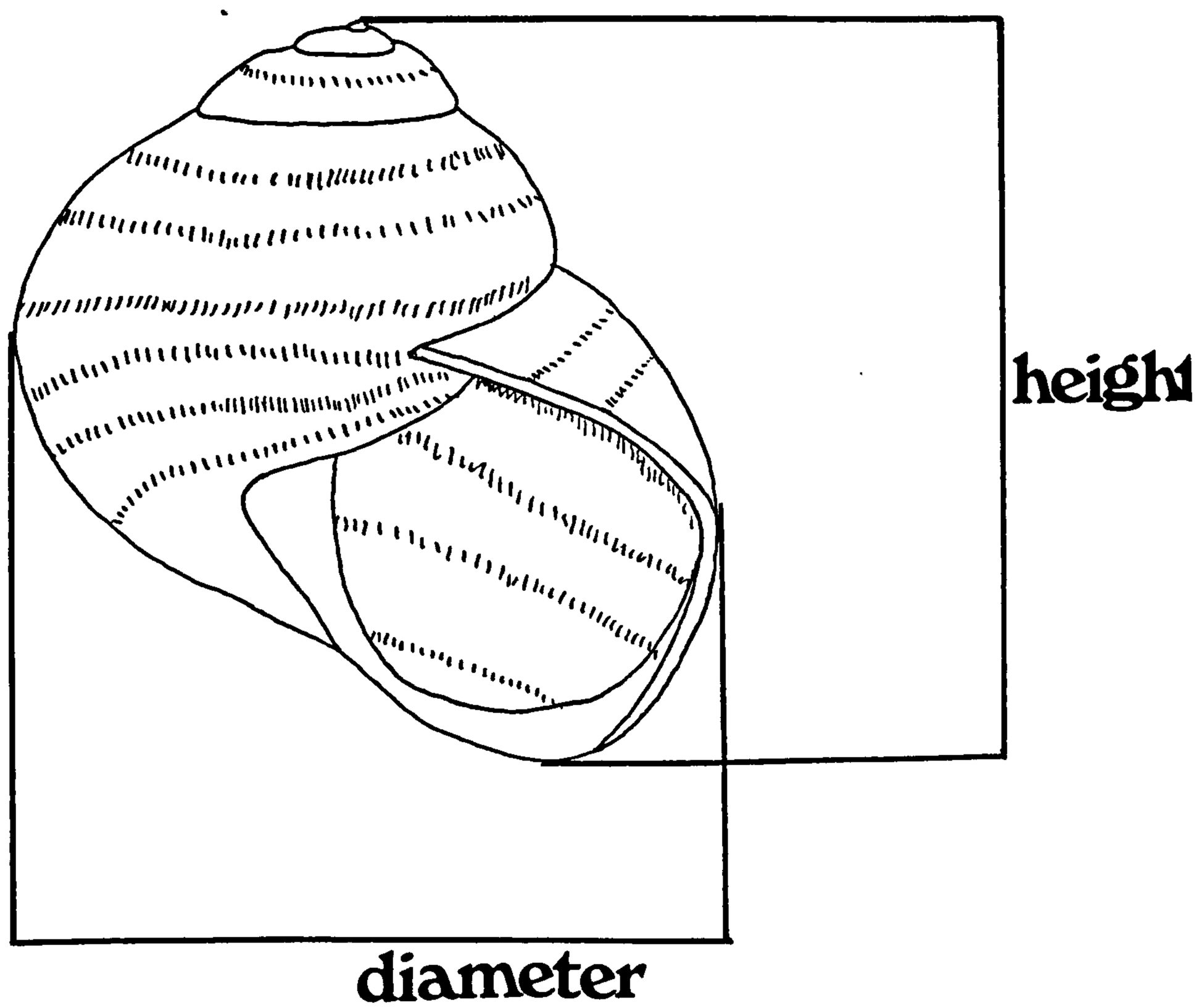
v. Histological observations on the gonad, albumen gland and lung of all groups.

vi. Presence of mites feeding in the and general condition of the lung.

7.2.1 Experiment A: Effect of High and Low Incidence of Mites on Isolated Snails.

As there appears to be some debate in the literature concerning the merits or otherwise of keeping snails in a high density cage, it was decided that in this experiment the snails would be kept separately. This also allowed some control over the mite infestation as they were not free to wander off to other hosts. It was not always possible to obtain a large number of mites from the culture and the experiment was set up using immature (approximately eight weeks old) snails over a period of weeks, as and when mite stocks became available.

Figure 7.1 : Measurement of shell dimensions.



The equipment consisted of sixty plastic plant propagators, measuring approximately 10cm x 7cm x 7cm with ventilation holes pierced into the lids. Each propagator was supplied with a small square of the same high absorbency gamgee wadding as used in the laboratory mite/snail culture. This provided a moist environment and a source of fluid for the snails to keep themselves at optimal hydration, thus preventing aestivation. Commercial snail food was placed in the propagator and removed when they were cleaned. Cleaning took place approximately every five days to prevent the build up of waste and mucus. Although this has been stated not to inhibit growth, it does attract flies. The uneaten food also encouraged the growth of fungus. Too frequent cleaning tends to damage shells as a result of too much handling. The propagators were placed on a tray and kept in an incubator at 20-25 °C with a 12 light:12 dark cycle.

Immature snails came from the culture of "clean" snails obtained on a regular basis from the snail farm. Sixty snails of about eight weeks in age and all with shell diameters of approximately 1cm were weighed then placed in individual boxes. The snails were divided into three groups of twenty animals each and mites transferred to them as follows:

1. High incidence of mites; twenty adult mites added initially to each snail, followed by a "top up" of ten mites a month later.

2. Low incidence of mites; five adult mites added to each snail initially, followed by a "top up" of five more a month later.

3. Control group; snails with no mites.

The snails were cared for as previously described. Mites were transferred to as many

snails as possible at one time and as little delay as possible incurred before setting up the rest. All snails were weighed at the same time and the general condition of the shell was assessed. The experiment was carried out for nine months to ensure that the snails had time to achieve sexual maturity. Under normal circumstances in the controlled environment of the laboratory, this would take about seven months, so any snails still sexually immature after nine months can be deemed to be abnormal. After nine months the snails were weighed for a final time, the shell heights and diameters measured with modified vernier calipers as described by Goodfriend (1983) and then the animals were killed and dissected in the manner described previously. All incidences of mites were noted and snails from all three groups were further divided into two groups to be treated as follows:

1. Whole animals (without shells) were wrapped in aluminium foil and frozen at -70 °C until it was convenient to carry out biochemical analyses (see Chapter 8).

2. Gonad, albumen gland and lung tissue was removed and prepared for light microscopy as previously described for mites. Because of the size of the pieces of snail tissue, penetration times for all solutions were doubled (except for initial fixation in Heidenhein's Susa, which remained 24 hours).

For infested snails, every attempt was made to obtain lung samples that had mites attached to assess whether there was any visible histological damage and to look for evidence of mite feeding. The development of the reproductive system was also assessed.

7.2.2 Experiment B: Effect of high incidence of mites on communally reared snails.

Snails were infested with mites as in Experiment A but using only two groups:

1. High incidence of mites.
2. Control snails with no mites.

Snails were kept communally in 30cm x 15cm x 10cm plastic boxes under the same conditions as for the previous experiment. One object was to compare weights, shell size and histology with the snails in the previous experiment in which they had been kept individually. Because of the limited time and also the fact that a snail farmer would normally sell his animals on at about five months, Experiment B was only run for five months as opposed to nine in Experiment A. The snails were weighed monthly as before and treated in the same way at the end of the experiment. The weights and histology of both groups were compared and also compared to similarly aged specimens from the previous experiment. In addition, the individual weights of the albumen glands were noted before any further treatments were carried out on the tissues.

7.3 RESULTS

7.3.1 General Observations.

It was noticed snails with high mite infestations had a tendency to enter periods of dormancy (they adhered to the side of the box with dry mucus). These periods often lasted for the whole of the five days between cleaning. At any one time approximately 50% of the animals would be dormant, though it was not always the same individuals. Snails with no mites always remained active and ate well while those in the infected groups often left food uneaten. In future studies it would be interesting to employ the use of video equipment to supply a record of the movements of snails throughout the study. As snails tend to be nocturnal feeders it would also remove the subjective element of observing them only during the daytime.

7.3.2 Experiment A.

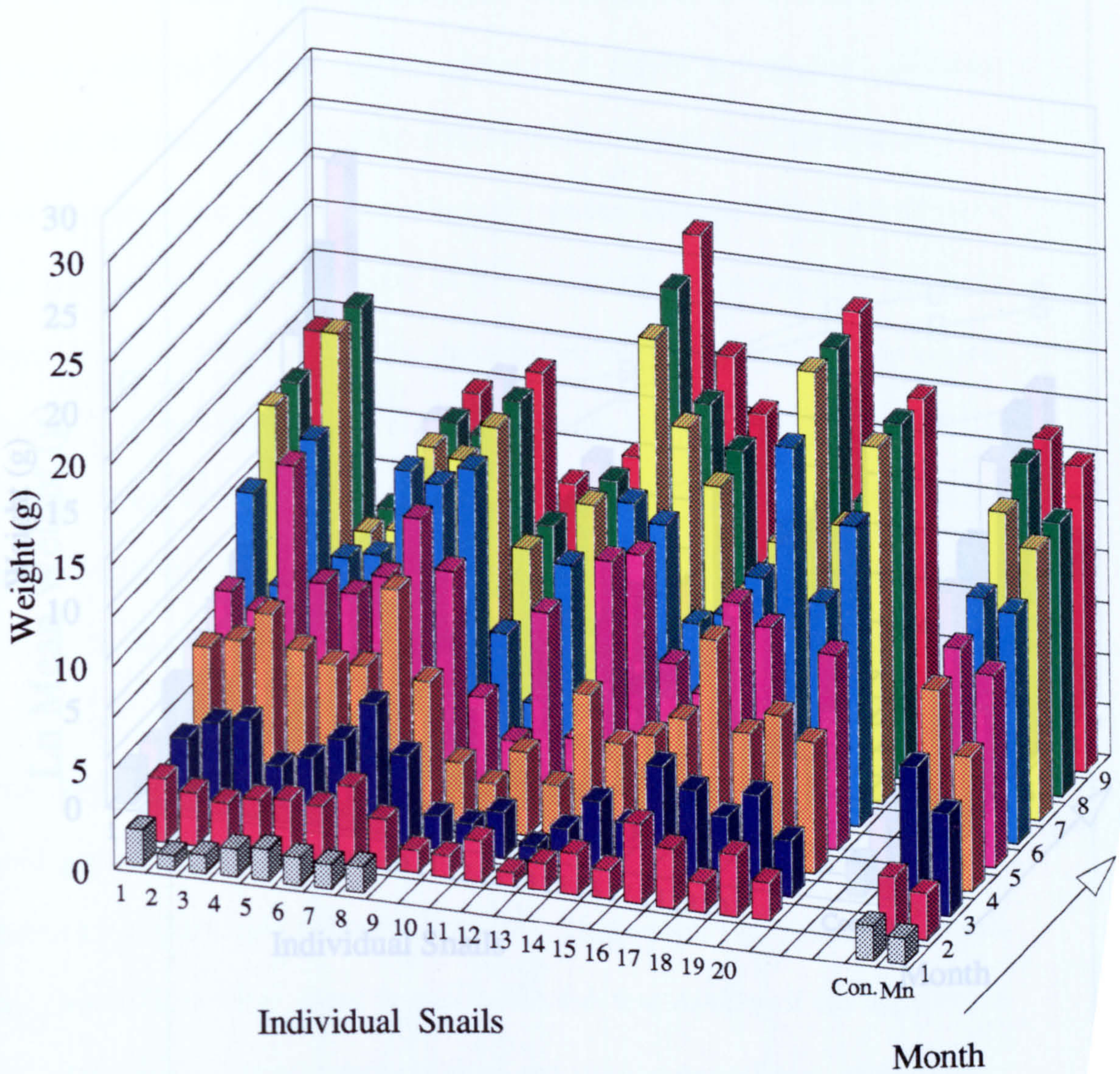
7.3.2.1 Final weights.

The average monthly weight of snails in each group are given in Table 7.1. These data are also displayed in Graphs 7.1 and 7.2. A direct comparison between the average monthly weights of all of the three groups can be seen in Graph 7.3. The final average weights, shell heights and shell diameters for snails in all the groups can be seen in Table 7.2. From these results it can be seen that there is a substantial difference between the weights of the high incidence group and those of the other two groups (see statistical analysis), with the control group weighing the most by a small margin. The average final weight of snails in the control group is practically double the weight of snails in the high incidence group of the same age.

Table 7.1 : Mean weights of all groups of snails

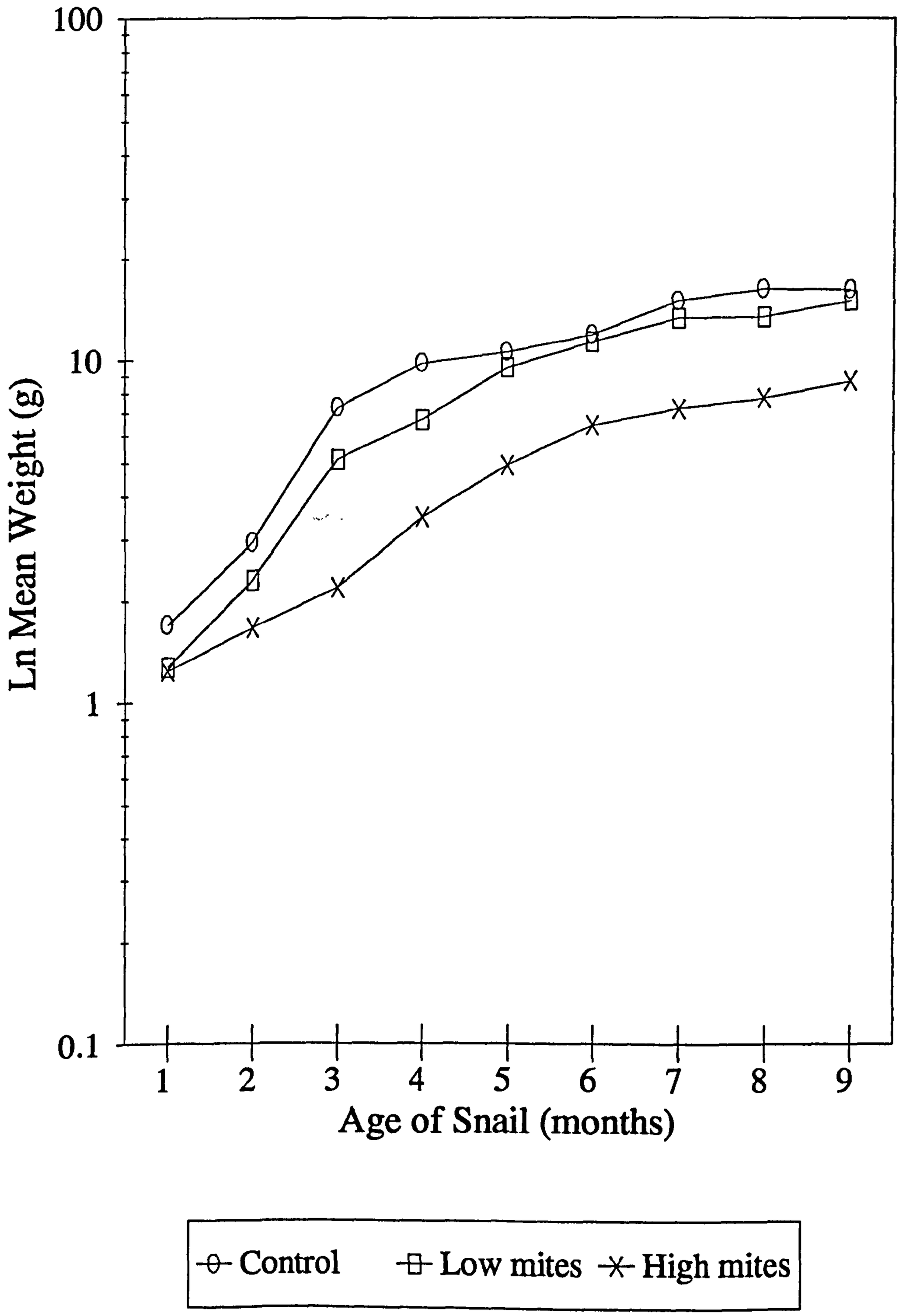
	Experiment A			Experiment B	
Months after start of experiment	**High Incidence group (g)**	**Low Incidence group (g)**	**Control group (g)**	**High Incidence group (g)**	**Control group (g)**
Initial	**1.24**	**1.27**	**1.69**	**0.93**	**1.16**
1	1.66	2.29	2.94	1.74	3.92
2	2.18	5.10	7.23	3.02	6.31
3	3.47	6.67	9.81	3.36	7.44
4	4.91	9.54	10.67	**4.66**	**9.13**
5	6.41	11.39	11.92		
6	7.20	13.36	15.01		
7	7.76	13.47	16.23		
8 (final)	**8.72**	**15.06**	**16.24**		

Graph 7.1- Low Mite Infestations
(monthly weights for 9 months)



Note:
Con.- Control
Mn. - Mean value for that month

Graph 7.3- Comparison of Snail Growth.
High and Low Infestations (Exp.A)



7.3.2.2 & 7.3.2.3 Shell characteristics.

The final shell dimensions can be seen in table 7.2. The shells of the control group were to all appearances "normal" in that they were smooth and shiny with a good colour. They exhibited no damage from breakages and were quite strong, the final dimensions indicated that although on average the low mite group had slightly larger shells than the control group, the difference was negligible when compared to the difference exhibited between the low and control groups and the high incidence group. All shells in the control group and low incidence group had the thickened rim around the growing edge indicative of sexually mature animals. The shells of snails in the high incidence group tended to be very brittle and weak, they regularly incurred damage from falling from the sides of their containers and the repairs resulted in irregular ridges. The shells of these snails did not appear shiny, were not so rich in colour, tended to be smaller and generally did not look as healthy as the control animals or those in the low incidence group. None of these individuals possessed the thickened rim to the shell.

7.3.2.4 Histological observations.

Control group snails: The reproductive organs in these snails were in the "normal" condition for a nine month old snail (Plate 12). These snails had obviously matured as they would have done naturally and in the last few months of the experiment several of the snails showed a pale coloured and swollen genital pore, characteristic of a sexually mature snail in breeding condition.

When 3μm sections of the gonad were examined using a light microscope, the appearance was also one of a normally mature snail. The germinal epithelium was intact and formed the boundaries of the acini without being at all swollen in appearance or having pulled away from the basement membrane. Various stages of

Table 7.2 : Final shell heights, shell diameter, and snail weights for Experiment A.

	Shell Height (mm)			Shell Diameter (mm)			Weight of whole snail (g)		
	HIGH	LOW	CON.	HIGH	LOW	CON.	HIGH	LOW	CON.
	25.05	33.55	26.25	32.95	41.90	35.05	8.190	16.989	16.004
	31.05	26.95	27.39	44.15	32.00	29.15	23.310	9.823	16.001
	18.25	23.42	25.69	25.65	31.69	39.00	4.510	10.359	17.235
	30.00	32.85	28.10	32.65	38.90	35.60	10.797	14.900	18.001
	12.95	29.05	25.10	23.15	41.80	29.70	5.320	16.337	16.868
	24.96	26.20	26.30	28.10	32.70	35.90	13.411	11.056	15.213
	24.70	25.60	27.75	28.40	25.65	33.00	4.301	12.811	13.429
	28.20	30.10	29.10	30.95	44.70	37.00	9.779	18.450	16.259
	9.55	25.99	31.00	22.55	36.90	39.54	3.211	15.768	15.630
	25.75	25.10	32.14	28.30	33.20	39.89	4.220	9.612	18.867
	26.40	25.73	-	22.30	34.70	-	5.610	12.231	-
	27.95	31.50	-	38.55	44.40	-	12.727	21.410	-
	26.15	28.05	-	30.80	35.40	-	7.612	14.539	-
	23.7	28.65	-	39.65	40.70	-	14.542	17.511	-
Mean	**23.90**	**28.05**	**27.88**	**30.58**	**36.76**	**35.38**	**9.11**	**14.41**	**16.35**

spermatogenesis could be seen clearly within the acini (Plate 12b & 12c). There were large quantities of mature sperm either in the lumen of the acinus or attached in a highly organised manner to nutritive Sertoli cells about to be shed to the lumen (Plate 12b & 12c). There were also many mature oocytes, showing the characteristic darkly stained nucleus and extremely obvious nucleoli, distributed evenly throughout the gonad (Plate 12d). There were vesicular connective tissue cells scattered between the acini of the gonad and these stained well with PAS indicating the presence of large amounts of glycogen (Plate 12 b).

The albumen glands of control snails were also sectioned although not without considerable difficulty due to the presence of large amounts of galactogen lending a very crystalline structure to the tissue. Invariably the sections shredded during the process of cutting but enough whole sections were taken to be able to say that the structure in these snails was normal (Plate 12a). The gland consists of many follicles each with a tiny lumen which connects to an albumen gland duct. The cells of the follicles secrete the nutritive substance which passes into the lumen and away to nourish the developing eggs. The cells making up each follicle in a mature specimen are extremely large with a small basal nucleus, no other features can usually be distinguished. They stain bright pink with the periodic acid-Schiff's (PAS) reaction indicating a large amount of carbohydrate, in this case known to be galactogen.

High and low mite incidence groups: The sexual organs of the animals in these two groups of snails were definitely less mature (Plate 13), those in the low incidence group were smaller than the controls whilst in the high incidence group it was not always possible to locate them.

In the high incidence group the albumen glands were in the worst cases only just

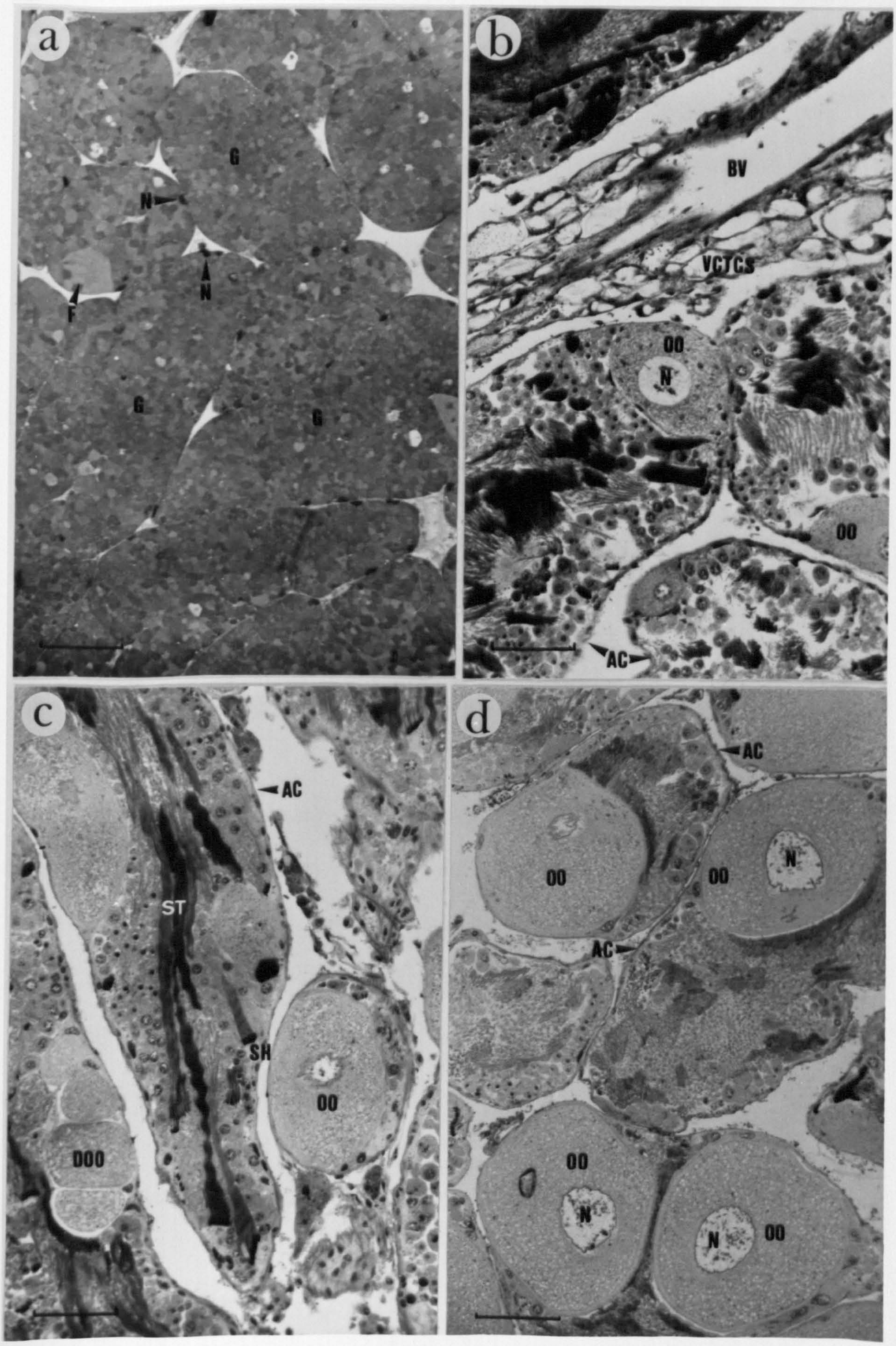
PLATE 12

a). Light microscope (LM) section (3μm) of "normal" albumen gland of *Helix aspersa* which had not been infested with *Riccardoella limacum* (control). Note nuclei (N) of cells occupy only a small proportion of the cytoplasm, central ducts to follicles hard to distinguish and marbled appearance of galactogen (G) deposits. Scale bar = 125μm.

b). LM (3μm) section of "normal" gonad of *H. aspersa* not infested with mites, taken in the region of a blood vessel. Note characteristically large number of vesicular connective tissue cells (VCTC'S) surrounding the blood vessel (BV), germinal epithelium of acinus (AC) intact and oocytes (OO) developing on the acinar wall, N = prominent nucleus of oocyte. Scale bar = 85.7μm.

c). LM (3μm) section of "normal" gonad of *H. aspersa* not infested with mites, acini (AC) are fully formed, highly ordered mature spermatozoa are present, SH = sperm heads embedded in a Sertoli cell and ST = sperm tails, as well as other stages of spermatogenesis. OO = oocyte and DOO = normal degeneration of oocyte. Scale bar = 93.75μm.

d). LM (3μm) section of "normal" gonad of *H. aspersa* not infested with mites showing healthy oocytes (OO) developing on the acinar wall (AC), N = nucleus. Scale bar = 93.75μm.


a
G
N
N
F
G
G
b
BV
VCTCs
OO
N
OO
AC
c
AC
ST
SH
OO
DOO
d
AC
OO
OO
N
AC
OO
N
N
OO

visible and in the remainder, not much larger. They were relatively easy to section compared to "normal" albumen glands of the same age as they had not accumulated as much galactogen. The cells were very small, the nucleus at this time filling most of the cytoplasm, (Plate 13a). The sections stained only very lightly with the PAS reaction indicating that there was not much stored galactogen a fact that was confirmed by the small size of the cells with no visible storage products. The gonad tissue occupied hardly any of the available room in the digestive gland and in some cases it was extremely difficult to identify it at all indicating that the volume of the gonad was probably considerably reduced. Where the gonad was sectioned the tissue was found to contain only very small acini and a few of the early stages of spermatogenesis and no fully mature sperm (Plate 13b &13c). In the more extreme cases the gonad tissue consisted of a mass of undifferentiated cells with no clear acinar structure visible. In general the high incidence group had a larger proportion of vesicular connective tissue cells and more oocytes than would normally be expected in a snail of this age or were found in the control group.

The snails in the low incidence group exhibited slightly less dramatic changes to their reproductive systems in that it was in all cases possible to locate the organs and obtain sections. The gonad tissue contained larger acini than the high incidence group but were still well below "normal" size, there were early stages of spermatogenesis and some mature sperm (Plate 13d). The albumen glands of these snails were not fully mature but were in a state somewhere between the two extremes, the presence of the follicles was clearly seen but the cells had not reached full size and had not accumulated the expected amount of galactogen for a snail of that age.

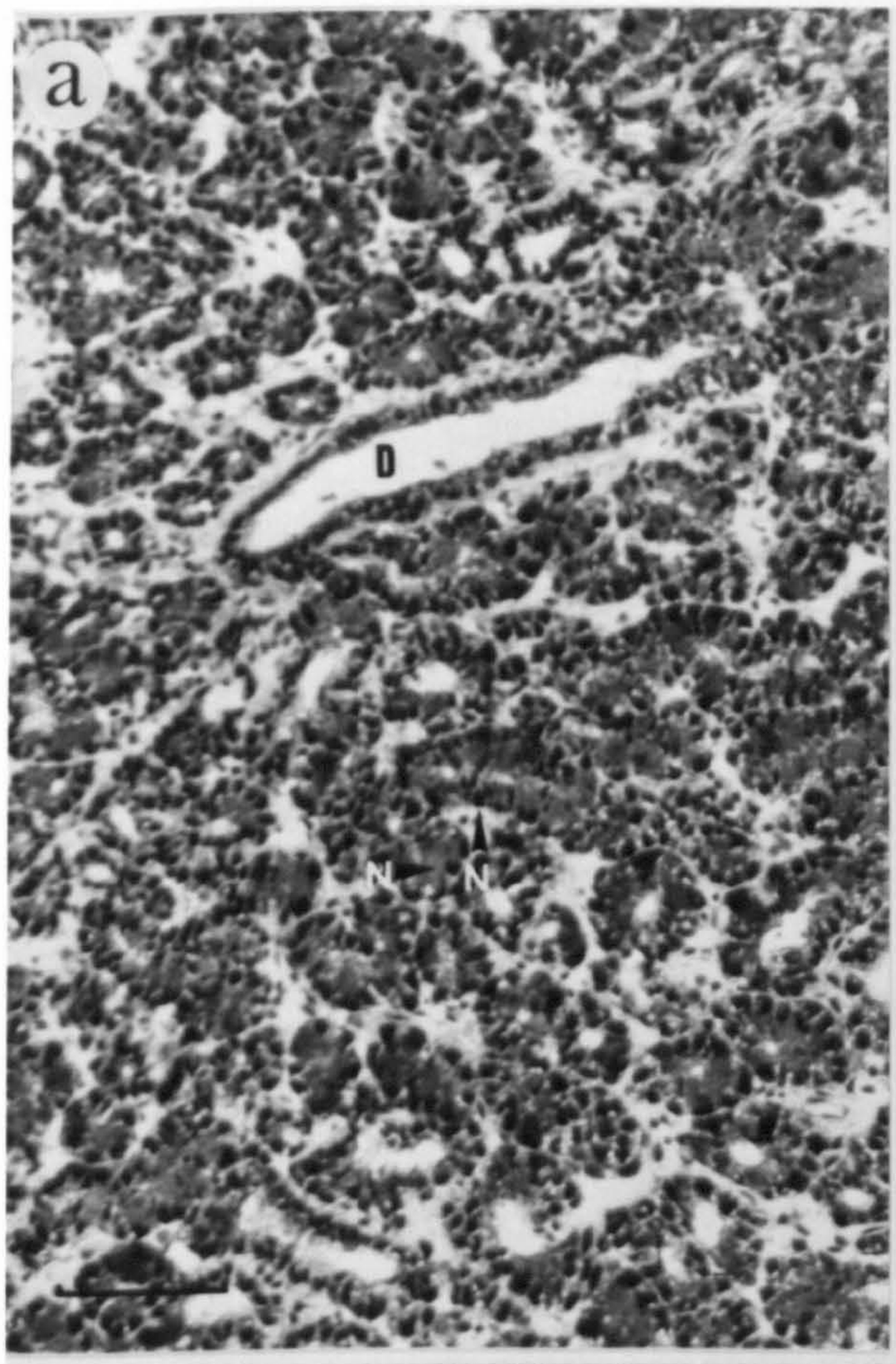
PLATE 13

a). LM (3μm) section of "abnormal" albumen gland of *Helix aspersa* infested with a high incidence of *Riccardoella limacum.* Note nuclei (N) occupy most of the cytoplasm as cells have not swollen to accommodate galactogen as is the case normally. Central ducts of follicles are visible as well as a large central duct (D). Scale bar = 91.2μm.

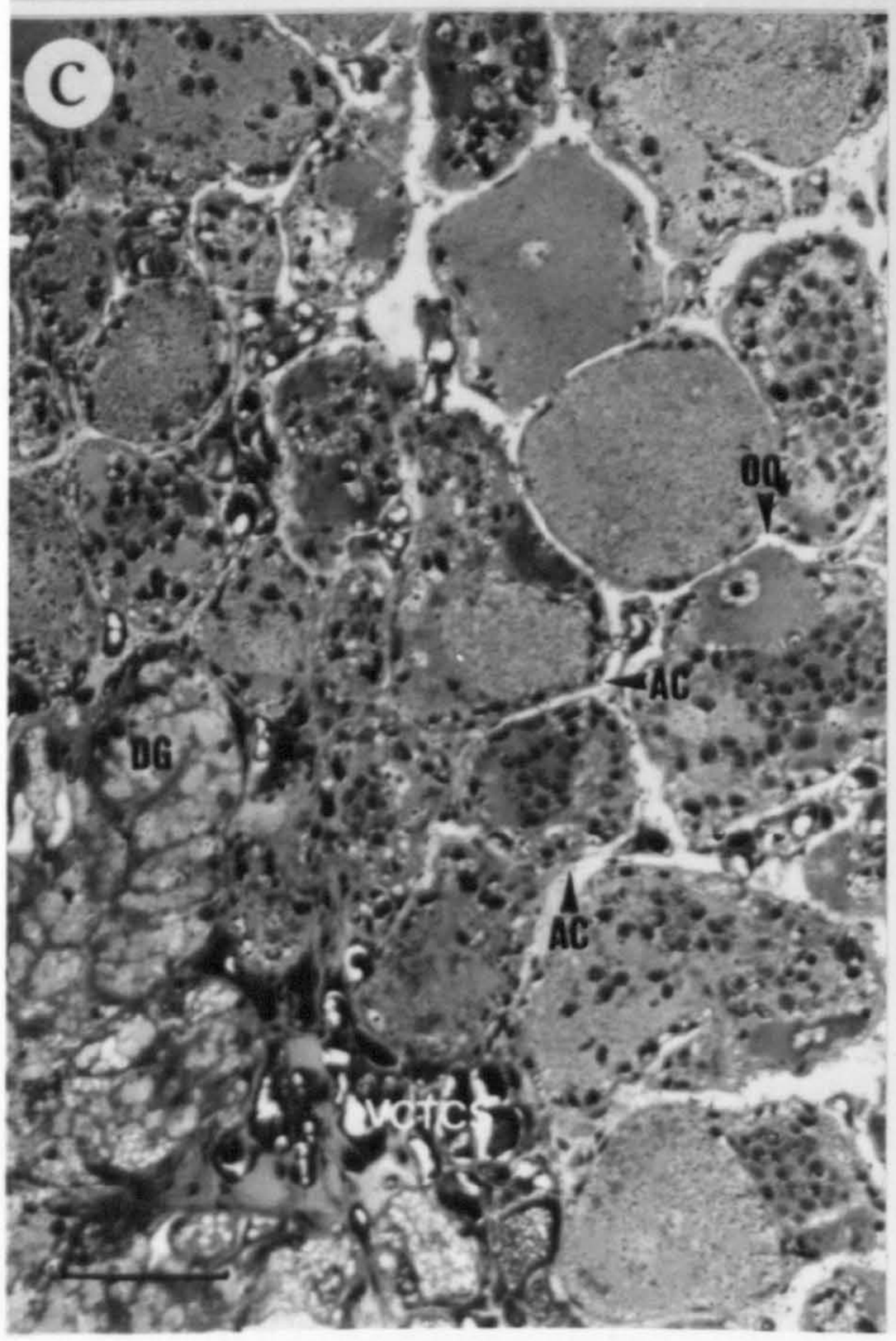
b). LM (3μm) section of "abnormal" gonad of *H. aspersa* infested with a high incidence of mites, taken in the region of a blood vessel(BV), note characteristically high number of VCTC's present as before (Plate 12 b) but very small acini (AC) with no mature spermatozoa resulting in an apparently large number of oocytes (OO). DG = digestive gland cells. Scale bar = 150μm.

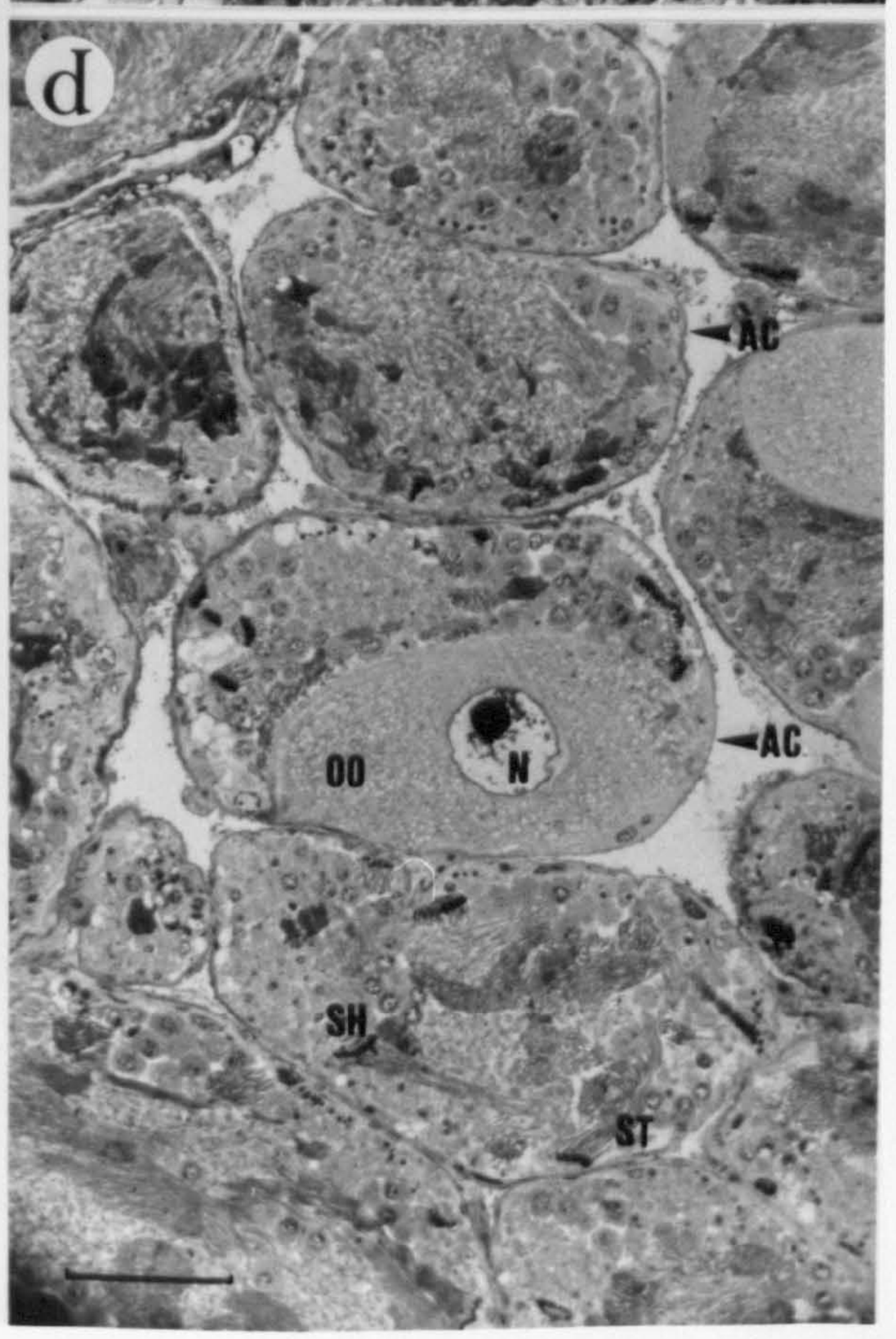
c). LM (3μm) section of "abnormal" gonad of *H. aspersa* infested with a high incidence of mites showing small under developed acini (AC) with no mature spermatozoa only spermatocytes. OO = oocyte and VCTC's = vesicular connective tissue cells. Scale bar = 100μm.

d). LM (3μm) section of slightly "abnormal" gonad of *H. aspersa* infested with a low number of mites, although mature sperm heads can be seen (SH) embedded in Sertoli cells, the sperm tails (ST) do not display the highly ordered arrangement of "normal" spermatozoa. AC = acinus, OO = oocyte and N = nucleus. Scale bar = 163μm.


a
D
N
N

b
DG
OO
OO
OO
OO
BV
AC
OO
OO
VCTCS
AC
OO
OO
DG


c
OO
AC
DG
AC
VCTCS


d
AC
OO
N
AC
SH
ST

7.3.2.5 Condition of the lung.

In control animals the lung had a "normal" appearance; the respiratory epithelium was not swollen or damaged in any way and there were numerous supporting elements (Plate 14 a &14b). The microvilli of the respiratory epithelium surface were all normal and had not formed large clumps. The thickness of the epithelium was estimated to be between 6-10μm. There were vesicular connective tissue cells (VCTC's) present and there was no alteration in the proportion of different cells present in the digestive gland near the gonad.

No mites were observed in sections of the lung of snails that had contained mites and no feeding structures such as tissue canals were seen. However the respiratory epithelium of these animals was highly vacuolated and there were very few supporting elements present (Plate 14c & 14d). The muscle tissue was breaking down and there was a higher proportion of excretory cells in the digestive gland than in the control animals though the VCTC's (=glycogen cells) appear normal. The microvilli of the respiratory epithelium had in some cases fused together and the estimated thickness of the epithelium varied more than the control animals, being between 1.5-16μm.

7.3.3 Experiment B.

7.3.3.1 Final weights.

The average monthly weights for the experimental and the control group snails can be seen in Table 7.1 along with those in Experiment A and are directly compared in Graph 7.4. the final weights of these snails are seen in Table 7.3. The mean final weights of the snails in the high incidence group are dramatically less than the control group snails, almost exactly half (see statistical analysis). This is similar to

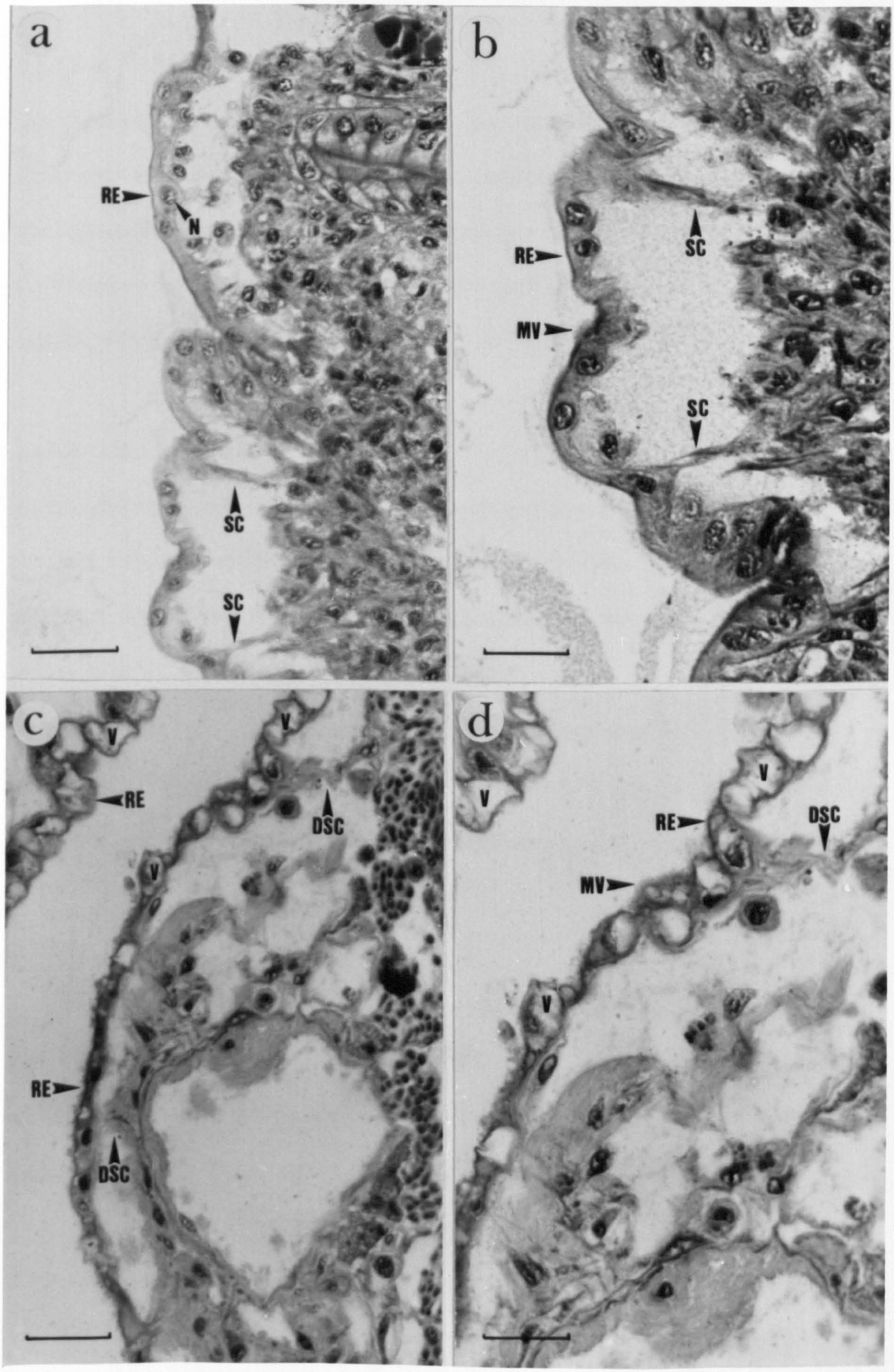
PLATE 14

a). LM (3μm) section through "normal" lung of *Helix aspersa* not infested with *Riccardoella limacum*, RE = respiratory epithelium, SC = supporting columns, mainly muscle and N = nucleus. Scale bar = 33.5μm.

b). High power view of above section of respiratory epithelium (RE) showing microvillus border (MV) and the same supporting columns (SC) as before. Scale bar = 22.9μm.

c). LM (3μm) section through lung of "abnormal" *H. aspersa* infested with large numbers of *Riccardoella limacum*, note highly vacuolated appearance of respiratory epithelium (RE) and degenerating supporting columns (DSC), V = vacuoles. Scale bar = 32.9μm.

d). High power view of above section of respiratory epithelium (RE) showing microvilli (MV) "clumped" together and degenerating supporting columns (DSC), the basement membrane also appears swollen, V = vacuoles. Scale bar = 20.9μm.


a
RE
N
SC
SC
b
RE
MV
SC
SC
c
V
V
RE
DSC
V
RE
DSC
d
V
V
RE
DSC
MV
V

the Experiment A result even though Experiment B was run for four months less.

7.3.3.2 & 7.3.3.3 Shell characteristics.

The shells of these snails were subject to the same variations as those in Experiment A; control snails were in much better general condition than experimental animals and had significantly larger final shell dimensions (see statistical analysis) along with the thickened rim. The experimental snails had much poorer shell quality and were smaller, which corresponds to the previous results, (see Table 7.3).

7.3.3.4 Albumen glands.

As the albumen glands were relatively easy to remove from the snails in one piece, the final weights of these organs were compared (Table 7.4). There was a major difference in these weights (see statistical analysis) and the condition of the sexual organs is correspondingly affected. Snails without mites had albumen glands that weighed almost twice as much as those that were infested with large numbers of mites. The difficulty in separating the gonad from the surrounding digestive gland tissue unfortunately made a similar comparison of that organ impossible.

7.3.3.5 Histological observations.

The results were similar to those obtained in Experiment A despite the fact that the snails were four months younger. Snails should be fully mature at six months old with little further development after that time; hence the two experiments were really looking at snails of similar ages in development terms.

Control group snails: These animals had very large acini with plenty of mature sperm, usually organised and embedded into nutritive Sertoli cells and many mature looking oocytes. The albumen glands of these snails were very mature with large

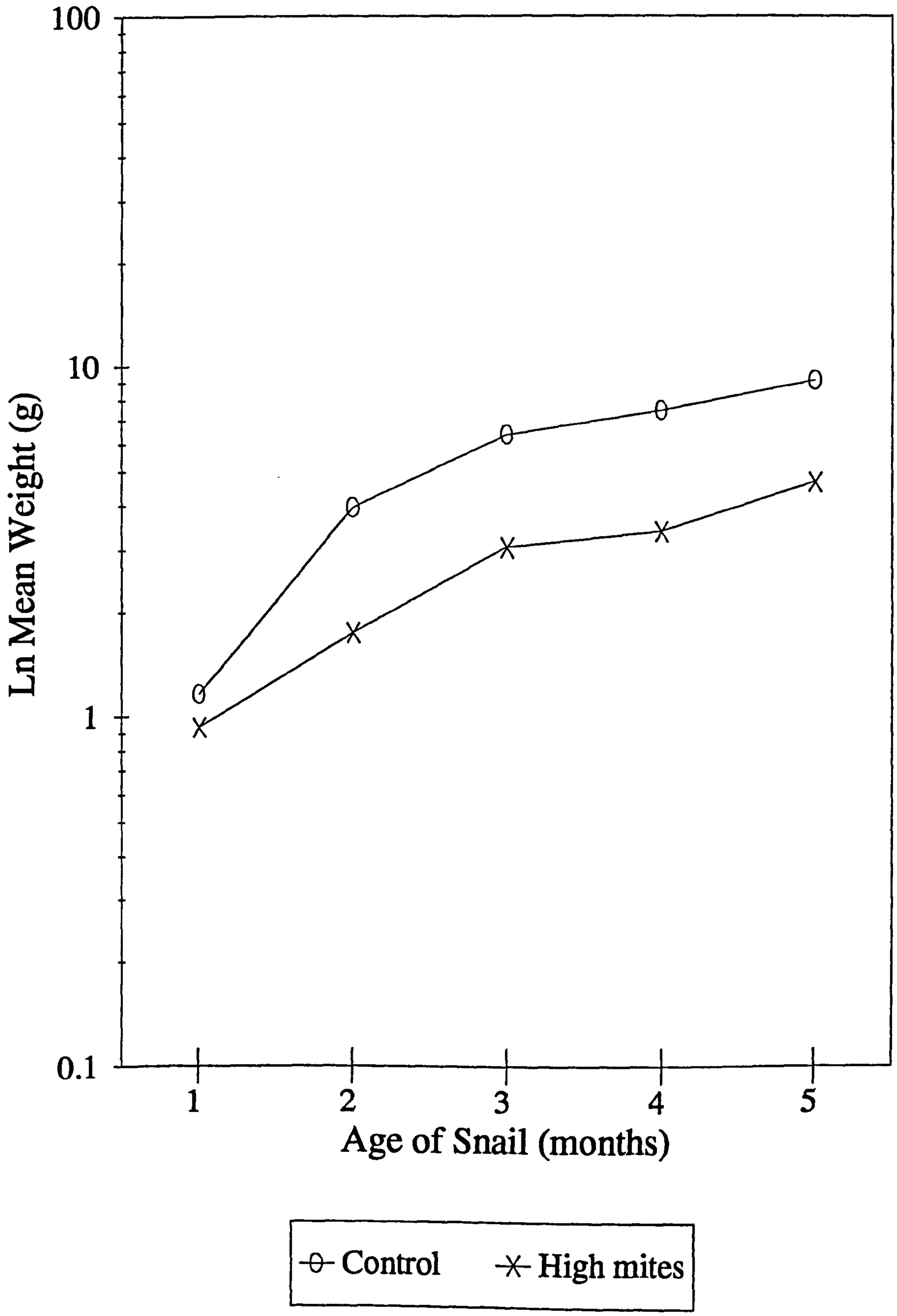
Table 7.3 : Final shell height, shell diameter, and snail weights for Experiment B.

	Shell Height (mm)		Shell Diameter (mm)		Weight of whole snail (g)	
	HIGH	CON.	HIGH	CON.	HIGH	CON.
	32.6	29.10	27.20	36.65	4.948	14.160
	22.55	30.05	20.75	38.05	6.606	13.366
	24.50	27.10	31.60	33.95	6.158	10.078
	23.30	25.40	27.50	30.30	5.215	7.023
	20.40	26.15	27.65	29.20	6.754	7.892
	23.25	32.45	27.55	37.15	4.808	13.136
	20.40	22.20	23.30	25.00	3.353	4.377
	20.80	38.55	23.75	33.30	3.499	10.386
	26.40	26.85	31.55	36.55	7.224	10.601
	21.45	33.55	21.45	39.10	4.383	15.129
	25.10	24.35	30.50	29.05	5.908	7.206
	21.60	20.95	27.85	21.05	6.223	5.466
	29.30	22.2	24.30	27.15	2.929	5.736
	21.00	21.30	26.30	24.45	4.193	4.958
	16.60	27.85	21.40	31.85	1.917	9.707
	18.60	26.65	21.80	33.65	2.444	9.435
	28.00	29.05	22.35	39.10	2.609	12.923
	-	26.20	-	31.20	-	8.621
	-	28028	-	37.90	-	6.307
	-	23.95	-	27.25	-	6.123
Mean	**23.29**	**27.10**	**25.69**	**32.10**	**4.657**	**9.132**

Table 7.4 : Wet weight of snail albumin glands in Experiment B only.

	Wet weight of albumin gland in Control Group (g)	Wet weight of albumin gland in High Infestations (g)
	0.53	0.37
	1.72	0.33
	1.48	0.25
	0.42	0.61
	0.92	0.80
	0.81	0.72
	1.07	0.30
	0.78	0.52
	0.89	0.51
	0.95	0.40
	0.81	0.63
	1.21	0.29
	1.03	0.54
	0.89	0.46
	0.67	0.31
	0.59	0.29
	1.03	0.45
	1.01	-
	0.95	-
	0.78	-
Mean	**0.9225**	**0.4576**

Graph 7.4- Comparison of Snail Growth
High Infestations (Exp.B).



follicular cells packed with galactogen tending to lead to characteristically poor quality sectioning.

High mite incidence group: These snails were definitely sub-mature animals showing the retarded sperm development seen in the previous experiment and higher than expected number of immature oocytes. The albumen glands were exceptionally immature consisting of many undifferentiated cells with little or no galactogen stored.

7.3.3.6 Condition of the lung.

This was as reported for the snails in Experiment A.

7.3.4 Statistical Analysis.

All of the data obtained in the above two experiments were subjected to two-sample t tests at the 95% level and all the P values were noted.

7.3.4.1 Experiment A.

SHELL HEIGHTS: The mean value (23.9mm) for the shell heights of the high incidence group was significantly lower than the mean (28.05mm) for the low incidence group (P = 0.037) and the mean (27.88mm) for the control group (P = 0.042). There was no significant difference between the low incidence group mean and the control group mean (P = 0.88).

SHELL DIAMETER: The mean value (30.58mm) for the shell diameters of the high incidence group was significantly lower than the mean (36.76mm) for the low incidence group (P = 0.013) and the mean (35.38mm) for the control group (P =

0.036). There was no significant difference between the low incidence group and the control group means (P = 0.48).

SNAIL WEIGHTS: The mean value (9.11g) for the final weight of the high incidence group was highly significantly lower than the mean (14.41g) for the low incidence group (P = 0.0064) and the mean (16.35g) for the control group (P = 0.0003). There was no significant difference between the low incidence group and the control group means (P = 0.087).

7.3.4.2 Experiment B.

SHELL HEIGHTS: The mean value (23.29mm) for the shell heights of the high incidence group was very highly significantly lower than the mean (27.10mm) value for the control group (P = 0.0093).

SHELL DIAMETER: The mean value (25.69mm) for the shell diameters of the high incidence group was very highly significantly lower than the mean (32.10mm) value for the control group (P = 0.0001).

SNAIL WEIGHTS: The mean value (4.66g) for the final weights of the high incidence group of snails was extremely, highly significantly lower than the mean (9.13g) value for the control group (P = 0.0000).

WEIGHTS OF ALBUMEN GLANDS: The mean value (0.458g) for final weights of albumen glands of snails in the high incidence group was extremely, highly significantly lower than the mean (0.927g) value for the control group (P = 0.0000).

7.4 DISCUSSION

The results indicate that a definite disadvantage is incurred in those snails which have high infestations of mites. There is a substantial difference between control and low infestation snails and those with high infestations. The threshold for mite damage is unclear since after the introduction of mites there was no control over the numbers that built up. It is certain that once the numbers of mites on a snail rise above an initial stocking density of five, then severe damage to the snail can occur. This damage takes the form of a slower growth rate, sexual immaturity beyond the age when they would normally be expected to be in breeding condition and a brittle shell which breaks often and repairs badly. All of these signs were seen in the experimental animals to varying degrees.

The most striking result is the difference in final weights of highly infected snails and the other groups, which differ by so much that the difference could not be attributed to the known normal variability of snail weights (Sampelayo *et al.*, 1990) or the weight variation in individual snails from the field reported by Howes & Wells (1933). A major component of this difference in total weight is the difference in the development weight of the reproductive system. There is a halving in the weight of albumen glands in the infested snails in Experiment B. Although the other parts of the reproductive tract were not isolated the differences in volume clearly parallel the differences in the albumen glands. The albumen gland is also the site of galactogen storage so that at least part of the weight loss caused by mites could arise from an alteration in the capacity for carbohydrate storage. Changes in the histology of the gonads, as well as the albumen glands, however, seems to suggest that it is the reproductive potential as much as food storage that is suffering. Sexual organs of snails without mites showed "normal" development with plenty of healthy sperm and

oocytes in the gonad while those with mites showed extremely suppressed development for the age of animal as well as an alteration of the ratio of male and female gametes. In the worst affected snails it was hard to identify any gonad at all and in others the number of oocytes far exceeded the numbers found in normal gonad for a comparable amount of gonad tissue. There was little difference in weight, condition or sexual maturity between animals of the same age in the same treatments in Experiment A and Experiment B. This indicates that the effect of better reproductive potential when animals are kept separately as opposed to communally, noted in *P. gyrina* by DeWitt (1954) does not exist in *H. aspersa* under the conditions of culture used in these experiments.

Snails with mites tended to have regular periods of dormancy when they consume and assimilate less food, contributing to lower weights and slowed development times. Bonnefoy-Claudet & Deroy (1984) stated that short periods of hibernation in *H. aspersa* lead to a generally lower fecundity with fewer eggs laid. These short periods of inactivity can presumably result from the stress imposed by the mites constantly feeding on haemolymph. This must be a considerable energetic drain on a snail, aside from the physical distress caused by having mites piercing the lung surface. Although it was not possible to find mites embedded in the surface of the lung in any of the sections taken, from previous work (Chapter 4) and the work of other authors (Lawrence, 1951; Baker, 1967, 1970b, 1971) it is clear that the mite feeds on the blood of the snail. One hypothesis for the method of carrying this out is the formation of a feeding tube as described by Jones (1950b) in *T. autumnalis* which is thought to be a permanent structure (Evans, 1992). If the tube is permanent then there must be a considerable amount of irritation (Fain, 1969) in the surrounding area which would presumably cause a localized immune response in the snail and affect the health in general. However the lung of a pulmonate is an ideal place for

a blood feeding mite to live as the blood vessels are not lined and a simple scraping action would be sufficient to effect penetration through the thin epithelial covering of the respiratory surface of the lung. This would result in a leakage of blood which the mite could then easily ingest without necessitating the formation of a feeding tube as previously imagined. It is possible that this loss of blood is not only energetically inefficient but it may also lower the hormone levels in the snail blood and thus delay maturation of the reproductive system. It is likely that a wound thus inflicted would probably heal quickly and not cause the snail any further problems, however the physical presence of sometimes large numbers of mites in the lung would be a severe disadvantage for any snails so afflicted. As many as sixty live mites and several hundred eggs as well as the cast skins from the development of all the nymphal stages have been found in a single snail. In such lungs there must be a reduction in the efficiency of the respiratory surface and the capacity for oxygen uptake. This would have an affect on the general health of the animals.

Observations on shell formation in all the groups of snails confirm those of Oldham (1931) in *A. arbustorum* in that when mites are present they affect shell growth and shape. *H. aspersa* with heavy mite infestations had very weak shells that were constantly being damaged and repaired, although in this case there was little effect on shell shape.

The striking difference in weight of control and experimental group snails, particularly of the albumen glands, prompted the decision to examine further the connection between weight and whether significant differences are caused by organ growth or storage product remobilization. The histology of these animals conclusively shows that mites cause slower, abnormal development of the sexual organs and therefore reduce the fecundity of such a population. It may also be that

some of the weight deficit in the infested snails is due to an effect on the proteins and carbohydrates of the snails. This would in turn affect the quality of the snail as a food item, with far reaching consequences to the snail farmer especially as the overall weight of snail meat production would fall if the farm was badly infested with mites. This, coupled with poor breeding stock could cause the premature closure of smaller enterprises.

To assess the quality of snail meat in snails in the experimental groups, biochemical analyses of the composition of the snails was carried out on those animals frozen earlier.

CHAPTER 8

THE BIOCHEMICAL ANALYSIS OF SNAILS INFESTED WITH MITES

8.1 INTRODUCTION

It has been shown that the presence of large numbers of mites on a snail results in poorly developed reproductive system, a weak shell and greatly reduced growth, all of which are highly undesirable to a farmer attempting to build a business with a high turnover of fattened snails. It is also desirable to know the effect of mites on the quality of the snail meat produced. A series of biochemical analyses were performed on the flesh of snails that had been heavily infested with mites.

The biochemical composition of snails and molluscs in general has been widely studied in the past in relation to the main metabolic compounds. The role of galactogen in particular has been studied to try to discover the part it plays in metabolism (Baldwin & Bell, 1938), as it is a characteristic compound of slugs and snails (Kugler, 1965). Other carbohydrates in these species, namely glucose and glycogen, are thought to have similar functions to those in mammals (Baldwin & Bell, 1940). Interest has concentrated on carbohydrate and lipid research (Gabbot & Peek, 1991) and protein metabolism has been comparatively poorly studied. Glycogen is found widely throughout the tissues of the snail *Achatina fulica* especially in vesicular connective tissue cells (VCTC's = Glycogen cells) in the digestive gland, where it serves as the main storage carbohydrate to be used as an

energy source in metabolism (Emerson, 1967; Monney, 1992).

Research into other areas of the biochemical composition of pulmonates has lead to the conclusion that in general carbohydrates show greater variation in concentration than any other component. Lipid in pulmonates is in general a very stable component and is subject to little variation. This is certainly the case in *Cepea nemoralis*, which undergoes no seasonal changes in lipid content indicating that the lipids are probably more structural than storage products (Van der Horst & Zandee, 1973). The lipid stores are not used during the winter hibernation of this species and are not affected in any way by the environment, in fact it is certainly the glycogen stores that are utilized at this time. However, since the water content of slugs and snails is subject to wide variations; for instance 2-10% in *H. aspersa* in culture (Klein-Rollais & Daguzan, 1990) it is difficult to know whether changes in the concentration of biochemical components are due to seasonal and daily fluctuations in water content, or whether they are genuine differences caused by mite infestation. Therefore when choosing the elements of the biochemical composition of *H. aspersa* to be examined it was obviously wise to discount lipids on the grounds that they show very little change and water content because it changes too much. For this reason in many studies it is only the carbohydrate and protein concentration fluctuations that are studied. It was also essential to base all estimates of biochemical composition on dry weight to avoid the effects of changing concentrations due to water content.

The presence of both glycogen and galactogen in the slug *Philomycus carolinianus* has been described by Kugler (1965). Glycogen occurs throughout the reproductive tract (as well as in other body tissues) whereas galactogen, a polymer of galactose, is restricted to the albumen gland and is later deposited in the eggs (Goudsmit &

Ashwell, 1965; Goudsmit & Neufeld, 1966). Corrêa *et al.* (1967) examined structure and distribution of galactans and galactogen in the albumen glands of *Biomphalaria glabrata* and *H. aspersa.* In *H. pomatia* Baldwin & Bell (1938, 1940) discovered that galactogen was confined to the albumen gland and that this organ did not contain any glycogen. They believed glycogen to be derived from the breakdown of plant cellulose using a rare cellulase. This has since been proved otherwise by Goudsmit (1975) who found that both galactogen and glycogen were produced by the albumen gland and could be stored in the same cells.

Fluctuations of protein and carbohydrates are to be expected throughout the year as the metabolism in the snail is based on these compounds (Monney, 1992). However, the proportions in which they occur at different times in relation to the development of sex organs and hibernation patterns is not clear. Kemp & Newell (1989) stated that in the slug *Arion hortensis*, the levels of glycogen drop in the breeding season due to the conversion of glycogen into galactogen for the development of the albumen gland. However Monney (1992) reports that although the levels of stored glycogen do fall during the breeding season in the snail *A. fulica*, this glycogen is not converted into galactogen for the development of the albumen gland. In general it appears that galactogen accumulates in the albumen gland before egg laying and the glycogen in the vesicular connective tissue cells accumulates as a result of feeding throughout the summer months (Goudsmit, 1975). During the winter hibernation the glycogen levels fall as they are utilized in metabolism and in the development of the reproductive system and gametes (Van der Horst & Zandee, 1973; Monney, 1992). In *Mytilus edulis* (Gabbot & Peek, 1991) the protein stores are conserved and remain high during the winter, despite rapidly falling glycogen stores, until the full reproductive condition is attained. When breeding condition is reached, oocytes develop and protein stores are dumped into them along with the

accumulation of glycogen from the VCTC's. Consequently the levels of protein and carbohydrates are low just after the end of the breeding season but are quickly regenerated afterwards (Peek, 1987). In *H. aspersa* during the breeding season, galactogen may account for up to 85% of the total polysaccaride in the snail (Nieland & Goudsmit, 1969). Galactogen is used up in egg production and the newly hatched embryo may metabolise all galactogen stores in as little as four days in *Bulimnaea megasoma* and *Lymnaea stagnalis*, (Goudsmit, 1976).

These fluctuations are found in normal healthy animals throughout the year, but in animals such as *M. edulis*, which are stressed for whatever reason, the metabolism of both carbohydrates and proteins is affected and stores will be metabolised, (Gabbot & Peek, 1991).

Mite infestations appear to cause the snails considerable stress and it was of interest to discover if this was reflected in the biochemical composition of the snails.

8.2 MATERIALS AND METHODS

Carbohydrate analysis was carried out using a modification of the methods used by Keppler & Decker (1974), to determine the glycogen and free glucose content. Protein was assessed by a modification of techniques developed by Lowry *et al.* (1951) using Folin reagent, this has limitations but is very sensitive, requires no predigestion and can be adapted easily for use with small samples. The amount of colour that develops is variable from protein to protein but in an assay of mixed protein, it is very useful, especially as absolute values for protein content are not as important as the comparison between different experimental groups.

8.2.1 Preparation of Samples.

The soft body parts of some snails in all of the experimental groups described in the previous chapter (low incidence of mites, high incidence of mites and no mites/control for both Experiments A and B), were frozen at -70°C until it was convenient to carry out biochemical analysis of the tissues. They were then lyophilized (freeze-dried) to remove all the water and ground to a fine powder using a pestle and mortar and a food blender, both of which were cleaned carefully after each sample, with alcohol. Samples for analysis were weighed out using a Sartorius balance to obtain accurate 2mg and 4mg quantities (for protein and carbohydrates respectively) which were then placed in individual air tight bags. A total of eighteen bags from each sample were used to enable carbohydrate and protein assays to be carried out three times each with three replicates in each run.

8.2.2 Total Protein Assay.

This was carried out using a Sigma Diagnostics Protein Assay Kit (Procedure No. P5656). This assayed the soluble proteins using the Lowry reagent and utilizes sodium dodecyl sulfate to facilitate the dissolution of insoluble lipoproteins. The kit contained a protein standard solution prepared from bovine serum albumen that was reconstituted with the required volume of distilled water and stored for up to three months at 0-5°C. The other reagents; Lowry Reagent Solution and Ciocalteu's Phenol Reagent Working Solution, were prepared in a similar manner by adding distilled water and stored at room temperature. The following procedure was followed:

1. Standards were prepared first from bovine serum albumen solution with 0.1M NaOH to a volume of 1ml in an Eppendorf tube appropriately labelled:

Protein Standard Solution (ml)	0.1M NaOH (ml)	Protein Concentration µg/ml
0.125	0.875	50
0.250	0.750	100
0.500	0.500	200
0.750	0.250	300
1.000	0.000	400

2. A blank was prepared from 1.0 ml 0.1M NaOH.

3. The ground tissue samples were added to 1ml of distilled water and homogenised with an Ultraturrax Homogeniser for one minute. 0.1ml of the sample homogenates were added to Eppendorf tubes and diluted with 0.9ml 0.1M NaOH in each and left for 30 minutes in a water bath at 57°C.

4. 0.5mls of Lowry Reagent Solution was added to all standard, sample and blank tubes, mixed well and allowed to stand at room temperature for 20 minutes.

5. 0.25mls of Ciocalteu's Phenol Reagent working solution was added to each tube and mixed thoroughly before being transferred into 2ml cuvettes.

6. The cuvettes were allowed to stand for 30 minutes to let the colour develop and then the absorbance of samples and standards read on a spectrophotometer at 750 nm against the blank. Protein concentrations can then be established.

8.2.3 Glycogen and Free Glucose Assay.

This was carried out using a Boehringer Mannheim Glucose GOD Peridochrome Test Combination P525. This assays both the stored glycogen and the free glucose if the enzyme solution is used, and only the free glucose if not, in this instance it was the former method which was employed. Solutions were reconstituted as before using the recommended amount of distilled water and stored at 2-8^{0}C. The following procedure was followed:

1. The enzyme solution was prepared using 1ml of amyloglucosidase (stock 20 mg/ml supplied) added to 99ml of 0.2M sodium acetate buffer pH4.8.

2. The 4mg of lyophilized sample was added to 1ml of distilled water and homogenised for a minute using an Ultraturrax Homogeniser.

3. 100µl of the homogenate was placed in an Eppendorf tube with 500µl of the amyloglucosidase enzyme solution and incubated for two hours at 37 °C with mixing every fifteen minutes.

4. Samples were deproteinized by precipitating out the protein using 100µl of 70% perchloric acid, 200µl of 7.5M potassium hydroxide and 100µl of distilled water and mixed thoroughly.

5. The samples were then centrifuged at 15000g for two minutes to remove the protein precipitate and the resulting supernatant used in the assay.

6. Standards were prepared in the range of 1.0 - 0.0mg/ml of carbohydrate using the stock solution provided (9.1mg/ml) and placed in appropriately labelled Eppendorf tubes.

7. The above standards and 100µl of each of the sample supernatants were all incubated for 25-50 mins at 20-25 °C with 1ml of the glucose reagent provided.

8. The absorbance of the samples and standards were read at 610nm with a spectrophotometer against a blank prepared with 1ml of the glucose reagent and 100µl of distilled water. The glycogen\free glucose concentration was then calculated for the samples.

N.B. Although it was highly desirable to assay the concentrations of galactogen stored in the albumen glands of the various groups of snails, this was not possible. At the time of study no simple and convenient assay test kit was available, in future work it would be interesting to carry out this test if available.

8.3 RESULTS

8.3.1 Total Protein Assay.

The absorbances of the standards used were plotted on a standard calibration curve and the corresponding concentrations of protein in µg/ml read off. These were multiplied by ten for the dilution factor used and then divided by the sample weights. The mean concentration of protein in mg/g dry weight for each group of snails, i.e. high infestation, low infestation and control for both Experiments A and B, can be seen in Table 8.1 and in graph 8.1..

8.3.2 Glycogen and Free Glucose Assay.

Again a standard curve was obtained and used to calculate sample concentrations in mg/ml which were then multiplied by the dilution factor, this time it was one hundred, and then divided by the sample weight. The mean concentration of glycogen and free glucose in mg/g dry weight for each of the sampled groups can be seen in Table 8.2 and in graph 8.1.

8.3.3 Statistical Analysis.

An analysis of variance was carried out at the 95% level of significance.

8.3.3.1 In Experiment A there was no significant difference in the amounts of protein present between the three treatments ($P = 0.263$). There was also no significant difference in the amounts of protein in Experiment B ($P = 0.095$).

8.3.3.2 The analysis of variance also showed that there was no significant difference in the amounts of glycogen/free glucose present in the three groups of snails in Experiment A, (P = 0.341) or in the two groups in Experiment B, (P = 0.349)

Table 8.1 : Mean protein concentrations for each snail tested

	Low Mite Incidence group		High Mite Incidence group		Control Group	
	Mean (mg/g dry weight)	S.D.	Mean (mg/g dry weight)	S.D.	Mean (mg/g dry weight)	S.D.
Experiment A	370	13.0	540	61.0	518	71.0
	560	51.5	219	15.0	621	63.0
	613	72.6	363	29.0	463	52.0
	489	45.0	710	86.0	319	37.1
	411	41.0	451	39.5	272	25.0
	329	29.0	299	22.8	459	49.3
	601	82.0	341	36.7	369	42.2
	297	13.5	401	49.0	720	59.1
	438	19.9	500	62.1	755	63.1
	521	39.4	297	19.0	698	51.0
Mean conc. per group (mg/g dry weight)	**462.9**	**107.8**	**412.1**	**143.5**	**519.4**	**172.8**
Experiment B			361	48.7	511	90.9
			492	49.5	602	71.0
			501	56.0	709	62.4
			262	22.1	394	43.2
			291	15.6	426	52.0
Mean conc. per group (mg/g dry weight)			**381.4**	**111.1**	**528.4**	**129.3**

Table 8.2 : Mean Glycogen and Free Glucose concentrations for each snail tested.

	Low Mite Incidence group		High Mite Incidence group		Control Group	
	Mean (mg/g dry weight)	S.D.	Mean (mg/g dry weight)	S.D.	Mean (mg/g dry weight)	S.D.
Experiment A	26	7.6	31	10.0	109	19.5
	34	6.0	49	8.0	129	18.0
	42	12.9	106	12.9	96	10.1
	96	25.0	92	18.5	51	6.9
	116	26.0	89	19.0	49	5.0
	120	21.0	76	15.0	39	7.0
	45	15.6	115	19.1	116	13.1
	39	12.1	20	7.6	91	11.0
	67	15.3	21	12.1	85	9.0
	58	12.0	35	10.0	71	7.0
Mean conc. per group (mg/g dry weight)	**64.8**	**34.6**	**63.4**	**36.3**	**83.6**	**30.5**
Experiment B			25	6.0	95	17.5
			31	8.9	111	15.0
			96	16.0	59	12.9
			78	17.1	45	6.0
			59	15.5	69	7.5
Mean conc. per group (mg/g dry weight)			**57.8**	**30.2**	**75.8**	**26.9**

Graph 8.1- Mean Concentration of
Protein and Glycogen and Free Glucose.

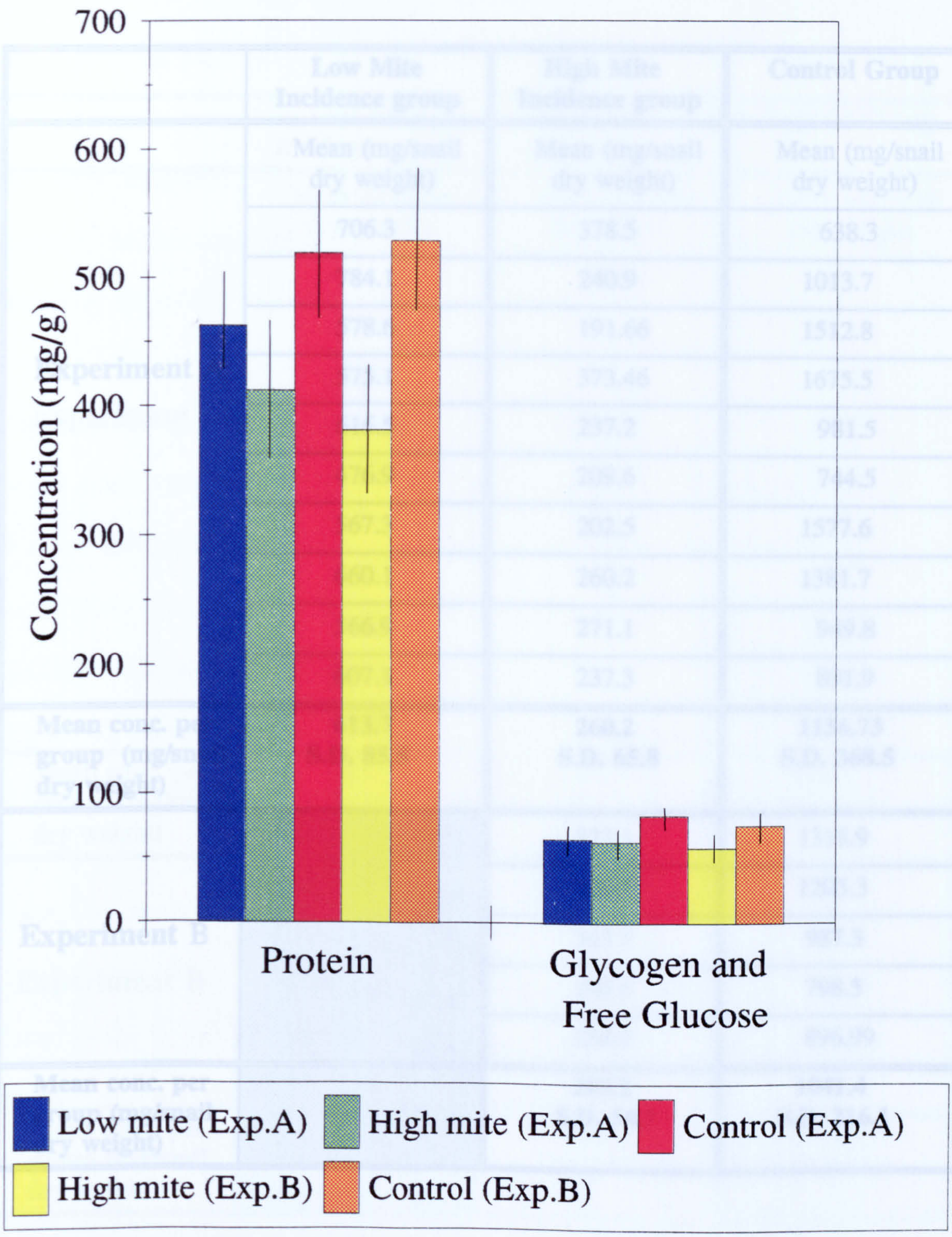


Table 8.3 Actual weights of protein per snail assayed.

	Low Mite Incidence group	High Mite Incidence group	Control Group
Experiment A	Mean (mg/snail dry weight)	Mean (mg/snail dry weight)	Mean (mg/snail dry weight)
	706.3	378.5	638.3
	784.1	240.9	1013.7
	578.6	191.66	1512.8
	573.1	373.46	1675.5
	616.5	237.2	981.5
	476.9	209.6	744.5
	567.3	202.5	1577.6
	660.1	260.2	1381.7
	566.9	271.1	949.8
	607.1	237.3	891.9
Mean conc. per group (mg/snail dry weight)	**613.7 S.D. 85.5**	**260.2 S.D. 65.8**	**1136.73 S.D. 368.5**
Experiment B		323.1	1318.9
		308.9	1205.3
		345.7	987.3
		209.6	798.5
		258.9	896.99
Mean conc. per group (mg/snail dry weight)		**289.2 S.D. 54.7**	**1041.4 S.D. 216.1**

Table 8.4 Actual weights Glycogen and Free Glucose per snail assayed.

	Low Mite Incidence group	High Mite Incidence group	Control Group
Experiment A	Mean (mg/snail dry weight)	Mean (mg/snail dry weight)	Mean (mg/snail dry weight)
	46.3	24.8	163.9
	34.9	49.1	245.1
	57.8	63.0	193.92
	56.9	85.9	178.5
	92.8	50.1	156.8
	89.1	29.9	238.9
	60.1	45.8	119.1
	79.8	31.0	106.3
	91.6	28.7	201.9
	98.9	26.5	221.6
Mean conc. per group (mg/snail dry weight)	**70.8 S.D. 22.3**	**43.5 S.D. 19.6**	**182.6 S.D. 47.1**
Experiment B		28.9	203.7
		31.6	198.8
		41.5	176.5
		52.1	212.3
		20.8	217.4
Mean conc. per group (mg/snail dry weight)		**34.9 S.D. 12.1**	**201.74 S.D. 15.9**

8.4 DISCUSSION

The results show no significant difference in the concentrations of the protein and carbohydrate components of snails that have had a high infestation of mites and those which have been mite free. However, quite dramatic differences were seen in the histology of the snails in the three different groups and in all of the other variables measured and examined also showed significant differences. The overall weights of the control compared to the experimental group snails was dramatically higher and therefore the total amounts of protein and glycogen/free glucose present per snail were very different (Tables 8.3 & 8.4).

The reason for the lack of significant differences in the concentration of proteins may be that most of the protein content of snails is structural, and is not stored to be used in metabolism, or is conserved for the production of oocytes (Peek, 1987; Gabbot & Peek, 1991; Monney, 1992). The main exception to this is the protein stores in specialized adipogranular cells in the mantle of *M. edulis* (Gabbot & Peek, 1991). The protein concentration in *H. aspersa* is therefore relatively stable and even in animals that have been given an external stress it does not appear to fluctuate outside of fairly constant limits. The glycogen level on the other hand is expected to vary much more as it is the main storage compound in snails. As soon as any stress is placed on the snails by a high infestation of mites it could be expected that glycogen stores would be the first resource utilized to maintain the metabolic balance. However, this is obviously not the case. The highly significant difference in total wet body weight between the snails that were infested with mites and those that were "clean", showed that on a basic level the mites have a detrimental effect on the growth of the snails by reducing their total body weight. Also the wet weight of the albumen glands of both groups of snails in Experiment B showed again that there

was a difference. The reason behind the differences seen in the albumen glands therefore lies in the fact that they store only galactogen and unfortunately could not be assayed in this study. The other indication is that while the total proteins and glycogen/free glucose concentrations remain unchanged, in mg/g dry weight concentration values, the effect the mites have on the snails is one of retarding their overall growth with the quality of the smaller amounts of snail flesh being the same as in larger, healthier snails.

If, for whatever reason, the snails go into a state of temporary semi-hibernation, as was observed during the experiment, it would probably not necessitate the utilization of carbohydrate stores as would normally be expected in hibernation, as these states only lasted for a few days at a time and some feeding did occur during the active phases. This type of behaviour would lead to generally smaller snails. In this particular experiment the reproductive development was slowed due to retarded weight gain as enough body mass had not been accumulated to support the maturation of the reproductive tract. However in some conditions animals can mature at a smaller size than in the wild and other effects on reproductive maturation seem likely as the relative proportions of male and female gametes are altered.

Mites do not cause a major change in the metabolism of their hosts. In this instance large numbers of mites are energetically bad for the snails, preventing normal weight gain, growth and therefore reproductive maturation. In conclusion, if mites are present in large quantities they cause a major reduction in growth and a severe delay in sexual maturation leading to a major difference in the distribution of carbohydrates, in particular galactogen in immature and adult snails. Mites do not affect the relative concentration of the biochemical components measured, they therefore do not cause a storage but a growth problem.

CHAPTER 9

FACTORS AFFECTING THE CONTROL OF *RICCARDOELLA LIMACUM*

9.1 INTRODUCTION

Too often the obvious way of dealing with any pest is to apply a pesticide. This unfortunately has several disadvantages, namely that people are understandably intolerant of eating anything treated with chemicals in addition, the pest invariably develops a resistance to the pesticide. More serious effects can occur when pesticides inadvertently cause a population increase in the pest. Carey (1982) noted this in *Tetranychus urticae* when acaricides applied to populations of mites not only affected the target mite but also all their natural predators. This, coupled with the resulting increase in females caused by the chemical caused a population explosion of *T. urticae*.

Resistance to acaricides is well known and was classified into various categories by Tellier *et al.* (1991). Differences in response to acaricides has also been reported by Schulze *et al.* (1991) who found that spray applications of acaricide to *Ixodes dammini* had little effect due to the fact that the sub-adult stages live in the leaf litter and thus avoid contact with the chemical. This would also be the case if acaricides were applied to *R. limacum* as most of the time it lives within the protected environment of the pulmonary cavity. The result of an application on the population also depends on the numbers of different stages present, Schulze *et al.*

(1991) noted that the greatest effect was on the nymphal stage while the least variation in level of control was in the larval stage. In *R. limacum* there would certainly be no control exhibited over the eggs which are laid inside the lung.

The effect of chemicals on other organisms in the environment is also important but is especially so on a snail farm where the mites in question are living in extremely close association with their host which is also ultimately destined for human consumption. The use of chemical molluscicides was reviewed by Runham & Hunter (1970) and in particular the effects of carbaryl on slugs was observed by Ruppel (1959 In: Runham & Hunter, 1970). The effect on snails of carbaryl, which was used by Schulze *et al.* (1991) in their experiments, has been reported by Singh & Aarwall (1986) working on chemical control of the snail, *Lymnaea acuminata.* They note that at sub-lethal doses there was no effect on the snail though at the higher end of the suggested scale there was a reduction in fecundity. Seuge & Roger (1981) showed that a small dose (4mg/l) of Fenitrothion applied to snails caused a reduction in fecundity, though a degree of protection was offered to the eggs by the egg membranes. Obviously if chemicals such as these were used on a snail farm, although some degree of control over the mites might be gained, it would be highly unsatisfactory as far as the productivity of the snails was concerned.

Alternative control methods must be considered to keep the numbers of mites at a minimum. Environmental factors can have a great influence on the life cycle of a mite, for instance it is possible to enhance the egg production of certain trombiculid mites by increasing the temperature in the culture for two or three days but if this was sustained then the results can be fatal to the mite (Melvin, 1946). Other workers report optimum temperatures for the development of mites, in *Tetranychus mcdanieli* it is 35 $\pm 2^{0}$C while in other spider mites it is between 24-29 °C (Tanigoshi *et al.*,

1975). Herbert (1981) notes that in *Panonychus ulmi* at 21°C there are twice as many eggs laid as at 15°C, this implies that if a snail population was kept at a sub-optimal temperature for the mites concerned, then at least a degree of control would be attained. Altering the humidity at which a mite has to live is also a factor that must be taken into consideration, Hazan *et al.* (1973) found that the survival of the carmine spider mite was always best at relative humidities around 38%. The mites reportedly lived longer and had greater fecundity at the lower humidities, whilst at 100% humidity no eggs were laid.

The potential of predatory mites as biological control agents has been examined in several species in the past but there are limitations. *Amblyseius potentillae* is known to prey on all stages of *P. ulmi* except for the eggs, but it exhibits a preference for the immature stages (McMurty & Van de Vrie, 1973). The protection conferred to the population by eggs (and females to an extent) results in an inefficient control system. Huffaker *et al.* (1970), produced an extensive review of the biological control of *T. urticae* as this mite is of considerable economic importance. They name a wide diversity of predators but conclude that insects are generally more efficient as they have a greater daily consumption. A common, successful use of predatory mites is the mesostigmatid mite, *Macrocheles muscaedomesticae*, preying on the common house fly. They are known to be capable of finding the eggs of the fly even if they are hidden in the substrate (Rodriguez & Wade, 1961) and stages as early as the protonymph will feed on the eggs while adults have been seen to cluster around an adult fly and suck out the body fluids (Wade & Rodriguez, 1961). Axtell (1963) has also reported this species piercing the body and removing the fluids from other species of soft bodied mite under laboratory conditions. It seems as though *M. muscaedomesticae* detects prey using chemoreceptors on the palps and tarsi of the front legs (Farish & Axtell, 1966) mites have well adapted receptors opening through

small pores, thus minimising water loss (Hodgson *et al.*, 1955; Slifer, 1970). *M. muscaedomesticae* can detect the presence of either fresh, frozen or macerated flies but prefers live immobilized specimens to those freshly killed (Farish & Axtell, 1971) it therefore seems that they are not detecting movement, but using their highly sensitive chemoreceptors to home in on the scent of the fly or perhaps carbon dioxide gradients. This opens the possibility of using chemical attractants to lure parasitic mites away from their hosts. Despite the clear advantages of using a species such as *M. muscaedomesticae* in biological control there is a problem that in some cases the predatory mite will only feed on a particular species or indeed specific life stages. The answer that has been suggested by several workers is one where chemical and biological control complement each other. Some of the problems in accidental elimination of natural predators can be solved by careful screening of possible acaricides before application, to eliminate those which adversely affect predators, and then combine the effects of both acaricide and predator. This approach was suggested by Axtell (1963) for use with *M. domesticae* against the house fly and also more recently by Tellier *et al.* (1991). Dover *et al.* (1979) made a predator-prey model for *P. ulmi* and discovered that pesticides could be selectively applied to remove the pest species but conserve the predatory one, thus resulting in a more efficient elimination of the problem.

As farmed snails are for human consumption, the use of pesticides is not feasible. Therefore, the likelihood of using a predatory mite in the control of *R. limacum* was investigated along with the possibility of using attractants to lure the mites off snails.

9.2 MATERIALS AND METHODS

9.2.1 Use of a Predatory Mite.

Originally when the permanent mite culture was set up in the laboratory, the aquaria that the snails were kept in were filled with soil. A chance observation revealed that the soil contained numbers of quite large, reddish coloured mites that appeared to be capable of living freely in the soil. It was not certain where they came from but it is likely that they were present either as eggs or in the adult form in the soil used. These mites were seen to carry specimens of *R. limacum* around in their chelicerae and thus it was thought that in the absence of any other food stuffs, they may be gaining nourishment from *R. limacum.*

Before any further observations could be made it was necessary to identify the second species of mite; this was achieved by preparing a series of whole mounts using the same method as that employed in Chapters 3 & 4. The mite was identified as a member of the Mesostigmata; *M. muscaedomesticae.* This is the well-known predator of the house fly; *Musca domesticae.* This mite usually feeds on the eggs of the fly or first instar larvae (maggots) although observations of it piercing and feeding off other mites has been reported. There was a feasible possibility that this mite could be used to control *R. limacum*, especially as initial indications were that *M. muscaedomesticae* was at least attracted to *R. limacum.*

It was thought that the best way to establish whether *M. muscaedomesticae* would control *R. limacum* would be to carry out trials away from the snail to ensure that the predator could react to the possible prey, a situation which would be complicated if experiments were carried out *in situ* due to the parasitic nature of *R. limacum.*

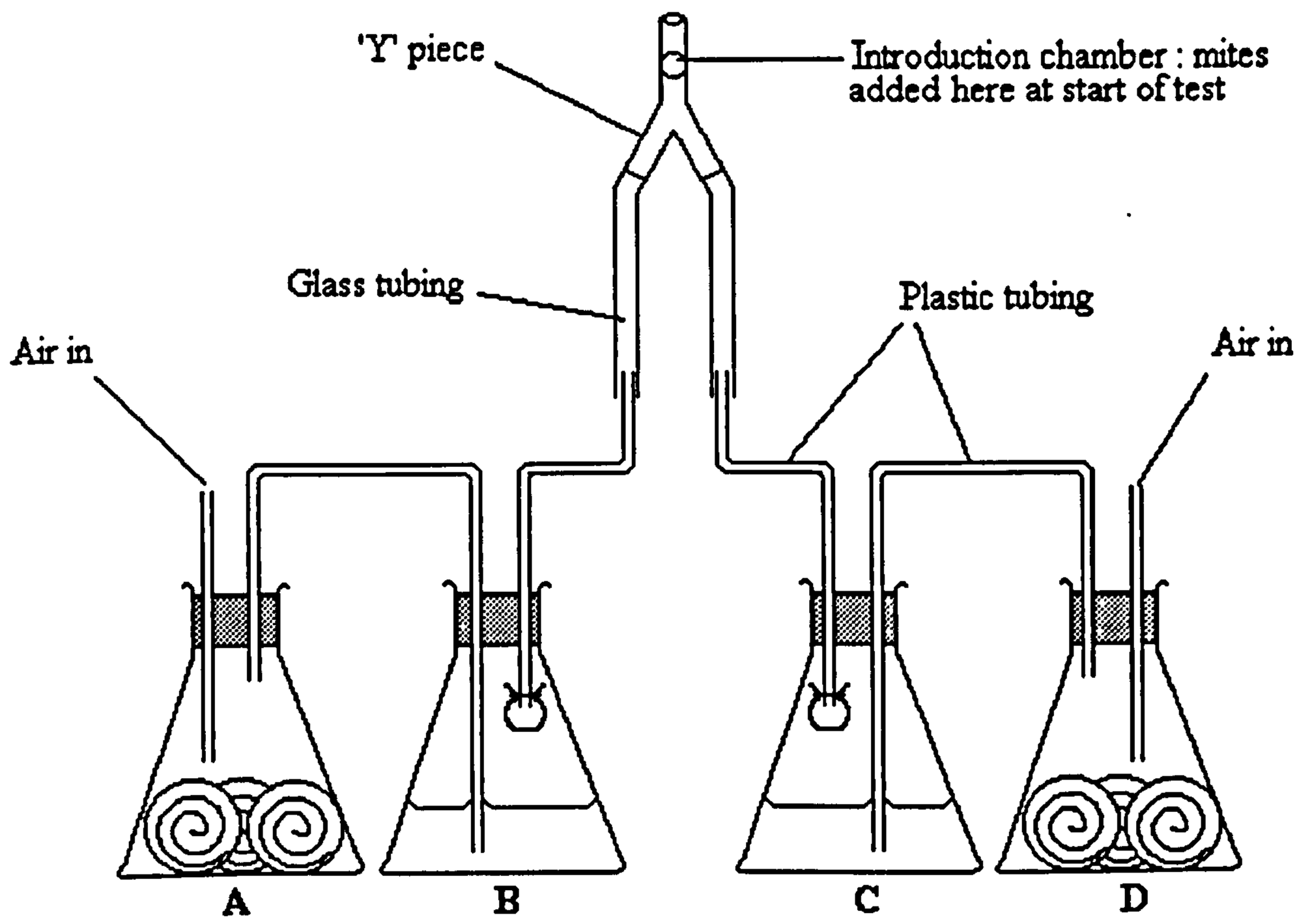
Predator-Prey Trials:

A petri dish was lined with highly absorbent filter paper which was then moistened to create a high relative humidity. One macrochelid mite and ten *R. limacum* were introduced and the lid replaced over the petri dish and secured with an elastic band. Controls were set up in the same way omitting the predatory mite. The petri dishes were placed in an incubator at 20°C with 12:12 hours light:dark cycle and examined every two hours before eventually being left overnight and examined in the morning. This was repeated several times and the numbers of both species of mites varied to see the effect of changing predator-prey densities.

9.2.2 Agents attractive to *R. limacum.*

A choice experiment was designed to test the response of the mite to different stimuli. The apparatus used consisted of an introduction chamber leading into a "Y" piece, each arm of which in turn had a tube leading into a conical flask. The two flasks were sealed with bungs which also had an air inlet pipe for ventilation. Each tube had a small square of nylon wrapped around the open ends to collect the mites that chose that particular tube (Fig. 9.1). The experiment was conducted over 24 hours after which time the mites collected in this manner were examined and counted.

Figure 9.1- Experimental set-up for testing the attractiveness of certain stimuli to mites.



A&D : These were used to contain test stimuli (i.e. snails)

B&C : These were used to contain pottassium hydroxide or silica for the removal of specific stimuli (i.e. carbon dioxide and water)

The choices which were tested during this experiment were as follows:

1. Snails : Blank
2. Water : Blank
3. Mucus : Blank
4. Carbon dioxide : Blank
5. Snails : Water
6. Snails : Carbon dioxide
7. Snails : Mucus
8. Snails : Snails - Water
9. Water : Snails - Water
10. Snails : Snails - Carbon dioxide
11. Water : Snails - Carbon dioxide

Where necessary carbon dioxide was removed from the apparatus using potassium hydroxide, and water with silica gel crystals. Where mucus was required, snails were allowed to crawl around in the conical flask for several hours prior to the trial before being removed. Carbon dioxide was supplied in the form of a large piece of "dry ice" which slowly sublimed to give a steady stream of gas.

Each trial was carried out five times using twenty mites that were initially placed in the introduction chamber.

9.3 RESULTS

9.3.1 Use of a Predatory Mite.

In all trials when the mites in the experimental cell where examined after the initial two hour period, all were seen to be alive. The macrochelid was observed carrying *R. limacum* around in the chelicerae but not killing it. This was the case for up to eight hours after the start of the trials after which time several of the *R. limacum* exposed to *M. muscaedomesticae* were always dead, while all the control mites were still alive. All ten of the *R. limacum* exposed to the predator were found dead after 24 hours in all trials and on examination all appeared shrunken. After the same period of time most of the control mites were also dead, though in each trial at least two control mites remained alive (in one case four).

Therefore it is unclear whether the death of *R. limacum* was due to the removal of body fluids by the machrochelid mite or whether they had died through excessive time spent away from their snail host and consequent starvation and dehydration. When these mites were later carefully examined using the SEM their shrunken appearance was quite obvious. However, no sign of a pierced hole which would attribute death to the predatory mite could be found. This is not surprising as the *R. limacum* were all badly crumpled. When the trials were repeated using more macrochelid mites and the same number of *R. limacum* similar results were observed. Likewise, when greater numbers of *R. limacum* were used with just the single predator the same results were obtained. Evidently further more detailed trials are required to establish whether or not the predatory mite was responsible for the death of the snail mites. They do not however rapidly kill *R. limacum* and therefore can have only limited use in controlling them.

9.3.2 Agents attractive to *R limacum.*

The results of the five repetitions of each trial are summarised below with the mean number of mites quoted for each:

1. Snails	: Blank	20 : 0
2. Water	: Blank	20 : 0
3. Mucus	: Blank	19 : 1
4. Carbon dioxide	: Blank	0 : 0#
5. Snails	: Water	20 : 0
6. Snails	: Carbon dioxide	0 : 0#
7. Snails	: Mucus	20 : 0
8. Snails	: Snails - Water	18 : 2
9. Water	: Snails - Water	2 : 18
10. Snails	: Snails - Carbon dioxide	19 : 1
11. Water	: Snails - Carbon dioxide	19 : 1

All the mites were found dead in the introduction chamber at the end of the test period, this is presumably due to the anaesthetizing properties of the carbon dioxide gas and therefore different concentrations need to be tested in future. It is also possible that sublimation of the "dry ice" caused the temperature to fall below acceptable levels for the mites contributing to their death.

After 24 hours all of the mites had moved to the nylon traps at the ends of the choice tubes and there was a 100% recovery. The results obtained for each replicate, of each trial were subject to very little variation. They exhibited such a definite trend that it was thought unnecessary to carry out any statistical analysis.

9.4 DISCUSSION

While a degree of success was attained in the trial of the predatory mite *M. muscaedomesticae* as an agent for the control of *R. limacum,* in the situation of a snail farm, the results are far from definitive. Trials to see if this was a viable option were somewhat difficult to arrange due to the parasitic nature of the lifestyle of *R. limacum.* As *R. limacum* spends a high proportion of the time inside the pulmonary cavity of the snail host, *H. aspersa* and the larger size of *M.muscaedomesticae* makes it unlikely that it would follow the smaller, parasitic mite inside, the encounter rate of the predator prey system is consequently quite low. For any biological control system to work there must be ample opportunities for the predator to come into contact with the prey. There is an additional problem encountered by any snail parasite: that of copious quantities of highly viscous mucus in which to become entrapped. *R. limacum* copes well with the mucus considering the numerous hairs covering its body, but it is unlikely that a foreign predatory mite introduced to the alien environment would cope as well. The performance of a particular predator also varies with the prey densities and dispersal (Huffaker *et al.,* 1970) and consequently in a snail farm the searching efficiency of any predatory mite used would be related not only to the dispersal and distribution of *R. limacum* but also to the behavioural movements of the snails resulting in highly variable distributions of parasitic mite.

It was not possible to tell whether snail mites were killed by the predator, or by removal from their hosts resulting in dehydration and starvation. This leaves open the possibility of further research into the biological control of this species, although it is a highly unusual situation in which to apply this method. Biological control is normally used against free living leaf feeding mites, (McMurty & Van de Vrie, 1973; Huffaker *et al.,* 1970), where predatory mites have been highly successful and

provide a commercially viable option. Use of predatory mites is essential in enterprises such as glasshouse agriculture (Hussey *et al.*, 1969). Red-spider mite populations run continuously in the controlled environment instead of ceasing in the autumn for hibernation and thus their numbers build up, a predatory mite is therefore used. The efficiency of a hypothetical predator is likely to be reduced when the prey is feeding inside the lung of snails. Control might be possible as *R. limacum* often runs freely over the snail surface opening itself up to predation. A predatory mite that did not become trapped in mucus and was small enough to enter the snail lung and attack the snail mites would be more efficient and this would also expose the eggs of *R. limacum* to predation. This would be an advantage as many of the known predatory mites, including *M. muscaedomesticae*, feed preferentially on the egg stage. Whether or not this is a possibility is not known but evidently further trials need to be carried out before any firm conclusions can be drawn concerning the successful biological control of *R. limacum* using *M. muscaedomesticae* or any other predatory species.

The tests on possible attractants for *R. limacum* showed definite preferences when a choice was provided. When faced with the choice of an empty flask with no stimulus or a flask which contained either mucus, water or snails, the mite always chooses the flask with the test substance. Carbon dioxide however had a highly detrimental effect on the mite.

When a choice of different stimuli was offered, snails were the most powerful attractant. The mites were preferentially attracted to the odour of snails with nothing removed, compared with snail odour with water vapour or carbon dioxide removed. However they prefer the odour of snails with the water vapour removed, to water vapour alone and so there is obviously another factor involved besides the presence

or absence of water. The snail odour that was passed through potassium hydroxide before reaching the mites, was not as attractive as water alone.

Clearly the snails produce a volatile attractant. Emission of chemicals from the pneumostome glands has been studied in the past by Lloyd (1969) working on *Oxychilus allarius*, the garlic snail. This snail emits an odour from cells near the mantle flap when irritated, this substance is thought to be for defence and is almost certainly sulphurous and dietary in origin. It is possible to isolate the odour producing cells in this species and therefore future work on *H. aspersa* might include the isolation of similar cells with a view to breeding a snail without them. Alternatively identification of the substance could allow synthesis of the attractant for commercial use in baiting traps to kill the mites. The effect of the attractant odour in *H. aspersa* is possibly reinforced by the carbon dioxide that they exhale, while water vapour is a less significant modifying factor.

Since normal respiring snails were clearly the most powerful attractant, a manufactured attractant to attempt to lure mites away from farmed snails may be too simple to overcome the normal snail stimulus. However some artificial mixture including mucus and traces of odour (if present) might offer promise in confusing the mites and reducing host location efficiency if incorporated into traps of some kind.

The most effective systems of control are those that combine different methods, indeed this is the suggestion of workers such as Axtell (1963) and Dover *et al.* (1979), although they were implementing the use of a biological agent and an acaricide rather than a chemical "lure". If using a "lure" along with a predatory mite was possible, a highly effective control system could perhaps be developed.

CHAPTER 10

GENERAL CONCLUSIONS AND DISCUSSION

10.1 GENERAL CONCLUSIONS

1. *Riccardoella limacum* and *Riccardoella oudemansi* are two similar but separate species living on different hosts; *R. limacum* preferring snails and *R. oudemansi,* slugs.

2. The two species can be separated by the differences in structure of the body setae, *R. limacum* has elongate hairs whilst *R. oudemansi* has swollen, rounded hairs, and by the fact that *R. oudemansi* has an extra hair on each of coxae 1 and 3.

3. The two species have different life cycles; *R. limacum* has an overwintering egg enabling it to survive while its snail host is hibernating, while *R. oudemansi* does not appear to have one and it is unclear how they survive the winter as many slugs die at this time.

4. Historesin plastic sections for light microscopy give good resolution of the internal anatomy of *R. limacum.*

5. The internal anatomy observed, mostly confirms work already done by previous authors on this and related species.

6. The main difference was the presence of a paired oviduct not seen in this species before.

7. Oviducal eggs were seen in specimens of the adult female.

8. Three-dimensional computer-aided reconstruction of the mite showed the internal organs and how they relate to each other.

9. The stages in the life cycle were confirmed as egg > larva > protonymph > deutonymph > tritonymph > adult.

10. Larvae are hexapod and nymphs octopod.

11. Nymphs can be separated from each other on the basis of increasing size and increasing number of genital setae.

12. The male form is rare but there is no evidence, as previously suggested, that it is regressing from the life cycle.

13. Larvae and nymphs are also rare, this is due to the fact that they are short-lived stages.

14. The snail hosts in the wild do not hibernate as early or for as long when they are immature.

15. Immature snails are less likely to have mites than adults possibly due to the limiting aperture size of the pneumostome.

16. Mites are quick to disperse to new host snails and large numbers can build up quickly.

17. Numerous eggs are laid in the lung by the mite on first entering a new host.

18. Immunological techniques show that haemocyanin from Helix aspersa is present within the mites.

19. It is probable that *R. limacum* ingests snail haemolymph as a food source.

20. *R. limacum* lives mainly in the lung where humidity is high, a food source is readily available and consequently ideal conditions prevail for the proliferation of the mite population.

21. The haemocyanin detected in the mite does not originate in the snail mucus.

22. Mucus cannot be discounted as a food source of this mite on the strength of evidence gained in this study.

23. Snails infested with large numbers of mites have poor quality shells, slow growth and delayed sexual maturation.

24. The respiratory epithelium of snails with large numbers of mites is vacuolated and the cells damaged.

25. There is no evidence of a feeding tube produced by the mite in the lung or indeed is there any real need for one on such a thin surface.

26. There is no difference in the concentration of proteins and glycogen/free glucose in the tissues of snails with and without mites.

27. There is a dramatic difference in total protein and glycogen/free glucose in snails with and without mites when the dry weight of the whole snail is considered.

28. Excessive numbers of mites cause a growth not a storage problem.

29. Low numbers of mites can be tolerated without too much ill effect.

30. The predatory mite *Macrocheles muscaedomesticae* showed an interest in *R. limacum* and it is possible, but by no means certain, that it was responsible for the deaths of some specimens of *R. limacum.*

31. Any truly efficient predator would need to be small enough to enter the lung of the snail through the pneumostome in order to gain access to the eggs of *R. limacum.* Such a predator would also need to be able to walk over the snail mucus without getting trapped.

32. *R. limacum* is attracted to certain stimuli, snails being the most attractive of all.

33. It is possible that snails emit an odour from certain glands that the mites find attractive, it may be possible to isolate this and use it to confuse the mites thereby reducing host relocation efficiency.

10.2 GENERAL DISCUSSION

It is clear from these studies on the mite *R. limacum* that this particular species, and indeed the whole of the family Ereynetidae in general, has been very poorly studied in the past. The most extensive works available on the family Ereynetidae are those of Thor (1932 & 1933) and Grandjean (1939). Where work has been carried out more recently on *Riccardoella spp.* it has been brief with errors of identification.

Many authors have worked on the slug mite which has since been shown to be *Riccardoella oudemansi* (Baker, 1945; Turk & Phillips, 1946; White, 1959; Baker, 1967, 1970a, 1970b; Arutyunyan, 1972; Mienis, 1984). Few of these seem to have been entirely accurate in their descriptions; André & Lamy (1930); Thor (1932); Karbarz-Wiktorowicz (1973); Fain & Van Goethem (1986) being among the few who correctly identified the mites they were working on. This confusion has been observed quite recently by Polaco & Mendl (1988) who noted that in one work a mite collected from some snails was mistakenly called *R. oudemansi* when the accompanying figures showed it to be *R. limacum.*

The stages in the life cycle were confirmed as egg > hexapod larvae > protonymph > deutonymph > tritonymph > adult. These observations agree with those of Baker (1970) although his descriptions of the proportions of the different life stages present at any one time were not the same as in the present study, presumably because he was working on *R. oudemansi.* The life cycles of these two species of mite are different as would be expected of species with different hosts. The snail hosts of *R. limacum* hibernate from October to March in the wild during which time overwintering eggs carry the mite population through to spring. The slug hosts of *R. oudemansi* usually die out altogether making overwintering eggs useless.

However, although this is the situation in the wild, the whole object of a snail farm is to fatten as many snails as possible in as short a time as possible and therefore the environment of a farm is maintained such that snails do not hibernate. It is possible that in a modified environment such as this the life cycle of the mite is altered and overwintering eggs may not be laid. This situation arises in glasshouses where the life cycle of red-spider mites runs continuously without the break for hibernation that normally occurs in the wild (Hussey *et al.*, 1969). Though it is possible that *R. limacum* still lays overwintering eggs in a farmed situation, it would depend on the exact criteria required for the mite to lay such eggs.

Conditions on a snail farm must be carefully monitored as environmental factors have long lasting effects on snail development, particularly of the reproductive system. Temperature is one of the main influences on growth (Carrick, 1942) and the development of the reproductive system. In *Limicolaria martensiana*, the actual breeding season is correlated with the temperature (Owiny, 1974). Smith (1966), noted that maturation of the slug can be retarded by extremes of temperature and this in turn causes abnormalities in the gonad such as resorption of gametes and an immature reproductive tract. The gonad appears to be the main organ affected by the environment, this is the case in *Arion ater rufus* which displays a preponderance of gametes of one sex or the other according to the prevailing temperatures (Lüsis, 1966; Smith, 1966). It seems that in low temperatures, conditions favour the production of oocytes and suppress male development, whilst the reverse is also true (Parivar, 1978) although DeWitt (1954) states that all gamete production was inhibited by low temperatures in *Physa gyrina*. The incubation period of eggs laid by the snail *Achatina (Calachatina) marginata* (Plummer, 1975) was also affected by the temperature. It is possible that the effects brought about by adverse temperatures are due to the reduced food assimilation rates at extreme temperatures.

In *Helix aspersa*, percentage assimilation of food is temperature dependant between 5-15 ^{0}C (Mason, 1970).

The amount of light at any particular time also has a strong affect on development, this can be seen in the slug *Limax maximus* in which the maturation of the reproductive system is influenced by the photoperiod (Sokolove & McCrone, 1978). In *H. aspersa* the change to a constant temperature and a light regime of twelve hours light, when brought into the laboratory caused earlier maturation of the reproductive system than is expected in the wild with the natural variation in environmental conditions (Bailey, 1981). Alternative light regimes affect both growth and reproduction in *H. aspersa* and *H. lucorum* (Lazaridou-Dimitriadou & Bailey, 1991) growth rates were best in 24 hour regimes whilst reproductive potential was maximised in a 20 hour cycle. A long day cycle such as 18 hours light: 6 hours dark promotes reproduction (Gomot, 1990), though sensitivity depends on the species. *H. aspersa* is more sensitive to light regimes than *H. pomatia*, but in general is easier to culture (Lazaridou-Dimitriadou & Bailey, 1991).

Obviously there are many factors involved in the growth, maturation and general well being of the snail; not all deleterious effects are due to a mite infestation. They are also very particular about the crowding factor; high densities cause slow growth rate, smaller snails at maturity and the formation of the shell lip is delayed (Lucarz & Gomot, 1985). It has been suggested that keeping more than two snails together will give inconsistent results, long periods of inactivity resulting in irregular growth and less frequent reproduction (Hertzberg, 1983). In the environment of a snail farm, this is impossible and therefore stocking densities must be higher even if it is at the expense of some growth. Although in the past it was thought that the reason that snails showed reduced growth in crowded conditions was due to the repelling affect

of stale mucus left by the snails, this is not the case. Dan & Bailey (1982) showed that even with mucus removed regularly juvenile *H. aspersa* grow more slowly in crowded conditions and if a larger container is used this presents problems in keeping the humidity high enough for this not to be an inhibiting factor. However in *Physa gyrina*, DeWitt (1954) showed that isolated animals actually take longer to reach maturity but they were bigger and laid three times as many eggs, though the viability increased when snails were kept together. Isolated snails also have a shorter life span than grouped animals probably due to the increased reproductive potential that they seem to show. However in a farm concentrating on fattening vast quantities of snails, the life span is of no consequence, reproductive potential would also usually take second place to growth.

If the points discussed above are taken into consideration, there is no reason why a generally healthy population of snails should not be maintained. However in making recommendations to a snail farmer concerning the problem of mites, it is suggested that the following precautions be taken.

If snails are brought into a farm in the winter months and they have been hibernating for any reason, it is possible that any mites present will have laid overwintering eggs before dying. It is likely that a random check of all the imported animals will reveal that they are mite free and without killing the snail it is not possible to tell whether it contains mite eggs. It is therefore advisable that new animals only be purchased in the summer months when any mites present will be active, unless it can be categorically stated that the snails in question have not been in hibernation thus denying mites any opportunity of laying overwintering eggs.

From observations made in this study it is advisable to purchase snails as young as

possible as they have rarely been found to be infected with mites or eggs whilst still immature, presumably because the pneumostome is too small to effect entry in such a snail without the snail responding. As soon as a snail is irritated by anything, such as a relatively large mite trying to gain access, copious quantities of mucus are exuded through the pneumostome thus flushing out potential intruders. Immature snails are also less likely to have undergone any hibernation period prior to purchase as they tend to delay this until a larger body mass is attained, therefore they are less likely to contain mite eggs.

As soon as the new stock is purchased, they should all be examined, obvious contamination will usually be easy to spot and such snails discarded immediately. A quarantine period of approximately three weeks should be applied to the remaining animals, this will allow any eggs that are present (excluding overwintering eggs) to hatch. After this time the snails should be reexamined and further expulsions made if necessary. While removing a snail on the grounds that it is contaminated with only a few mites may seem extreme, it must be remembered that there are usually many more mites inside the lung of the snail than will be immediately visible on the outside. It is also worth noting that if mites are present, they are more likely to be found on the outside during times of dormancy of the snail.

Once a mite-free stock has been obtained it is necessary to maintain it and this is obviously difficult as much of the available literature shows that *R. limacum* is one of the main pests to be found on a commercial farm (Jackson *et al.*, 1974; Morand, 1985). One point of utmost importance is that of preventing contamination of farmed animals by wild snails. All too frequently doors and windows are left open, especially during the summer when the heat can be oppressive, and this gives easy access to wild snails. The humid conditions inside the farm, ideal for fattening the

commercial snails, also provide an ideal sanctuary from the dry, summer heat outside for wild snails that are likely to be infested with mites. Doors and windows must therefore remain shut at all times and a watchful eye kept for any wild snails that manage to enter by any other means. If mites do appear in the farm it would be wise to monitor the population spread. Morand (1988), when looking at the nematodes parasitising *H. aspersa*, used a computer modelling program and was able to conclude that there were several mechanisms at work within the host-parasite system.

The above recommendations are all for preventative measures, it is advisable that other methods are considered as well. If a predatory mite, were found, that was small enough to enter the snail lung to reach the eggs of *R. limacum* and could cope with viscous snail mucus, then successful biological control might be possible. If *H.aspersa* has an odour gland similar to the one found in *Oxychilus allarius*, and the odour could be isolated and used in conjunction with a trap, it would be possible to at least confuse the mites and keep mite numbers to a minimum. The use of "Hawkyard" cages for snails utilizes a daily spraying with a jet of water to remove stale food and mucus. This would be likely to remove any mites that happen to be on the outside of the snail at the time and thus exert some control over the mite population. Unfortunately conditions found in intensive snail farms are also ideal for mite populations to expand and it is not certain at this time whether it is possible to keep the mite numbers under control in this way.

However, it has already been stated that is possible for a snail to grow well and mature while infested with a very low mite parasite load. So if all the above recommendations are followed and the snails constantly checked externally for mites, those with them being removed immediately, it ought to be possible for a farmer to

keep mites down to a level that does not affect the growth of the snails, and thus maximise his profits.

APPENDIX

Solutions used during the course of experiments in Chapter 8:

1. 30% Acrylamide Stock Solution

Acrylamide	29.2g
Bis-acrylamide	0.8g
Distilled water	100ml

Mix and filter through 0.22μm Millipore filter.

2. Bottom Tank Buffer

50mM Tris pH 8.8

3. Electroblotting Buffer

Tris	25mM	$3.025 gl^{-1}$
Glycine	190mM	$14.260 gl^{-1}$
Methanol	20%	$200 ml l^{-1}$

4. Loading Buffer

Glycerol	50%
SDS	2%
BME	5%

Make up in 0.315M Tris pH 6.8, add a hint of bromophenol blue.

5. Resolving Buffer

Tris	36.4g

Distilled water 100ml

Adjust with 5M HCL to pH 8.8, filter through 0.22μm filter.

6. Secondary antibody

Goat-anti-rabbit IgG (Sigma) 40μl

TTBS 20ml

BSA (Cohn Fraction V) 1%

7. Silver Stain

Silver nitrate 0.2% in distilled water; keep in the dark.

8. Sodium Dodecylsulphate Stock Solution 10%

SDS 1g in 10ml distilled water.

9. Stacking Buffer

Tris 43g in 100ml distilled water, filter through 0.22μm filter, adjust to pH 6.8 with 1M HCL.

10. Enzyme substrate solution

1-4 Chloronaphthol 20mg in 4ml methanol

TBS 20ml (warmed)

Hydrogen peroxide 10μl

11. Transblotting Solution (TBS)

Tris 20mM $2.42gl^{-1}$

Sodium chloride 0.9%

Adjust to pH 7.2 with 1M HCL.

12. Tween Transblotting Solution (TTBS)

Tris	20mM	2.42 gl^{-1}
Sodium chloride		0.9%
Tween 20		0.1%

Adjust to pH 7.1 with 1M HCL.

13. Upper Tank Buffer

Tris	50mM
Glycine	190mM
SDS	0.1%

DO NOT adjust with HCL, filter through 0.22μm filter.

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