STUDIES IN GALL INDUCTION
WITH SPECIAL REFERENCE TO THE *PONTANIA*-*SALIX* SYSTEM

ROGER NEWELL HIGTON

WADHAM COLLEGE

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

HILARY TERM 1991

ABSTRACT

An investigation was conducted into gall induction in the leaves of *Salix fragilis* L. var. *russelliana* (Sm.) Koch by *Pontania proxima* (Lepeletier) (Hymenoptera: Tenthredinidae). The work was divided into four parts:-

a) Reviews of the available literature were undertaken on: hypotheses concerning cecidogenesis of galls initiated by insects, the history of cecidology as it related to galls formed by insects, the ontogeny and morphology of galls produced by sawflies, ecological aspects of gall biology and the effects of galling on the host plant.

b) Methods of rearing both insect and host were established; these resulted in an extension of the insect's flight period from five to eight months with three broods per year instead of the usual two. Further work established the topological specificity of oviposition. Preliminary studies were completed on the tissue culture of *Salix* leaf-disc explants and the fusion of protoplasts released from normal and galled tissue.

c) Using light and electron microscopy, a study was made of the reproductive system and associated glands of *Pontania proxima* females, together with the first stages of the procecidium they initiated. Using these techniques, no microorganisms, viral or otherwise, were observed in the gall, in the lumina of the reproductive system and associated glands or in surrounding tissues of the insect. Evidence for a secretory rôle for the lateral oviducts was found. A structure termed the vaginal valve was described and it was hypothesized that this functioned to separate the fluids produced by the accessory glands from the contents of the oviduct, until oviposition. In the plant, it was observed that the gall effect was limited and that the presence of an egg or larva was not required for the formation of a procecidium. Gall growth was mainly due to periclinal divisions of the provascular tissues of the leaf.

d) A bioassay, based on microinjection techniques, was developed . This demonstrated that the cecidogen was contained in the colleterial fluid produced by the accessory glands. Further analysis showed that the cecidogen had a molecular weight of less that 3 kDa.

STUDIES IN GALL INDUCTION

WITH SPECIAL REFERENCE TO THE PONTANIA-SALIX SYSTEM

Roger Newell Higton

Wadham College

A thesis submitted for the degree of Doctor of Philosophy

University of Oxford

Hilary Term 1991

PREFACE

Gall biology is a subject that covers a wide range of topics. From these it was decided that an investigation into the mechanism of gall induction, by an insect, should be chosen as the major theme of this thesis. From an initial survey of the literature it was soon apparent that not only was work in this area limited, but much of it suffered from an overconcentration on one partner in the relationship, with little attention paid to the other.

It was decided, therefore, that the approach employed would cross the traditional boundaries of botany and zoology, in an attempt to produce a synthesis of all that was known and could be investigated in three years about one particular gall former and its host.

This decision has dictated the format of this thesis. Thus, the first part is a review of the available literature, not only of gall biology in general, but in particular an emphasis has been placed on galls produced by sawflies in an effort to identify those areas that have suffered from a lack of attention. The history of gall biology, the ontogeny of the gall and insect, the ecological aspects and the effects of galling, as they are related to the theme of this thesis, are all reviewed in Chapter Two. The hypotheses that are concerned with an explanation of how galls are induced are discussed in Chapter Three. The contents of these two chapters serve to illustrate the lack of progress that had been made by any mechanistic investigation into gall induction.

The work for this review also proved to be invaluable in providing a background for the experimental studies undertaken as part of this project. It identified areas of ignorance and those that would benefit from further study: it ensured the formulation of a research programme leading to an integrated approach for the remainder of the thesis.

One aspect of this integration was the development of methods that would allow a wide range of hypotheses to be tested, since it was felt that the review showed that many previous studies had concentrated on far too narrow a front of investigation.

ACKNOWLEDGEMENTS

During the three years I have spent at Oxford, I have received instruction, advice and co-operation from many people. Without exception, everyone I have met has given of their time and expertise to my advantage. I must thank them not only for this, but also for making my studies at Oxford such a pleasant experience. In these acknowledgements, I hope that I have not forgotten anyone who has supported me in this work; if I have, I apologise for my error now.

Firstly I would like to express my thanks to Dr. D. J. Mabberley and Dr. G. C. McGavin. Both, in the course of this work, have been my Supervisors. They not only introduced me to a fascinating subject, but through their expertise and professionalism have provided me with support and guidance, that can only be described as "second to none".

I must thank Professor F. R. Whatley and Professor C. J. Leaver, both of whom contributed to the success of this project, also the Science and Engineering Research Council who funded my studentship. As well as encouraging me to become a mature student, Professor Whatley was my Supervisor for one year, during which he brought a new dimension to my work.

I also have to thank my colleagues - Anne Sing, Dr. M. Cheek, Mr. T. Clark, Dr. B. M. P. Singhakumara, Simon Bennett, and Rowan Jenkins - who shared Room 203, although much of their work was very different from mine: they were very patient with the upheaval I caused, and by their presence made my working environment a happy one.

Within the Department of Plant Sciences a special acknowledgement must be made of the assistance I received from Dr. J. Coleman who encouraged and supported my efforts in much of the biochemistry and tissue culture work; Mr. C. Merriman, who instructed me in electron microscopy techniques; Dr. J. M. Whatley for advice on electron microscopy and permission to use the micrographs shown in Plates 5.29a and 5.31; Dr. A. Banham who introduced me to the HPLC apparatus; and Mr. J. Baker who instructed me in photographic techniques. Also within the Department, I must thank Dr. F. A. L. Clowes, Ms. L. Cole, Mr. B. Collins. Dr. F. M. Dewey, Dr. D. Durell, Dr. M. Fricker, Dr. A. Fry,

Mr. R. Gardner, Dr. W. Greenaway, Ms. R. Haworth, Mr. D. Headford, Dr. C. R. Huxley-Lambrick, Ms. L. Lockhart, Ms. S. Marner, Dr. B. A. McLean, Dr. D. J. Osborne, Dr. R. Pearce, Dr. R. Priestly, Mr. F. Roche, Mr. F. Topliffe and Ms. R. Wise (especially for Figure 4.1) - all have, either through advice or technical assistance, made my work more profitable. I would also like to thank the staff of the Plant Sciences Library and Information Service, who did much to help me find and acquire literature; Ms. L. Garbrick, Mr. D. Powell and Ms. J. Powell for providing translations of foreign papers; the computing staff, especially Dr. C. Goodwin Bailey; the secretarial and administrative staff of the Department, especially Ms. E. A. Stedman whose advice was always worth following.

Outside the Department I received much good advice from a number of sources. In correspondence Dr. M. Anthony (Laboratoire de Cecidologie), Mr. J. Atkinson and Ms. S. Zabarich (International Bee Research Association), Dr. C. I. Carter (Forestry Commission), Dr. D. F. Cutler (Royal Botanic Gardens, Kew), Dr. M. Fay (Kew Micropropagation Unit), Dr. J. Good (Bangor Research Station); the Rt. Hon. Michael R. D. Heseltine MP; Dr. D. R. McCalla (McMaster University), Dr. J. Meyer (Bebe-beraq, Israel), Dr. H. Parry (University of Aberdeen) and Dr. P. W. Price (Northern Arizona University) - all took time to answer my letters and replied with useful information.

Others to whom I am indebted include Dr. R. Buxton and The Northmoor Trust who kindly allowed me to collect galls at Little Wittenham Nature Reserve - these collections formed the bulk of the biological material used in this thesis; Dr. S. Hartley (University of York) and Dr. I. Leitch (University of Cambridge), who had both worked on Pontania, and meeting them provided an opportunity for discussion and debate. Within the environs of the University of Oxford, Dr. R. T. Aplin; Dr. R. Beddington (Imperial Cancer Research Institute); Dr. M. L. Birch; Dr. J. Cory and Mr. P. Entwistle (Institute of Virology); Dr. K. Dyke, my College Tutor, who introduced me to Wadham; Dr. C. O'Toole; and Dr. M. Speight. All allowed, either the use of their facilities, or provided encouraging discussions.

Finally, I must thank my family (extensions as well) - Ian and Neil

who showed great patience with their father while he was occupied with other things; Tamsin, Xavier and Andrew who supported, collected galls and humoured me; and Dreena, who above all others shared this experience at every stage and is the person to whom I owe the most.

CONTENTS

PAGE

PLATES.....viii

TABLES.....xv

FIGURES.....xvii

ABBREVIATIONS.....xx

INTRODUCTION.....1
1.1 **Definitions**.....1
1.2 **Aims and objectives**.....5

THE PONTANIA/SALIX SYSTEM.....8
2.1 **Introduction**.....8
2.2 **History**.....10
2.3 **Synonymy**.....20
2.4 **Occurrence**.....21
2.5 **Emergence of imago**.....22
2.6 **Oviposition**.....24
2.7 **Reproductive strategies**.....31
2.8 **The nature of the gall**.....34
2.8.1 The dynamics of interactions.....37
2.8.2 Impacts.....43
2.9 **The adaptive nature of the gall and evolution**.....45

HYPOTHESES CONCERNING CECIDOGENESIS IN GALLS INITIATED BY INSECTS.....50
3.1 **Introduction**.....50
3.2 **Hypotheses**.....52
3.2.1 Cytokinin hypothesis - Elzin (1983).....53
3.2.2 Auxin hypothesis - Miles (1968a & b).....54
3.2.3 Amino acid hypothesis - Anders (1961).....55
3.2.4 Cynipine-virus mutualism hypothesis - Cornell (1983).....56
3.3 **Further considerations**.....58
3.3.1 Lignin formation as a response to chitin.....59
3.3.2 Anthocyanin affecting polar auxin transport (PAT).....59
3.3.3 Abscisic acid.....60
3.3.4 Ecdysone and gibberellins.....60
3.4 **Conclusions**.....63

REARING AND CULTURING.....65
4.1 **Introduction**.....65
4.2 **Propagation of the host**.....66
4.2.1 Introduction.....66

4.2.2 Establishment of cuttings....69
4.3 **Rearing Pontania proxima**....74
4.3.1 Introduction....74
4.3.2 Collection and rearing....74
4.4 **Observations of oviposition**....83
4.5 **Topological specificity**....87
4.5.1 Introduction....87
4.5.2 Methods....88
4.5.3 Results....90
4.5.4 Discussion....95

MORPHOLOGY AND ULTRASTRUCTURE....98
5.1 **Introduction**....98
5.1.1 Aims....98
5.1.2 The insect....100
5.1.3 The gall....104
5.2 **Methods**....111
5.2.1 Introduction....111
5.2.2 Negative staining....111
5.2.3 Embedding schedule....112
5.3 **Results**....119
5.3.1 The structure of the internal reproductive system and associated glands of Pontania proxima....119
5.3.2 The gall....148
5.4 **Discussion**....159

THE CECIDOGEN....168
6.1 **Introduction**....168
6.2 **Methods of injection and the development of a bioassay**....170
6.2.1 Introduction....170
6.2.2 Methods....172
6.2.3 Results....178
6.2.4 Discussion....184
6.3 **Initial studies of the colleterial fluid**....187
6.3.1 Introduction....187
6.3.2 Methods....190
6.3.3 Results....194
6.3.4 Discussion....202
6.4 **Further separation**....203
6.4.1 Introduction....203
6.4.2 Methods....205
6.4.3 Results....208
6.4.4 Discussion....216

DISCUSSION....218
7.1 **Introduction**....218
7.2 **Gall-induction by insects**....219
7.3 **Research undertaken**....220
7.4 **Experimental limitations**....221
7.5 **Future research**....222
7.6 **Methodology**....222
7.7 **Conclusions**....223

REFERENCES.....224

APPENDIX ONE.....260
Tissue Culture.....260
A1.1 **Introduction**.....260
A1.1.1 The rôle of tissue culture techniques in the study of insect-gall induction.....260
A1.1.2 Methods used throughout.....262
A1.2 **Induction and maintainance of calli**.....268
A1.2.1 Introduction.....268
A1.2.2 Methods.....271
A1.2.3 Results.....273
A1.3 **Micropropagation of plantlets**.....277
A1.3.1 Introduction.....277
A1.3.2 Methods.....280
A1.3.3 Results.....280
A1.4 **Protoplast extraction**.....283
A1.4.1 Introduction.....283
A1.4.2 Experiment to estimate optimum plasmolyticum for leaf and gall tissue.....284
A1.4.3 Reagents used for protoplast extraction.....287
A1.4.4 Methods.....288
A1.4.5 Results.....289
A1.5 **Discussion**.....291

APPENDIX TWO
Locations of field sites used.....294

APPENDIX THREE
Weather observations for the local area.....299

APPENDIX FOUR
Results of studies on topological specificity of oviposition....303

APPENDIX FIVE
Cocoon size.....319

APPENDIX SIX
Insect Ringer.....320

APPENDIX SEVEN
Measurements of leaf and gall growth.....321

APPENDIX EIGHT
Measurements.....322
A8.1 Colleterial sac.....322
A8.2 Diameter of accessory gland tubule.....323

APPENDIX NINE
HIGTON. R. N., (1989). Historical perspectives in the development of cecidology. Cecidology. 4(3): 5-9.....324

PLATES

PAGE

Plate 2.1. Genital apparatus of Pontania proxima in water (from Beyerinck, 1888); vs. colleterial sac; gv. accessory gland; ovr. ovaries with ripe and unripe eggs; sg. saw; ar. last abdominal segment.....19
Plate 4.1. Pollarded trees by flowing water, the type of habitat favoured by Pontania proxima. Northmoor Nature Reserve, Little Wittenham (22.5.89 see Appendix Two).....68
Plate 4.2. Original cuttings of Salix fragilis. Note the small size of the leaves compared to those of the larger cutting shown in Plate 4.3.....72
Plate 4.3. Later cutting of Salix fragilis using a "truncheon". Note that the leaves are larger and more numerous than those shown in Plate 4.2.....73
Plate 4.4. Pontania proxima (Lepeletier) (Hymenoptera: Tenthredinidae) (scale divisions mm).....75
Plate 4.5. Cocoon of Pontania proxima (scale divisions mm).....82
Plate 4.6. Third brood galls produced by Pontania proxima and maintained under the conditions detailed in this Chapter. Photograph taken six weeks after oviposition. (scale divisions mm).....85
Plate 4.7. Third brood gall produced by Pontania proxima and maintained under the conditions detailed in this Chapter. Photograph taken six weeks after oviposition (scale divisions mm).....86
Plate 4.8. Galls of Pontania proxima on the leaves of Salix fragilis.....87
Plate 4.9. Terminal bud of Salix fragilis showing the outer leaves just separating (Mag. x2.0).....95
Plate 5.1. Internal reproductive organs and associated glands of Pontania proxima; ag. accessory gland; cs. colleterial sac; e. egg; lo. lateral oviduct; mo. median oviduct; ovl. ovarioles; s. saw (Mag. x40).....120
Plate 5.2. Section through lateral oviduct and ovarioles of Pontania proxima. Thickness five microns. Fixation using protocol one. Stain p-phenylenediamine; c. calyx; e. egg; f. follicular epithelium; lo. lateral oviduct; oo. developing oocyte; ovl. ovariole; tr. trophocytes (Mag. x140).....121
Plate 5.3. Unstained squash preparation of the colleterial sac, spermatheca and median oviduct of Pontania proxima, mounted in water; cs. colleterial sac; mo. median oviduct; spe. spermatheca (Mag. x134).....122
Plate 5.4. Section through the distal portion of the accessory gland of Pontania proxima. Thickness two microns. Fixation using protocol two. Stain p-phenylenediamine; cu. cuticle (duct to secretory cell); en. end-apparatus (of secretory cell); ics. intercellular spaces; lu. lumen (of accessory

gland tubule); n. nucleus (of secretory cell) (Mag. x168).....125
Plate 5.5. Transverse section through the distal region of the accessory gland of Pontania proxima, similar to Figure 5.2. Fixation using protocol two; bm. basement membrane; cu. cuticle; en. end-apparatus; go. golgi complex; lu. lumen (of tubule); m. mitochondrion; mv. microvilli; n. nucleus; nu. nucleolus; pm. plasma membrane; rer. rough endoplasmic reticulum; ser. smooth endoplasmic reticulum (Mag. x6280).....126
Plate 5.6. Section through part of the cuticle-lined duct that passes from the epidermal cells into the invaginated apical portion of the underlying secretory cells of the accessory gland of Pontania proxima. Fixation using Protocol two; cu. cuticle; cy. cytoplasm (of epidermal cell); go. golgi complex; lu. lumen (of duct); m. mitochondrion; pm. plasma membrane; rer. rough endoplasmic reticulum (Mag. x15000).....127
Plate 5.7. (a) Secretion from the tubule of the distal portion of the accessory gland of Pontania proxima. (b) Secretion contained within the colleterial sac. Fixation using Protocol two (Mag. x450K).....129
Plate 5.8. Sections through the accessory gland of Pontania proxima, just before it enters the colleterial sac. Thickness two microns. Fixation using protocol two. Stain p-phenylenediamine; cs. colleterial sac; cu. cuticle (lining duct); en. end-apparatus; ens. densely staining end-apparatus; lu. lumen (of accessory gland tubule); mu. muscle (Mag. (a) x1600, (b) x1066). Light microscopy.....130
Plate 5.9. A cell from the accessory gland of Pontania proxima, seeming to be undergoing lysis. Fixation using Protocol two; h. haemolymph (Mag. x3750).....131
Plate 5.10. Wall of an abdominal ganglion lying on dorsal surface of median oviduct of Pontania proxima. Fixation using Protocol two; g. glial cells (with processes passing between cell bodies of neurons); h. haemolymph; mu. muscle; nl. neural lamella; np. neuropile; pk. perikaryon; pn. perineurium (Mag. x4630).....133
Plate 5.11. Developing oocyte with associated trophocytes of Pontania proxima. Thickness two microns. Fixation using protocol two. Stain p-phenylenediamine; f. follicular epithelium; nup. nutritive pore; oo. oocyte; tr. trophocytes (Mag. x570).....134
Plate 5.12. Longitudinal section through the wall of the distal portion of the lateral oviduct of Pontania proxima. Fixation using Protocol one;

mf. muscle fibrils; h. haemolymph; lu. lumen (of oviduct); nu. nucleolus; p. peritoneal membrane (Mag. x12500).....136

Plate 5.13. Apical surface of cells lining the distal portion of the lateral oviducts of Pontania proxima. Fixation using Protocol one; go. golgi complex; lu. lumen (of oviduct); m. mitochondrion; ma. desmosome (macula adhaerens); mv. microvilli; se. desmosome (septate); ser. smooth endoplasmic reticulum; zo. zonula occludens (Mag. x25K).....137

Plate 5.14. Pore in the cuticle lining of the lumen of the median oviduct of Pontania proxima. Fixation using Protocol two; cu. cuticle; lu. lumen (of oviduct); mv. microvilli; po. pore (Mag. x70K).....138

Plate 5.15. Wall of the median oviduct of Pontania proxima. Fixation using Protocol two; cu. cuticle; de. desmosome; h. haemolymph; mc. muscle cell; lu. lumen (of oviduct); m. mitochondrion; n. nucleus; sp. spine; mv. microvilli (containing mitochondria) (Mag. x11200).....139

Plate 5.16. Folded wall of the lateral oviduct of Pontania proxima, showing the start of the cuticle lining at(*). The micrograph shows a single portion of the oviduct folded back on itself. A comparison of the distal wall, without a cuticle, shows fewer mitochondria, more smooth endoplasmic reticulum and numerous vacuoles. The nucleus labelled n in the Plate was situated in the proximal region of lateral oviduct where the cuticle layer has thickened, and mitochondria are more numerous. Fixation using Protocol two; cu. cuticle; de. desmosome; h. haemolymph; lu. lumen (of lateral oviduct); m. mitochondrion; mf. muscle fibrils; mv. microvilli; n. nucleus; sp. spine (Mag. X11200).....140

Plate 5.17. Junction at start of cuticle lining of lateral oviduct of Pontania proxima. Fixation using Protocol two; ap. apical surface (of cells lining the lateral oviduct); cu. cuticle; lu. lumen (of lateral oviduct) (Mag. x62.5K).....141

Plate 5.18. Section through the median oviduct of Pontania proxima. Thickness five microns. Fixation using Protocol two. Stain p-phenylenediamine; cu. cuticle; ep. epithelium (with microvilli at apical surface); lu. lumen of median oviduct; mu. muscle; mv. microvilli; sp. cuticular spine (Mag. x1400).....142

Plate 5.19. Cuticular spine in the median oviduct of Pontania proxima. Fixation using Protocol two; ap. apical surface (of cells lining

the median oviduct); cu. cuticle; lu. lumen (of lateral oviduct); mv. microvilli; sp. spine (containing processes from microvilli) (Mag. x14.5K).....143
Plate 5.20. Section through the spermatheca of Pontania proxima. Thickness five microns. Fixation using Protocol two. Stain p-phenylenediamine; a. abdominal ganglion; cs. colleterial sac; cu. cuticle; mo. median oviduct; mu. muscle; sd. spermathecal duct; vc. vaginal clamp; vf. vaginal fold; vv. vaginal valve (Mag. x560).....144
Plate 5.21. Section through the vaginal pouch region of Pontania proxima. Thickness two microns. Fixation using Protocol two. Stain p-phenylenediamine; cd. colleterial duct; cs. colleterial sac; mo. median oviduct; mu. muscle; sd. spermathecal duct; spe. spermatheca; vc. vaginal clamp; vf. vaginal fold; vl. vaginal lip; vp. vaginal pouch; vv. vaginal valve (Mag. x515).....146
Plate 5.22. Section through the vaginal pouch region of Pontania proxima showing the change in shape of the vaginal valve, so possibly closing the colleterial duct. Label vp corresponds to the same area in Plate 5.21. Thickness five microns. Fixation using Protocol two. Stain p-phenylenediamine; cd. colleterial duct; cs. colleterial sac; mo. median oviduct; mu. muscle; sd. spermathecal duct; spe. spermatheca; vc. vaginal clamp; vf. vaginal fold; vl. vaginal lip; vp. vaginal pouch; vv. vaginal valve (Mag. x560).....147
Plate 5.23. Leaf tissue 24 to 36 hours after oviposition by Pontania proxima, but away from its site. Thickness 0.5 microns. Fixation using Protocol one. Stain toluidine blue; le. lower epidermis; lp. lower parenchymal layer; pp. palisade parenchyma; pr. provascular tissue; ue. upper epidermis (Mag. x400).....149
Plate 5.24. Leaf tissue 24 to 36 hours after and at the site of oviposition by Pontania proxima. Thickness 0.5 microns. Fixation using Protocol one. Stain toluidine blue; ar. area of rapid cell division; ca. cavity; e. egg; hy. hypertrophy of vascular tissue; le. lower epidermis; lp. lower parenchymal layer; pp. palisade parenchyma; pr. provascular tissue; ue. upper epidermis (Mag. x140).....150
Plate 5.25. Leaf tissue 24 to 36 hours after and at the site of oviposition by Pontania proxima. Thickness 0.5 microns. Fixation using Protocol one. Stain toluidine blue; ar. area of rapid cell division; ca. cavity; e. egg; pp. palisade parenchyma; pr. provascular tissue;

ue. upper epidermis (Mag. x400).....151
Plate 5.26. Leaf tissue 24 to 36 hours after and at the site of oviposition by Pontania proxima; ar. area of rapid cell division; ca. cavity; e. egg; le. lower epidermis; lp. lower parenchymal layer; pr. provascular tissue (Mag. x750).....152
Plate 5.27. Provascular cells lining the cavity adjacent to the egg; ce. chorion of egg; ch. chloroplasts; cw. cell wall; ed. electron-dense material; fb. fibrillar material; rcw. remains of cell wall (Mag. x5000).....153
Plate 5.28. Eggless procecidium after an abortive attempt at oviposition by Pontania proxima; ar. area of rapid cell division; le. lower epidermis; lp. lower parenchymal layer; pp. palisade parenchyma; pr. provascular tissue; ue. upper epidermis (Mag. x140).....154
Plate 5.29. (a) Young chloroplasts developing in leaf tissue of Theobroma cacao (Mag. x22000). Reproduced by kind permission of Dr. J. Whatley, University of Oxford. (b) Young chloroplasts developing in normal leaf tissue of Salix fragilis (Mag. x7500); ch. chloroplasts; cw. cell wall; gr. granum; v. vacuole.....155
Plate 5.30. Chloroplast of a cell from the central region of the gall after approximately 60 hours of development; ev. plastid envelope; gl. globule; in. invagination (Mag. x50000).....156
Plate 5.31. Chloroplast of Photinia x fraseri 'Red Robin', showing the same characteristic as those of a young chloroplast in the gall of Pontania proxima; cw. cell wall; ev. plastid envelope; gl. globule; in. invagination (Mag. x50000). Reproduced by kind permission of Dr. J. Whatley, University of Oxford.....157
Plate 5.32. Chloroplasts from the central region of the gall; ch. chloroplast; cw. cell wall; er. endoplasmic reticulum; isc. intercellular space; m. mitochondrion; v. vacuole (Mag. x8000).....158
Plate 5.33. Procecidium 24 to 36 hours after oviposition. Abaxial surface of leaf. an. anthocyanin ring; e. egg. sc. scar of oviposition. (scale divisions mm).....166
Plate 6.1. Completed injection into the leaf tissue of Salix fragilis. A glass bead has been placed in the wound to keep the mesophyll layer apart. The wound has then been sealed with lanolin and a fragment of glass coverslip; fr. fragment of glass; gb. glass bead; la. lanolin (scale divisions mm).....176
Plate 6.2. Use of a micropipette to remove colleterial fluid from the colleterial sac of Pontania proxima; cs. colleterial sac; e. egg; mp. micropipette; ol. ovary ligaments;

rc. released colleterial fluid; s. saw;
te. tear made with pin (Mag. x40).....177
Plate 6.3. Callus tissue induced by the implantation of colleterial fluid. View of adaxial surface of leaf after eight weeks (scale divisions mm).....182
Plate 6.4. Callus tissue induced by the implantation of colleterial fluid. View of abaxial surface of leaf after eight weeks (scale divisions mm).....183
Plate 6.5. Callus produced on the abaxial surface of a leaf, by an abortive attempt of a sawfly to oviposit. Growth after eight weeks (scale divisions mm).....184
Plate 6.6. Native-PAGE. Lanes: (1) and (8) molecular weight markers. (2) colleterial fluid. (3) colleterial fluid, dilution 1+1. (4) colleterial fluid, dilution 1+2. (5) colleterial fluid. (6) colleterial fluid, dilution 1+1. (7) colleterial fluid, dilution 1+2. Silver staining.....199
Plate 6.7. SDS-PAGE. Lanes: (1) and (8) molecular weight markers. (2) colleterial fluid. (3) colleterial fluid, dilution 1+1. (4) colleterial fluid, dilution 1+3. (5) colleterial fluid (6) colleterial fluid, dilution, 1+1. (7) colleterial fluid, 1+3. Silver staining.....200
Plate 6.8. IEF. Lanes: (1) and (8) isoelectric point markers. (2) colleterial fluid. (3) colleterial fluid, dilution 1+1. (4) colleterial fluid, dilution 1+2. (5) colleterial fluid, dilution 1+3. (6) colleterial fluid, dilution 1+2. (7) colleterial fluid. Silver staining.....201
Plate 6.9. Callus tissue induced by the injection of the 10 kDa filtrate into Salix tissue. Nine days after inoculation (scale divisions mm).....211
Plate 6.10. Callus tissue induced by the injection of the 3 kDa filtrate into Salix leaf tissue. Eight days after injection. Same magnification as Plate 6.9.....212
Plate 6.11. Necrotic issue produced by injecting distilled water into Salix leaf tissue. Eight days after inoculation (scale divisions mm).....213
Plate 6.12. SDS-PAGE gel of filtrates produced by ultrafiltration. Lanes: (1) and (2) molecular weight markers, (3) and (6) 30 kDa filtrate, (4) and (7) 10 kDa filtrate, (5) and (7) 3 kDa filtrate. Methods used as in Section 6.3.2. Silver staining.....214
Plate A1.1. Calli developed from leaf-disc explants and grown on MS based media containing 0.5 mg/l Kn and 2.0 mg/l 2,4-D. Photograph taken after 48 days of development using 100D Professional

Fuji film (used throughout).....275
Plate A1.2. Rhizogenesis from callus material 34 days after subculture on WPM containing BAP 0 mg/l and NAA 0.2 mg/l (Mag. x9).....281
Plate A1.3. Rhizogenesis from leaf-disc 56 days after culture on WPM containing BAP 0 mg/l and NAA 1.0 mg/l with additional lysine 100 mg/l.....282
Plate A1.4. Gall protoplasts showing a large anthocyanin-containing vacuole, (a) fluorescence image, (b) Nomaski interference contrast image (Mag. x1300).....290

TABLES

PAGE

Table 2.1. Summary of information relating to the development times and conditions for the successful development of eggs and larvae of various species of Pontania, collected from literature published between 1914 and 1959.....29
Table 2.2. Summary of information relating to the development times and conditions for the successful development of eggs and larvae of various species of Pontania, collected from literature published between 1964 and 1986.....30
Table 4.1. Number and percentage of cocoons formed from sample collections of galls. Details of sites are given in Appendix Two.....82
Table 4.2. Results of counts of galls in each area of the leaf, for each of one to 12 galls per leaf.....90
Table 4.3. Results of counts of galls in each area of the leaf, for 13 and 14 galls per leaf, the total of all galls and the total of 10 to 14 galls per leaf.....91
Table 5.1. Constituents of embedding resin. These were combined and shaken until the mixture was fully homogeneous. The container holding the resin was then placed in a sonic water bath for 30 minutes until all air bubbles had been removed and the resin was clear.....113
Table 6.1. Results of the uptake of methylene blue from micropipettes into the tissues of the leaf.....179
Table 6.2. Results of the amount of physical damage caused to the leaf tissue by the insertion of micropipettes.....179
Table 6.3. Run conditions (maximum limits) for native-PAGE, SDS-PAGE and isoelectric focusing, using the PhastSystem developed by Pharmacia LKB Biotechnology.....193
Table 6.4. Absorption peaks in the 240 to 260 nm region of fractions collected during off-line purification (nm).....209
Table A1.1. Composition of media used in the isolation of protoplasts (I), micropropagation of plantlets (II), induction and maintenance of calli (III) and for the culture of mesophyll cells (IV). (Continued in Table A1.2. See text for full details).....266
Table A1.2. Composition of media used in the isolation of protoplasts (I), micropropagation of plantlets (II), induction and maintenance of calli (III) and for the culture of mesophyll cells (IV). (Continuation of Table A1.1. See text for full details).....267
Table A1.3. Summary of media and explants used in the production of callus tissue of species of Populus and Salix by earlier workers.....268

Table A1.4. Summary of media and explants used in the differentiation (diff.) of shoots and roots, regeneration (regen.) of shoots and roots, shoot proliferation (prolif.) and growth of species of Populus and Salix from explants and callus material by earlier workers.....279
Table A1.5. Percentage changes in mass of gall and leaf tissue after 30 minutes in mannitol solutions of differing molarity.....285
Table A4.1. Results of counts of galls in each area of the leaf, for each of one to 12 galls per leaf.....304
Table A4.2. Results of counts of galls in each area of the leaf, for 13 and 14 galls per leaf, the total of all galls and the total of 10 to 14 galls per leaf.....305
Table A4.3. Data from Table A4.1, leaves with one to seven galls, corrected to remove possible bias caused by the different sizes of areas 1A to 4D. (data from Table A4.1/mean size of area from Table A4.11).....306
Table A4.4. Data from Table A4.1 and A4.2, leaves with eight to fourteen galls, corrected to remove possible bias caused by the different sizes of areas 1A to 4D. (data from Table A4.1/mean size of area from Table A4.11).....307
Table A4.5. Data from Table A4.2, the total number of galls counted and the total number of galls on leaves with 10 to 14 galls per leaf. Data corrected to remove possible bias caused by the different sizes of areas 1A to 4D. (data from Table A4.1/mean size of area from Table A4.11).....308
Table A4.6. Number of galls in each area for one to seven galls per leaf. Data corrected to remove possible bias caused by the different sizes of areas 1A to 4D and expressed as a percentage frequency (data from Table A4.3/Totals from Table A4.3)x100.....309
Table A4.7. Number of galls in each area for eight to fourteen galls per leaf. Data corrected to remove possible bias caused by the different sizes of areas 1A to 4D and expressed as a percentage frequency. (data from Table A4.4/Totals from Table A4.4)x100.....310
Table A4.8. Total number of galls counted and the total number of galls on leaves with 10 to 14 galls per leaf. Data corrected to remove possible bias caused by the different sizes of areas 1A to 4D and expressed as a percentage frequency. (data from Table A4.5/Totals from Table A4.5)x100.....311
Table A4.9. The size of each of the areas 1A to 4D (x4 mm^2) and the total area (x4 mm^2) for ten randomly selected leaves.....312
Table A4.10. Areas from Table A4.9 expressed as running

means, continued on Table A4.11. (Size of areas 1A to 4D from Table A4.9/Sum of areas From Table A4.9)x100. Means calculated from these data.....313
Table A4.11. Continuation of Table A4.10, areas from Table A4.9 expressed as running means. Tenth mean used in Tables A4.3 to A4.5.....314
Table A4.12. Expected numbers of galls per leaf, for one to seven galls per leaf, calculated from the total numbers of galls on each leaf (Table A4.1) and the mean size of each area (Table A4.11, 10th mean). (Table A4.11x(Table A4.1/100)).....315
Table A4.13. Expected numbers of galls per leaf, for eight to fourteen galls per leaf, calculated from the total numbers of galls on each leaf (Table A4.1 and A4.2) and the mean size of each area (Table A4.11, 10th mean). (Table A4.11x(Table A4.1 and A4.2/100)).....316
Table A4.14. Expected numbers of galls per leaf, for the total number of galls and the total number for 10-14 galls per leaf, calculated from the total numbers of galls on each leaf (Table A4.2) and the mean size of each area (Table A4.11, 10th mean). (Table A4.11x(Table A4.2/100)).....317
Table A4.15. Results of chi-squared test between the observed counts of the number of galls on leaves (Table A4.1 and A4.2) and the expected results based on the size of each area 1A to 4D (Table A4.12 to A4.14).....318
Table A5.1. Results of the measurement of the length and breadth of 50 cocoons.....319
Table A6.1. Chemical composition of insect Ringer solution used throughout this thesis.....320
Table A7.1. The mean lengths of ten leaves and ten galls, measured over a period of 17 days.....321
Table A8.1. Data collected to determine the size and volume of the colleterial sac of Pontania proxima.....322
Table A8.2. Data collected to determine the mean diameter of tubules of the accessory gland of Pontania proxima.....323

FIGURES

PAGE

Figure 1.1. The artificial classification of galls according to Küster.....2
Figure 2.1. A summary of factors which affect the success or otherwise of sawfly attack and survival.....42
Figure 2.2. Relationships of factors affecting gall phenotype (from Weis and Abrahamson, 1986).....45
Figure 3.1. Hypothetical reactions leading to the production of IAA in the saliva of a plant-bug (from Miles, 1968b).....55
Figure 4.1. Position of areas 1A to 4D on leaves of Salix fragilis, used to plot the position of galls.....89
Figure 4.2. Number of galls expressed as a percentage frequency and corrected to remove possible bias caused by the difference in size of positional areas for leaves with one to eight galls per leaf.....93
Figure 4.3. Number of galls expressed as a percentage frequency and corrected to remove possible bias caused by the difference in size of positional areas for leaves with nine to 13 galls per leaf, the total number of all galls and the total number of galls for 10-14 galls per leaf.....94
Figure 5.1. Generalized diagram of the female reproductive system of Symphyta (dorsal view, not to scale). ag. accessory gland; c. calyx; cs. colleterial sac; e. egg; lo. lateral oviduct; mo. median oviduct; ol. ovary ligament; ov. ovary; ovl. ovariole; s. saw; sp. spermatheca; v. vaginal pouch.....103
Figure 5.2. Simplified diagram of a transverse section of an accessory gland tubule of Pontania proxima, taken from the distal portion of the gland and showing an inner epidermal cell and a single secretory cell; bm. basement membrane; cu. cuticle; ec. epidermal cell; en. end-apparatus; go. golgi complex; lu. lumen (of tubule); m. mitochondrion; mv. microvilli; n. nucleus; nu. nucleolus; pm. plasma membrane; rer. rough endoplasmic reticulum; ser. smooth endoplasmic reticulum.....124
Figure 5.3. Simplified diagram showing the relative positions of the median oviduct, spermatheca, exit of the colleterial sac and vaginal pouch of Pontania proxima. a. abdominal ganglion; cd. colleterial duct; cs. colleterial sac; mo. median oviduct; sp. spine; spe. spermatheca; vl. vaginal lip; vc. vaginal clamp; vf. vaginal fold; vp. vaginal pouch (leading to the saw); vv. vaginal valve.....132
Figure 6.1. U/V absorption spectra of (a) supernatant obtained from the colleterial fluid (1 = absorbance at 260 nm; 2 = absorbance at 280 nm), (b) pellet (dotted line = spectrum 6.1a), (c) resuspended freeze-dried supernatant

(dotted line = spectrum 6.1a). Arrows on 6.1b and 6.1c indicate absorption peaks (see text for details).....196
Figure 6.2. Absorption spectra for (a) frass obtained from section galls, (b) tissue from the inner layer of the gall. Arrows indicate absorption peaks (see text for details).....197
Figure 6.3. Calibration curves for (a) SDS-PAGE and (b) IEF. Details given in the text.....198
Figure 6.4. HPLC chromatogram of the 3 kDa filtrate. 0.08 ABU's, 265 nm, Solvent system 2. 10 μl sample. Chart speed 10 mm/min.....215
Figure 6.5. Absorption peaks in the 240 to 260 nm region of the fractions collected by ultrafiltration (nm).....215
Figure A1.1. Growth rate of subculture. Percentage increase in diameter of callus from original diameter (n = 33). Kn = 0.5 mg/1; 2,4-D = 1 mg/1.....276
Figure A1.2. Percentage changes in mass of gall and leaf tissue after 30 minutes in mannitol solutions of differing molarity.....286
Figure A3.1. Monthly mean of daily air temperatures for the years 1987 to 1990. Long-term means calculated from 1815 to 1989.....299
Figure A3.2. Total rainfall each month for the years 1987 to 1990. Long-term means calculated from 1767 to 1989.....300
Figure A3.3. Absolute minimum grass temperature each month for the years 1987 to 1990. Long-term means calculated to 1989.....301
Figure A3.4. Monthly means of the daily grass minimum temperature for the years 1987 to 1990. Long-term means calculated from 1881 to 1989.....302
Figure A7.1. The mean length of ten leaves and ten galls, measured over a period of 17 days.....321

ABBREVIATIONS

General.

2,4-D	2,4-dichlorophenoxyacetic acid
ACM	Aspen Culture Medium (Ahuja, 1983).
ABA	abscisic acid
BAP	benzylaminopurine
C	percentage by weight of total monomer T which is due to cross linker
DDH_2O	reagent grade water
DM	Durzan's Medium (Durzan et al., 1973).
DMSO	dimethyl sulfoxide
DOPA	dihydroxyphenylalanine
DTT	dithiothreitol
EDTA	ethylenediaminetetraaceticacid
e.p.u.	eye piece unit
GA	gibberellic acid
GD	modified Gresshoff and Doy (Chalupa, 1983).
HPLC	high pressure liquid chromatography
IAA	indole acetic acid
IEF	isoelectric focusing
Kn	kinetin
Mag.	magnification
μ	micron
MES	2-[N-Morpholino]ethanesulfonic acid
MS	Murashige and Skoog (Murashige and Skoog, 1962).
MW	modified White's (1942) (Hildebrandt et al., 1946).
MWS	modified Wolter and Skoog (Winton, 1970).
NAA	naphthaleneacetic acid
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine: ammonium lyase
PAT	polar auxin transport
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
SDS	sodium dodecylsulphate
SM	Saito's medium (Saito, 1980).
T	total concentration of monomer (acrylamide monomer + N,N'-methylene-bis-acrylamide) w/v expressed as a percentage,
Tris	Tris(hydroxymethyl)aminomethane
TWEEN 20	polyoxyethylenesorbitan
U/V	ultraviolet
WPM	woody plant medium (Lloyd and McCown, 1981).
WS	Wolter and Skoog (Wolter and Skoog, 1966).
w/v	weight by volume or mass concentration

Abbreviations for Plates and Figures.

a	abdominal ganglion
ag	accessory gland
an	anthocyanin ring
ap	apical surface
ar	area of rapid cell division
bm	basement membrane
c	calyx
ca	cavity
cd	colleterial duct
ce	chorion of egg
ch	chloroplasts
cs	colleterial sac
cu	cuticle
cw	cell wall
cy	cytoplasm
de	desmosome
e	egg
ec	epidermal cell
ed	electron-dense material
en	end-apparatus
ens	densely staining end-apparatus
ep	epithelium
er	endoplasmic reticulum
ev	plastid envelope
f	follicular epithelium
fb	fibrillar material
fr	fragment of glass
g	glial cell
gb	glass bead
gl	globule
go	golgi complex
gr	grana
h	haemolymph
hy	hypertrophy of vascular
ics	intercellular spaces
in	invagination
la	lanolin
le	lower epidermis
lo	lateral oviduct
lp	lower parenchymal layer
lu	lumen
m	mitochondrion
ma	desmosome (macula adhaerens)
mc	muscle cell
mf	muscle fibrils
mo	median oviduct
mp	micropipette
mu	muscle
mv	microvilli
n	nucleus
nl	neural lamella
np	neuropile
nu	nucleolus
nup	nutritive pore
ol	ovary ligament
oo	oocyte
ov	ovary
ovl	ovariole
p	peritoneal membrane
pk	perikaryon
pm	plasma membrane
pn	perineurium
po	pore
pp	palisade parenchyma
pr	provascular tissue
rc	released colleterial fluid
rcw	remains of cell wall
rer	rough endoplasmic reticulum
s	saw
sc	scar of oviposition
sd	spermathecal duct
se	desmosome (septate)
ser	smooth endoplasmic reticulum
sp	spine
spe	spermatheca
t	tonoplast
tr	trophocyte
ue	upper epidermis
v	vacuole
vc	vaginal clamp
vf	vaginal fold
vl	vaginal lip
vp	vaginal pouch
vv	vaginal valve
zo	zonula occludens

CHAPTER ONE

INTRODUCTION

> "In the first place this research requires a very close working relationship between a classical entomologist versed in the raising of insects and a botanist who can grow and manipulate the host plant".
>
> (Went, 1970).

1.1 Definitions.

Meyer (1987:1) considered galls as "all manifestations of growth, whether positive or negative, induced on a plant by animal or plant parasites". For the purpose of this thesis, a gall, by definition, must exhibit hypertrophy and hyperplasy over and above that exhibited by a wound response, of a plant, to mechanical injury. Excluded by this definition are domatia, which were shown by Monad and Schmitt (1968) to be formed, at least for some species of Acacia Miller (Leguminosae), independently from the presence of a parasite. Van Steenis (1976) summarized the issue as, "there is every reason to accept that domatia are an irrelevant structure in plants which is secondarily exploited by animals". Von Ihering (1907), went further and suggested that the well known 'ant-plant' genus Cecropia Loefling (Cecropiaceae) needed its ants as a dog needed its fleas. Other exclusions are the effects of

leaf miners, except those that produce a gall-like formation in the host e.g. Pegomyia chenopodii Rondani (Anthomyiidae) on Chenopodium album L. (Chenopodiaceae) (Mani, 1964: 4). Also excluded is the webbing and rolling of leaves by caterpillars (e.g. by moths of the genus Gracillaria Haworth (Gracillariidae). In each case the rôle of the plant is purely passive.

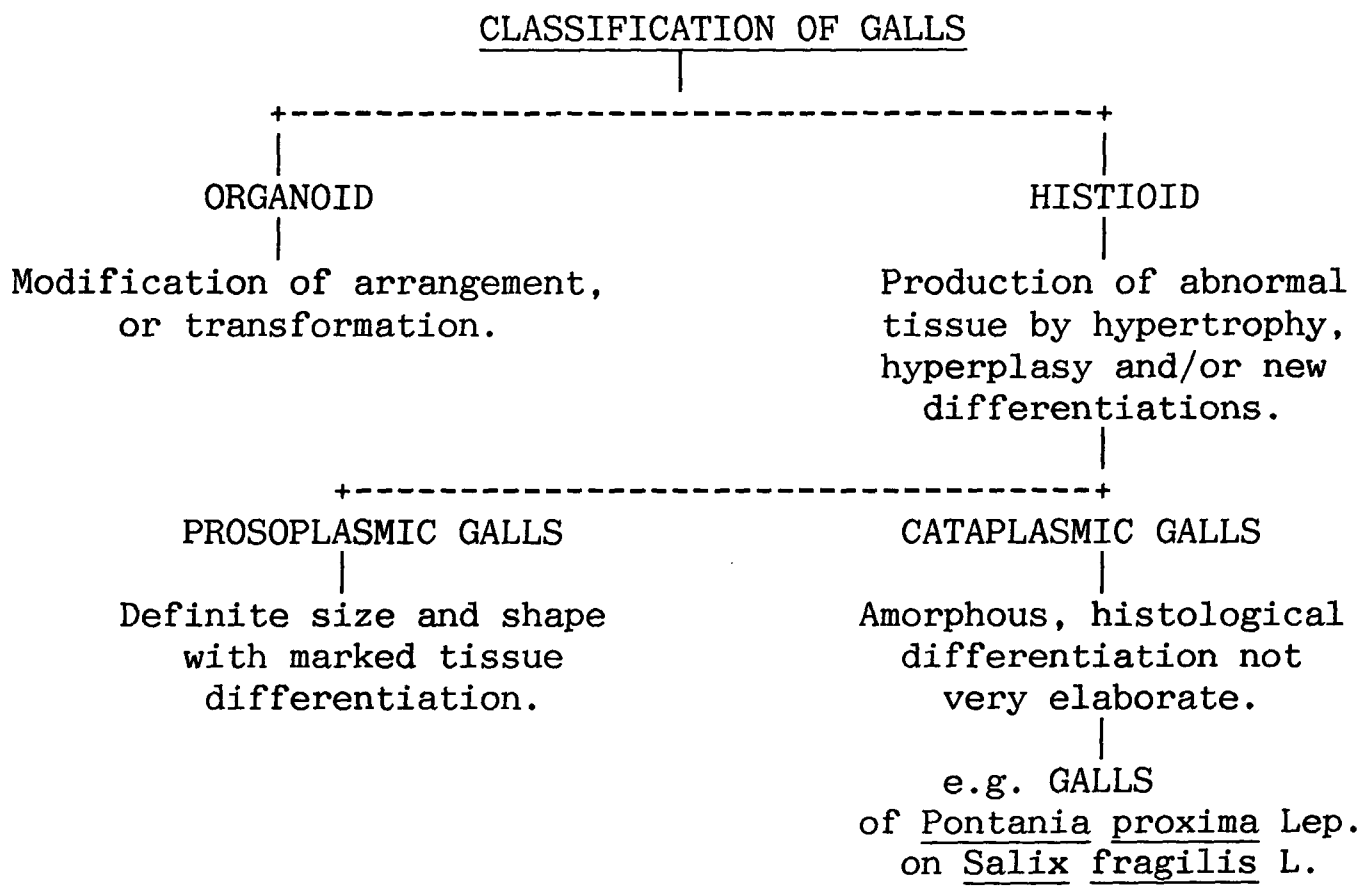


Figure 1.1. The artificial classification of galls according to Küster.

In 1911, Küster (Mani, 1964: 35) produced an artificial classification of galls which was based on their morphology (Figure 1.1). Galls were separated into two groups. The first group consisted of the organoid galls, which were characterized by an abnormal arrangement or transformation of plant organs, e.g. virescences, fasciations, abnormal leaves, stunted growth and witches-brooms, where vertical growth in branches and a reduction in leaf size predominate.

Histioid galls formed the second group; these were much more variable and in the main developed through hypertrophy, hyperplasy and metaplasy. Of these, prosoplasmic galls had a marked internal differentiation; belonging to this group are the majority of insect galls (e.g. those of the Cynipinae). Cataplasmic galls are much simpler and lacked the internal structure of prosoplasmic galls.

Smith (1970), described gall formation as following four stages:-

1) Inhibition of normal cell growth (hypoplasy).

2) Cell overgrowth (hypertrophy).

3) The abnormal proliferation of cells (hyperplasy).

4) Redifferentiation of cells (metaplasy).

Although less naturalistic, these correspond with the preproliferation, blastemomorphous, callusomorphous, gallomorphous and necrotrophic stages of Slepyan and Gabarayeva (1981).

Whatever the size, morphology or colour of a gall (cecidium), it is generally recognized that for a gall to develop, meristematic or undifferentiated tissues are required (Bastin, 1921; Benes, 1968; Dieleman, 1969; Slepyan and Gabarayeva, 1981; Ananthakrishnan, 1984). The transformation of differentiated tissue into meristematic tissue may also occur (Ananthakrishnan, 1984). Whatever the nature of the tissue, gall-forming insects synchronize their reproduction with periods of active growth of the host-plant, whose cells are released, under the influence of the insect, from their normal morphogenetic coordinating influences (Rohfritsch and Shorthouse, 1982; Ananthakrishnan, 1984). The structure that results from this synchrony contains no novel tissue, but arrangements of tissues may differ from the norm in time and space (Cosens and Sinclair, 1916; Yermakov, 1965

and Sachs, 1975), or even exhibit atavisms (Cosens and Sinclair, 1916; Corner, 1963).

Galls can be found, to a greater or lesser degree, in all major classes of plants and fungi, but the highest percentage (98%) is found on the Angiospermae, 93% of which is on the Dicotyledonae (Mani, 1964: 16). Just as varied and unrelated are the gall-inducers (cecidogenous organisms). Cecidogenesis is of polyphyletic occurrence (Plumb, 1953: 7). Meyer (1987) described galls induced by a range of organisms from viruses, bacteria and algae, to fungi (Archimycetes, Phycomycetes and Septomycetes). Of the animal gall inducers (cecidozoans), he included a diverse collection of representatives from Nematoda, Acarina and Insecta. Of these, the galls of Hymenoptera are among the most specialized, with notable gall-formers occurring in the superfamilies Tenthredinoidea, Cynipoidea and Chalcidoidea (Cornell, 1983; Askew, 1984; Narendran, 1984). Moreover, almost every part of a plant can be infected by a specialist cecidogenous organism. Roots, stems, leaves and inflorescences can all be galled, the galls (cecidia) manifesting themselves in a great variety of colours, sizes and shapes (Darlington, 1968: 16-17), their structure being determined by the species of gall-inducer and host-plant morphology (Smith, 1970). Galling is thus a widespread and variable response to a stimulus which may come from a variety of unrelated organisms. What they have in common is that they are "always positive, directional responses resulting in disharmonic growth effects, with the polarity related to the insect rather than the rest of the plant" (Ananthakrishnan, 1984). This response is highly specific with respect to host type, position and morphology of the gall and the life cycle of the inducer (Barnes, 1931; Smith, et al., 1983; Ananthakrishnan, 1984; Weis et al., 1988). A few gall-inducers are more

general in their range of hosts e.g. Phyllocolpa Benson (Tenthredinidae) which induce leaf-margin galls in Salix L. (Salicaceae) and Populus L. (Salicaceae) species, but a high degree of specificity of host-plant is the rule rather than the exception. It must be noted that the apparent degree of host-specificity may also reflect partly the depth of taxonomic knowledge of a particular group. In sawflies, gall-inducing species are certainly more host-specific than external feeders (Smith, 1970).

Much has been written on the subject of cecidology, but there is a lack of real substantive evidence that points toward the proximal cause of gall formation. The mystery of initiation remains. The only exception to this is the mechanism whereby the soil bacterium Agrobacterium tumefaciens (Smith and Townsend) induces crown gall disease on a wide variety of host plants (Ream and Gordon, 1982). Insect-induced galls, unlike crown gall, are non-infectious, and there remains little to choose between the many hypotheses, concerning gall induction (cecidogenesis) that have been expounded. Indeed, it is probably unwise to consider a universal cause, especially when so little is known about the subject and because of the phylogenetically diverse nature of the inducing organisms. A fuller appreciation is important in furthering our understanding of patterns of growth and the rôle of growth regulators in plants.

1.2 Aims and objectives.

The major aim of this project was the elucidation of the mechanism behind gall induction in just one species pair i.e. Pontania proxima (Lepeletier) (Tenthredinidae)-Salix fragilis L. (Salicaceae). Within

this stated aim, the objectives of this project concentrated on the establishment and development of systems that could be used to study gall induction.

Firstly, as neither of the two species had been studied at Oxford, it was necessary to devise and develop, from first principles, methods of collecting, culturing and breeding both organisms. Also, the seasonal nature of many inducers and their hosts had prevented continuity of research ("Unless one finds a way of having continuous gall and insect production, the gall research team would be out of work 50 weeks of the year" - Went, 1970). Therefore, the establishment of systems that ensured a constant flow of material for experimental purposes were developed.

Secondly, throughout this study every effort was made to adopt an holistic approach. Previous studies had concentrated on one organism of the species pair, while paying scant regard to the other (evidence for this will presented in the body of the thesis). This had led to an imbalance of knowledge, especially with respect to the anatomy and ultrastructure of the insect (Pontania proxima), compared with that of the gall it initiates. To this end a study of the internal reproductive organs and associated glands of Pontania proxima was made.

Thirdly, there was no suitable bioassay for the assessment of possible cecidogenic substances. To this end, experimental methods for the micro-injection of substances into leaves were devised. Previously, this had proved impossible ("ma main ne pouvait rivaliser avec l'outil de l'hymenoptère" - Beyerinck, 1887).

Fourthly, modern analytical techniques made the analysis and separation of compounds within the cecidogenic fluid more possible.

Previous attempts to carry out this type of analysis had met with confused and scant results.

Galling represents a highly specific link between organisms of different kingdoms, to such an extent that Cornell (1983) and Dawkins (1989, 218-219) both viewed the gall as an extention of the insect's phenotype. More recently Weis and Abrahamson (1986) proposed that, although the gall was plant tissue, the insect coded for the stimulus and the plant for the response. Thus, the gall was considered a product of two genomes. This philosophy is reflected in the approach adopted to the work in this thesis.

CHAPTER TWO

THE PONTANIA-SALIX SYSTEM

> "Galls induced in plants by insects present many types of abnormal differentiation. How the insect initiates the gall, the balance of factors involved in the gall development, and what stops gall growth are questions that have not been answered satisfactorily".
>
> (Pelet et al., 1960)

2.1 Introduction.

Cataplasmic galls are induced by the oviposition of the sawfly Pontania proxima, into the young leaves of Salix fragilis, commonly called crack willow, which is a common lowland tree, frequently found by riversides. It is recorded in every English and most of the Welsh counties. Linton (1913: 14) considered that it was not indigenous in Scotland, giving its distribution as covering southern Ireland, Europe, Asia Minor, Syria, Persia, Caucasus, Armenia, Western and Central Siberia. Meikle (1984: 26) described crack willow as a "complex group of tree willows, consisting of several distinct segregates", and doubted whether it could be considered as indigenous to Britain and Ireland since it did not form part of any natural plant community.

Fossil evidence suggests Salix-type leaves were present during the Eocene, with salicaceous pollen being identified as belonging to the Oligocene epoch (Cronquist, 1981: 435). Although the origins of Hymenoptera are poorly understood, the earliest fossil records are of members of the extant superfamily, Xyloidea, from the Middle Triassic of Central Asia. By the Jurassic, Hymenoptera had radiated considerably with 21 families being represented (Gauld and Bolton, 1988: 93). The first insect galls appeared in the Early Cretaceous period (Strong et al., 1984: 36-41; Larew, 1986; Craig et al., 1988). In evolutionary terms, therefore, the galling habit of Pontania O. Costa species would seem to have been derived from free-living phytophagy. This view is supported by Togashi (1970), who, from a study of the reproductive apparatus of Symphyta, suggested that the earliest forms laid their eggs on the surface of leaves, while later forms inserted their eggs into leaves, petioles or stems. Ananthakrishnan (1984) described gall insect-host plant associations as seeming "to exemplify an advanced/specialized level of 'trophic strategy'".

The radiation of Angiospermae occurred during the Jurassic/Cretaceous boundary period, and that of the galling habit during the Early Cretaceous, it is interesting to speculate as to whether there is a link in this timing. Insects were already well established and the radiation of Hymenoptera had already begun. The question that springs to mind is, what is it about Angiospermae that has, during the course of evolution, led to it being galled more frequently than any other group?

2.2 History.

Galls have been recognized, at least, since the earliest literary times; being described by both Hippocrates (406-377 B.C.) and Theophrastus (372-286 B.C.). It was not until the middle of the seventeenth century that Malpighi established the link between the adult insect and the gall (Higton, 1989; Appendix Nine); he suggested that the stimulus for gall formation was a combination of the sting of the insect and the injection of a fluid, which Malpighi termed the "ichor", into the wound.

Böhner (1935) cited Gesner (1516-1565) as giving the earliest description of the galls of the tenthredinids (Caltagirone, 1964). Later writers were interested in not only describing the galls and their inhabitants, but also how they originated and whether this was through spontaneous generation. Redi (1626-98) completed a variety of experiments designed to challenge Aristotle's assertion that insects could be generated spontaneously from putrefaction (Redi, 1668; Singer, 1959: 440-441; Glass, 1968: 40). When discussing willow galls, however, Redi reverted to Aristotle's theory suggesting that both the gall and the insect were produced from the tissues of the willow leaf (Redi, 1668: 135-136, 141). It was left to his pupil Vallisnieri (1661-1730) to correct this error (Miall, 1912: 226-227). Réaumur (1737: 437-438), in his description of willow galls, referred to the different experimental methods used by Redi and Vallisnieri. He suggested that Vallisnieri succeeded, unlike Redi, in raising adult insects as he included moistened earth in his growth chambers.

Two of the earliest, yet most thorough, accounts of the galls of Pontania were given by Swammerdam (1637-1680) and Leuwenhoeck

(1632-1723). Swammerdam (1758) not only challenged Redi's view, but observed, in great detail, the structure of the willow leaf and how this was related to the structure of the gall. He noted how the egg "grew" before hatching, and described the structure of the larvae, parasites and inquilines (co-inhabitants of a gall: usually these do not kill the gall-former but when this does occur, the organisms are termed aggressive inquilines). He suggested that the rôle of the gall was purely for the benefit of the insect (cf. Dawkins, 1989: 218-219). Although he did not actually observe oviposition, he predicted that it was responsible for gall formation (probably before Vallisnieri) suggesting that the gall was produced by the "variety of the stroke" used by the insect during oviposition (Swammerdam, 1758: 81).

Leuwenhoeck (1701) described the gall, its inhabitants and observed parasites attached to the larvae. Like Swammerdam (1758) and Frisch (1766) he noted a small hole in the wall of the gall through which the larva disposed of frass. On opening galls, Leuwenhoeck observed larvae and cocoons containing the pupating larvae. From observations of the sawflies themselves, he concluded "From this Discovery, 'tis easy to conceive that such Flies don't only lay their Eggs on the Leaves of Trees, but that they do also make an opening in the Skin of the Leaf, and Convey an Egg into it, from whence comes the worm, which knawing the vessels for its sustenance, occasions the Sap to flow out of them, and to coagulate into that knotty substance". Thus, Leuwenhoeck described the life cycle of the insect and like Swammerdam, suggested a mechanism whereby galls may be formed. Other descriptions of galls on willows are given by Linnaeus (1761: 388-389) and Frisch (1766: 22-24, Plate 4), who bred out a male and female wasp. The female is possibly Scambus vesicarius (Ratzeburg) (Ichneumonidae) and Frisch suggested

that this female was the gall-inducer. It is not possible to suggest an identity for the male, although from Frisch 1766 Plate 4, it may be of the same species of Ichneumonidae as the female.

Towards the end of the nineteenth century, Adler (1894: 99) concluded, with respect to oak galls, that oviposition alone was not the stimulus that resulted in gall formation, neither was it the introduction of a secretion, but that proper development only began when the larvae emerged from the egg. He noted an exception to this, i.e. Nematus vallisnieri Hartig (= Pontania triandrae Benson) galling Salix amygdalina L. (= Salix triandra L.). For this case he described the wounding of the leaf and the addition of a glandular secretion along with the egg and concluded that, as the gall was fully grown before the egg hatched, both the wounding and the glandular secretion were the cause of gall formation. Cosens (1912) disagreed with this assertion. He found larvae present soon after oviposition and before the gall was fully grown. These observations, by the two authors, would seem to illustrate differences in development and hatching times of species of Pontania from Europe and North America (see Summary of Development Times).

Further understanding came with the work of Beyerinck (1887 and 1888 - both papers are very similar in substance), he described in detail the gall of Pontania triandrae, the life cycle of the insect, how it reproduced by parthenogenesis, oviposition, the shape of the wound, how the female secreted a fluid into the wound, the development of the larvae and the reproductive anatomy of the wasp. He concluded that gall formation was due to the action of the fluid of the colleterial sac, a secretion produced by the accessory gland (see Section 5.1.2; Plate 2.1), and that the egg and the larva played no

part in the production of the gall. In support of these ideas, he observed that other non-galling Tenthredinidae would oviposit into leaves without galls being produced. Instead larvae hatched out, ate their way to the surface of the leaf and continued to feed there. He also observed that sometimes Pontania wasps were unsuccessful in their attempts to lay eggs. The leaf was wounded in the usual manner, but no egg was placed in the wound. This action resulted in the production of a miniature gall similar in all other respects to a normal one. From his observations Beyerinck judged the final size of the gall to be proportional to the volume of colleterial fluid injected during oviposition. Beyerinck (1887) also killed newly deposited eggs with a fine needle, and found that a small gall still developed. He considered that this observation demonstrated that the cecidogen was not of larval origin. He was, however, unsuccessful in attempting to inject artificially, ~~inject~~ fluid from the colleterial glands into young leaves, and he obtained only doubtful results.

Beyerinck addressed the wider question of whether the changes induced by the cecidogen were permanent or not, either by the gall being able to reproduce itself or by an irreversible alteration of the character of the plant. He decided the effect of the cecidogen was not permanent, nor was it inherited. He recognized that the insect was, in some way, modifying the normal growth mechanisms of the plant.

Twenty-six years later, Magnus (1914: 55-88) studied, in depth, the relationship between the same two organisms. He used Beyerinck's work as a basis and attempted to repeat and extend his experiments. Magnus (1914: 58) observed that Pontania triandrae preferred fast-growing coppice shoots for oviposition, an activity which occurred during a long flight period lasting from May through to September. He described

oviposition thoroughly, also the stages of gall formation and gall anatomy. Unlike Beyerinck (1887), Magnus (1914: 61-62) thought the final size of the gall was more closely linked to the age of the leaf in which the egg was deposited, i.e. the size of the gall was dependent on the extent of leaf expansion, rather than on the amount of fluid injected into the leaf by the insect.

Magnus (1914: 63) suggested the possibility of microorganisms, contained within the colleterial fluid, as being the proximal cause of gall initiation, but he did not find any evidence of their presence. In extending the experiments of Beyerinck (1887), Magnus (1914: 76-77) completely removed the egg soon after oviposition, he argued that even if destroyed by a fine needle, the remains could still be exerting an influence. Once the egg was removed the gall continued to develop in a normal fashion, mostly in the area away from the puncture site, but in these experiments the central cavity of the gall failed to develop. Development was sufficient, however, for him to agree with Beyerinck (1887) that the presence of an egg was not essential for the formation of a gall. Magnus (1914: 81-82) attempted to inject the colleterial fluid into the leaves of Salix triandra, but was unable to effect this in a satisfactory manner. He repeated the same experiments, but in other meristematic regions of the plant, such as the roots, but obtained no conclusive results. Further work led him to the opinion that the function of the colleterial fluid was restricted to sealing the wound caused by the ovipositor; the gall being a product of the wounding process alone.

The biology of Pontania proxima was studied in detail by Carleton (1939). Previously, it had been described in such standard works as Cameron (1885: 202-4), Connold (1901: 253-254) and Swanton (1912:

21-23). Carleton collected galls from Salix triandra and Salix fragilis and suggested that the wasps from these two hosts represented two biological races. Evidence for this hypothesis came from differences not only in the timing of the emergence the first broods, but also from the greater number of adults which emerged during the autumn and winter from the galls of Salix triandra, compared to the smaller number from galls of Salix fragilis. Experimental evidence was provided when sawflies reared from Salix triandra were placed in cages which contained cuttings of Salix fragilis and vice versa. In all cases oviposition was either unsuccessful, or the galls that formed were undersized. Of the few adults, obtained from these experiments, none was able to lay eggs. With this information and after an examination of a long series of wasps from both Salix triandra and Salix fragilis, Benson (1941) separated the species into two, creating Pontania triandrae on the basis of it possessing a shorter ovipositor-to-hind-tibia ratio than Pontania proxima. Benson (1940) had already separated Pontania bridgmanii (Cameron) from Pontania proxima, thus the proxima group (sensu Benson 1941) contained three species i.e. Pontania bridgmanii which galled Salix caprea L., Salix atrocinerea Brotero (= Salix cinerea ssp. oleifolia Macreight) and Salix aurita L., Pontania proxima which galled Salix fragilis and Salix alba L. and Pontania triandrae Benson which galled Salix triandra. Throughout the literature, even after this date, confusion arises unless the host's name is given, as often the name Pontania proxima is used sensu lato (cf. Benes, 1968). In this study all the experimental work, unless expressed otherwise, refers to Pontania proxima sensu stricto.

Hovanitz (1959) published an account of an unnamed sawfly, which according to Osborne (1972) was Euura pacifica (Marlatt), but from

Hovanitz's description is undoubtedly Pontania pacifica Marlatt, as oviposition occurred in the mid-rib of the leaf; this confusion may have been caused by the transfer of pacifica to the genus Euura E. Newman by Ross (1951: 50), a move that has not been accepted (Caltagirone, 1964). Hovanitz suggested a virus as being the proximal cause of gall formation (see also Cornell, 1983). Hovanitz (1959) described how the egg was laid and the ontogeny of the gall concluding that the egg was not necessary for the first phase of gall development, but that the colleterial fluid was. The first phase of growth of the gall lasted for approximately eight days, after which, continued growth required the presence and activity of a larva. Similar observations for Pontania proxima were made by Murphy (1929) and Lazareva et al. (1986). These views contrast with those of other authors and for other species. Caltagirone (1964) studied the same species as Hovanitz and maintained that gall growth was directly related to the growth rate of the leaf, with slow-growing leaves producing the largest galls. He believed that the presence of a larva was not necessary for the growth of the gall, as fully-grown galls were found even though the egg had failed to develop, i.e. "neither the size of the wound, nor amount of colleterial fluid injected nor presence of egg or larvae had influence on the final size of the gall". Smith (1970), who studied the bionomics of American species of Phyllocolpa, Pontania and Euura, stated that for galls of the type produced by Pontania proxima, hatching of the egg occurred after the gall had reached full size. For galls which included those of Pontania pacifica, 25% reached full size before hatching. For one species of Pontania allied to Pontania pacifica, but with galls of a different morphology and primarily a univoltine breeding strategy, the

gall maintained its growth long after the leaf had ceased its growth (Clancy et al., 1986).

To test the gall-inducing properties of the colleterial fluid, Hovanitz (1959) attempted to apply it to tissue cultures of willow as well as injecting it into willow leaves. Although some reaction was noted, he described his results as ambiguous, since similar growths occurred in merely damaged areas. He gave no details of the methods used for this work. When colleterial fluid was added to larger galls, from which the larva had been removed, growth continued, clearly suggesting a growth stimulating rôle. Analysis of the fluid suggested that it contained nucleic acid, proteins, carbohydrates and the precursors of silk. McCalla et al. (1962) continued this work and studied the effect of colleterial fluid on preformed galls and concluded that the active ingredients in the fluid were molecules of a low molecular weight of which "uric acid, two unidentified adenine derivatives, glutamic acid and possibly uridine as well, are present in the gland in large enough amounts and have sufficient growth-promoting activity to be of physiological importance". This work was not continued by McCalla (McCalla pers. comm.), neither is there any record of further research by this group.

Although work into the stimulus of gall formation is limited, numerous papers have been written concerning the bionomics of this species pair, or allied species pairs. Rey (1967 and 1968) studied the development and histology of the gall in great detail, adding to and extending the work of Magnus (1914: 55-88). The development and ultrastructure of chloroplasts in the gall tissue was also described by Rey (1972, 1973 and 1974).

Slepyan (1962) concluded from a study of the potential rate of photosynthesis of galled versus normal leaves that the pathogenicity of this sawfly was very slight. Later, Slepyan and Gabarayeva, in a series of papers (1976, 1978 and 1981), studied the tracheal system, morphology of the gut, Malpighian tubes and the salivary glands of the larvae, and finally the structure and development of the gall itself. Slepyan and Gabarayeva (1976) described how the tracheal system of the larva was adapted to hypoxia, a condition found in the gall, due to the limited gas exchange that could occur with the external environment. With reference to the digestive system and excretory organs, Slepyan and Gabarayeva (1978) described other adaptations to this particular habitat, "the presence of well developed salivary glands, the lack of a muscular proventriculus, the homogeneity of the ventricular enterocytes, cryptonephridia and the oligomerization of the Malpighian tubes are of an adaptive nature and indicate adaptation of the Pontania proxima larva to existence in a gall".

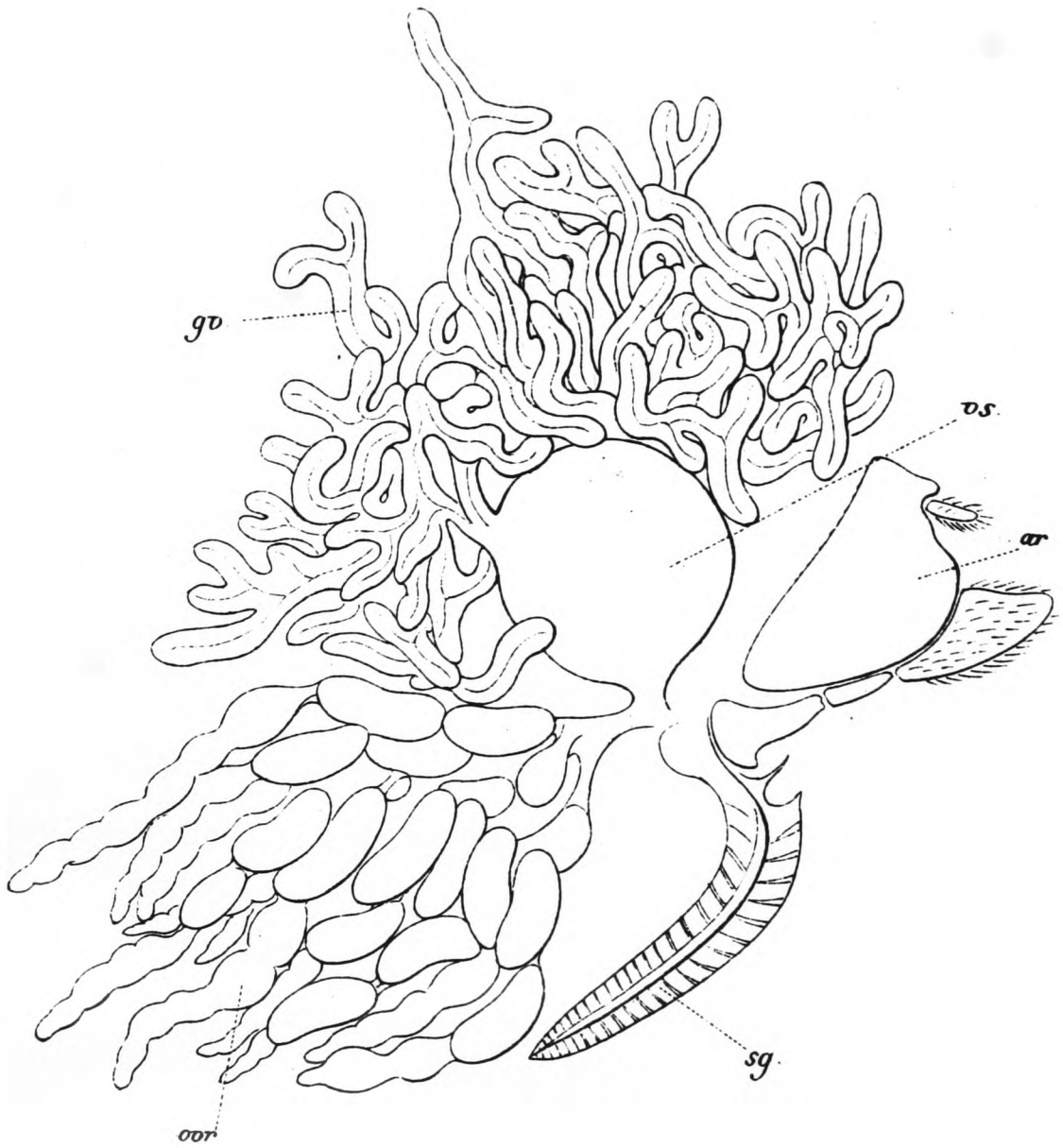


Plate 2.1. Genital apparatus of Pontania proxima in water (from Beyerinck, 1888); vs. colleterial sac; gv. accessory gland; ovr. ovaries with ripe and unripe eggs; sg. saw; ar. last abdominal segment.

2.3 **Synonymy.**

Genus Pontania Costa, O., 1859. Fauna del Regno di Napoli, Imen., pt 3, p. 20.

Type-species: Nematus gallicola Stephens = (Pontania proximus Lep.) Desig. by Marlatt, 1896.

Revision: Marlatt, 1896. U. S. Dept. Agr., Div. Ent. Tech. Ser. 3: 20-43.

Species Pontania proxima (Lepeletier), 1823

Synonymy according to Carleton (1939).

Nematus proximus Lepeletier, 1823. Mon. Tenthred., 67, No. 201.

Nematus gallicola Stephens, 1835. Ill. Brit. Ent. (Mand.), 7: 36.

Nematus crassispina Thomson, 1871. Hymenopt. Scandin., 1: 164, n. 99.

Nematus vallisnieri Hartig, 1873. Die familien der blattwespen...: 205

Nematus festivus Zaddach, 1883. Schrift. phys. ökon. Ges. Königs., 24: 146.

Nematus gallicola Stephens, 1835. Illus. Brit. Ent., 7: 36.

Nematus parvulus Holmgren, 1883. Ent. Tidskr., 4: 146. n. 12.

Synonymy according to Benson (1958: 204).

Hoplocampa gallicola Cameron, 1877. Ent. Monthly Mag., 14: 156.

Euura flavipes Cameron, 1885. Monog. Brit. Phytophagous Hym., 2: 211.

Pontania capreae (L.) auctt. nec L., in part.

Synonymy according to Kloet and Hincks (1978: 11).

gallicola Stephens, 1835. Illus. Brit. Ent., 7: 36.

gallicola Cameron, 1877. Ent. Monthly Mag., 14: 156.

flavipes Cameron, 1885. Monog. Brit. Phytophagous Hym., 2: 211.

Synonymy according to Smith (1979: 71-75).

Nematus proximus Lepeletier, 1823. Monog. Tenthred., p67. female.

Nematus gallicola Stephens, 1835. Illus. Brit. Ent., 7: 36.

Messa hyalina Norton, 1864. Ent. Soc. Phila., Proc. 4: 8. female.

Holocampa gallicola Cameron, 1877. Ent. Monthly Mag., 14: 156.

Euura flavipes Cameron, 1885. Monog. Brit. Phytophagous Hym., 2: 211.

2.4 Occurence.

Most sawflies associated with the formation of plant galls are confined to the families Tenthredinidae and Xyelidae. There are approximately 5000 species of Tenthredinidae distributed world-wide (Narendran, 1984). Benson (1950) estimated that, in the British Isles, there were approximately 460 native or naturalized alien species of the suborder Symphyta, while Kloet and Hincks (1978: 4-12) listed 479 possible species, of which 12 were species of Pontania. Over half have woody plants as hosts. Gauld and Bolton (1988: 106) provided an extensive list of food-plants of Symphyta. The most frequented plant families and the approximate numbers of sawfly species associated with these families are as follows:-

101 species on Salicaceae (Populus L. and Salix L.).

87 species on Rosaceae.

60 species on Betulaceae (Betula L. and Alnus Miller).

32 species on Pinaceae (Pinus L., Picea A. Dietrich and Larix Miller).

30 species on Gramineae.

16 species on species of Quercus L. (Fagaceae).

13 species on Cyperaceae.

10 species on species of Equisetum L. (Equisetaceae).

Of the Tenthredinidae, Gauld and Bolton (1988: 116) suggested an occurrence in Britain of just under 400 species. Benson (1958)

described the subfamily Nematinae as the dominant group of his Arctic zone with Pontania being distributed throughout his Siberian zone (Benson, 1950); with, in Britain, nearly 200 species in 21 genera (Gauld and Bolton, 1988: 117-118). Carleton (1939) listed the native distribution of Pontania proxima as being Great Britain and Ireland, most of Europe and extending into Siberia. It has been introduced into North America (Benson 1960b) and New Zealand. Sandlant (1979) and Kay (1980) noted the first observation of Pontania proxima in New Zealand as being at south Canterbury in 1929.

In Britain there are 180 species of the tribe Nematini divided into 16 genera (Benson, 1958). Of these the three gall-making genera are Phyllocolpa, species of which live in leaf-roll margins of Salix and Populus species (Benson, 1960a), Euura, whose species incite galls in the closed buds, petioles and stems of Salix species and Pontania species, which incite a wide variety of leaf galls in Salix species (Benes, 1968; Smith, 1970).

The life-cycle, parasites and inquilines of Pontania proxima and related species, together with their interactions, have been described by numerous authors. In this century these authors include, Magnus 1914, Bastin 1921, Carleton 1939, Benson 1943, 1950 and 1954, Smith 1951, Caltagirone 1964, Smith 1970, Sandlant 1979, Kay 1980, Kopelke 1985a, 1985b, 1986 and 1988 and Clancy et al. 1986.

2.5 **Emergence of the imago.**

Carleton (1939) stated that the Pontania proxima sensu lato female emerged from her winter cocoon in May or June. More specific times for oviposition were given by Murphy (1929), late April for the spring

brood and mid-July (especially the third week) for the summer brood. However, Kopelke (1985a) described the duration of larval instars as variable, resulting in a considerable overlap between broods. This is in agreement with my personal observations. When freshly emerged, sawfly adults remain where they are for some days while their cuticle hardens, after which the females begin to oviposit (Benson, 1950). The life span of the adult is quite short (Benson, 1943), Smith (1970) described sawflies in the subfamily Nematinae as "relatively fragile creatures, and adults usually do not live for more than two weeks....Emergence of the imago is synchronized with daylight, and most leave their pupal chambers in early morning. However, they may remain hidden in the immediate vicinity and will not attempt to fly until the temperature rises later in the day. Since humidity is low in midday, the 1st flights may not be made until late afternoon". He suggested a temperature of 28° C and 90% relative humidity are required for optimum survival in the laboratory. This agreed with Benson (1943), who described sawflies as living from two to 26 days depending on the species. In captivity, males of the small species survived for only three to four days and the females for seven to 14 days (Benson, 1950); my own observations relating to Pontania proxima would reduce this period to two to three days at 25°C with a high humidity (70%+).

Chapman (1982) described the larvae of the Tenthredinidae as polypod larvae, with abdominal prolegs as well as thoracic legs. These larvae were poorly sclerotized and relatively inactive. The Nematinae associated with Salix feed from the catkins in the spring and are rarely found on other flowers (Benson, 1950; Smith, 1970).

2.6 Oviposition.

For Pontania proxima the most complete account of oviposition was given by Magnus (1914: 59-60). He described the saw of the ovipositor as being homologous to the bristles of a bee sting. During oviposition, the saw was driven vertically downwards into the leaf, at a site just above a lateral vein. As the ovipositor penetrated the leaf it became more prominent and was gradually twisted, becoming parallel to the surface of the leaf. This movement resulted in a bean-shaped cavity, tapered at the tip and with the concave side facing the midrib. The egg was placed at the end of the cavity and colleterial fluid sealed the wound. Caltagirone (1964) referred to Pontania pacifica on Salix lasiolepis Bentham, which oviposits into the midrib, rather than the lamina of the leaf as does Pontania proxima. This evergreen willow is capable of being host to approximately six generations of sawfly each year (Smith, 1970), whereas most other species are bivoltine. For Pontania proxima, as with other species of Pontania, young actively expanding leaves are required as the presence of meristematic parenchyma is essential: it has the greatest potential to produce a physiological reaction to the stimulus of the sawfly (Slepyan and Gabarayeva, 1981).

Caltagirone (1964) described how the female searched for a suitable leaf, palpated the tender parts with her antennae, and sometimes her ovipositor which resulted in small incisions in the leaf. When a developing bud was found these movements became more intense, the female mounted the midrib of the abaxial surface of the outer leaf, her head facing the petiole of the leaf. The ovipositor was then driven into the vein and as soon as the mesophyll was penetrated it bent at an

angle of 90° away from the vein and the cut was continued parallel to the surface. The ovipositor moved radially downwards towards a vein and an egg laid in the resulting pouch. The cavity formed in this manner was concave and slightly tapered towards the top, the concave surface facing the midrib. Along with the egg, colleterial fluid was injected into the pouch; it bathed the egg and sealed the wound. The whole process took 60-65 seconds to complete. Ovipositing continued as the sawfly moved down the leaf, usually on alternate sides of the midrib. Murphy's observations (1929) related to the summer brood of Pontania proxima with oviposition occurring on still, sunny afternoons. Too much wind rendered the sawflies unable to oviposit, due to the movement of the branches . She stated that the female selected an unfolded terminal bud, where the outermost leaf had just separated at the bottom and around the edges, from those below. These observations are similar to those of Caltagirone (1964). Murphy recorded that the sawfly crawled to the tip of the leaf, then moved downwards, her abdomen moving up and down. One quarter of the way down the leaf, the sawfly pierced the lamina near the midrib. This made a slanting slit, into which the egg was placed. This process was continued down one side of the leaf, then up the other. By the following day the leaf was usually unfolded. Smith (1970) described oviposition as happening through a rapid vibration of the gonapophyses, rather than a more obvious sawing motion. The lamina was penetrated by the saw in a pneumatic drill-like fashion. Only a slight disruption of the plant tissues resulted, but this disruption increased as the host/parasite specificity decreased. Hovanitz (1959) described the ovipositor of Pontania pacifica as being pushed into the midrib, making a hole of considerable size. The egg was then pushed through the midrib to lie in the mesophyll space, with specificity

being based on the morphology of the willow tissue. Magnus (1914: 58) gave a time of two minutes for the completion of this process, but added that the wasp needed to rest several times in successive ovipositions. Slepyan and Gabarayeva (1981) argued that specificity was determined by the length of the ovipositor, together with the tannin content and mesophyll structure of the leaves.

For Pontania proxima, the site of oviposition lies between the midrib and leaf margin (Benson, 1954; Slepyan and Gabarayeva, 1981) of the five to ten apical leaves. Slepyan (1962) demonstrated that oviposition by Pontania proxima was topologically specific. Both Beyerinck (1887) and Magnus (1914: 59) stressed that oviposition started from a lateral vein, and Magnus (1914: 60) observed that the length of the slit caused by the saw was 1.25 mm. Shanahan (1957) observed that galls of the second brood were mainly in the upper branches of Salix fragilis and in a comment to Shanahan (1957), Benson pointed out that at this time of the year most growth would be concentrated in the upper parts of the tree, rather than all over, as it does in the spring. This point was echoed by Caltagirone (1964) who described the spring galls on Salix lasiolepis as being randomly distributed with one to two galls of Pontania pacifica per leaf. Late in the season, galls occurred in greater numbers on leaves confined to the tips of the twigs; the reason advanced was that oviposition occurred only on young leaves, i.e. in spring, practically all the leaves would be available, because the plant was growing rapidly, later in the season mature leaves were unfit for oviposition and only those at the growing tips were suitable.

Müller (1872) reported that females of Pontania proxima were able to lay more than 30 eggs, an activity which occurred in one session,

but on different leaflets of the leading shoot. Swammerdam (1758: 77), Magnus (1914: 74), Peacock (1928), Carleton (1939) and Benson (1950) all noted that, after being laid, the egg increased in size. Peacock (1928) working on Pristiphora pallipes (Lepeletier) (Tenthredinidae, a pest of Ribes L. spp., Grossulariaceae), found an abnormal female with crippled saws and unable to lay eggs. He dissected out the eggs and glued them onto gooseberry leaves. From 28 eggs treated in this way, five larvae were obtained. He concluded, significantly for this research, that the colleterial fluid produced by the female was not needed for the egg to hatch, because the eggs that he removed had not passed down the oviduct and so had not been exposed to this fluid. Further, he concluded that the expansion of the egg after oviposition was due to intra-ovum conditions, not from absorption from the leaf. Extra-ovum absorption, however, does occur, because in other studies Peacock (1928) found that when a cut gooseberry twig was placed in a weak eosin solution, normal eggs in leaves were stained red; though atmospheric moisture cannot be ruled out as playing some part in the swelling process. Benson (1950) linked the growth of the egg to the growth of the developing embryo. Chapman (1982: 400-402) discussed the absorption of water by the eggs of various Coleoptera, Heteroptera, Diptera and Orthoptera, a process which may result in a considerable increase in volume and mass. This occurred during a limited period of development which varied from species to species. Price and Craig (1984) noted eggs of the sawfly Euura lasiolepis Smith (Tenthredinidae) absorbed water during embryogenesis.

The stimulus for oviposition is unknown, but when placed on a suitable leaf, Pontania proxima will begin to attempt oviposition almost immediately (personal observation). Harborne (1988: 179)

suggested surface chemicals and visual clues were important stimuli in other cases; for example, for the carrot fly Psila rosae (Fabricius) (Psilidae), propenylbenzenes, coumarins and polyacetylene (which are powerful growth inhibitors of bacteria, fungi, plants and nonadapted herbivores) were found in leaf surface extracts of Daucus carota L. (Umbelliferae) and synergistically stimulated oviposition by Psila rosae (Städler and Buser, 1984). Zimmerman (1979), identified an oviposition-deterring pheromone which prevented overcrowding in tephritid flies.

Benson (1950) stated that the incubation time of eggs will vary according to temperature, humidity etc. and, citing Miles et al. (1933), gave the incubation time for the sawfly Hoplocampa flava (L.) (Tenthredinidae), the larvae of which are found in wild and cultivated plums, as four to 24 days.

AUTHOR	MAGNUS	MURPHY	CARLETON	HOVANITZ
DATE	1914	1929	1939	1959
NO. EGGS LAID			25-35	50-100
SIZE OF EGG (mm)	0.45 to 0.51	0.50 x 0.18	0.64 x 0.27	
HATCH AFTER (days)	12	6 *fragilis* 21 *triandra*	12-19	4-5
TIME SPENT AS: INSTAR 1st 2nd (days) 3rd 4th 5th 6th			 3 3 >3 >3 >3	
SUB TOTAL			15	30-45
PREPUPA (days) PUPA				
SUB TOTAL			9-22	14
TOTAL TIME			36-56	48-64
TEMPERATURE			ambient air	
SPECIES PAIR	*P. triandrae* *S. triandra*	*P. triandrae* *S. triandra* *P. proxima* *S. fragilis*	*P. triandrae* *S. triandra* *P. proxima* *S. fragilis*	*Pontania* sp. Willow sp.
GALL DIMENSIONS (mm)	12 x 6 x 5		7 x 4 x 5	
FULLY GROWN AFTER (days)	12		14	continuous

Table 2.1. Summary of information relating to the development times and conditions for the successful development of eggs and larvae of various species of *Pontania*, collected from literature published between 1914 and 1959.

AUTHOR	CALTAGIRONE		SLEPYAN & GABARAYEVA	LAZAREVA et al.
DATE	1964		1981	1986
NO. EGGS LAID	120-160		30+	3-60
SIZE OF EGG (mm)	0.56 x 0.16			0.55 x 0.25
HATCH AFTER (days)	4.3		12-13	7-8
TIME SPENT AS: INSTAR	MALE	FEMALE		
1st (days)	2.3	2.7		
2nd (days)	2.3	2.9		
3rd (days)	2.0	2.4		
4th (days)	2.0	2.2		
5th (days)	8.5	8.6		
6th (days)		4.4		
SUB TOTAL	17.1	23.2		
PREPUPA (days)	11.0	10.4		
PUPA (days)	5.5	5.8		18-20
SUB TOTAL	16.5	16.2		
TOTAL TIME	37.9	43.6		
TEMPERATURE	26.7° C			
SPECIES PAIR	*Pontania pacifica* *Salix lasiolepis*		*P. proxima* *S. fragilis*	*P. proxima* willow spp.
GALL DIMENSIONS (mm)	length 8.4		10 x 6 x 8	length 7-13
FULLY GROWN AFTER (days)	directly related to growth of leaf			

Table 2.2. Summary of information relating to the development times and conditions for the successful development of eggs and larvae of various species of Pontania, collected from literature published between 1964 and 1986.

2.7 **Reproductive strategies.**

Both facultative and obligate parthenogenesis are more common in Hymenoptera than in any other order of insects. Characteristic of Hymenoptera is haplo-diploidy, where unfertilized eggs yield haploid males, while fertilized eggs produce diploid females (Chapman, 1982: 442). This system is also known as haploid parthenogenesis or arrhenotoky. Whiting (White, 1954: 327 citing Whiting, 1945) preferred to use the term impaternate (fatherless) males as the terms haploid and diploid referred to germ line nuclei and the degree of endopolyploidy in the somatic cells was unknown.

Pontania species exhibit thelytokous parthenogenesis (i.e. parthenogenesis which results in females only), which Benson (1950) described as obligatory parthenogenesis. Both Hovanitz (1959) and Benson (1950) suggested that parthenogenesis in Pontania was mediated by temperature. Hovanitz found that the warmer the temperature, the higher the proportion of males was within the population. He based this assertion on the comparison of sex ratios between populations of Pontania species in Pasadena, where half the offspring were males, and Canada where this proportion dropped to 2%. Benson (1950) also linked thelytoky with temperature. He suggested that there was a tendency for these forms to appear in the north, with normally reproducing strains in the warmer areas to the south. Benson imagined some temperature threshold across which different forms of reproductive strategies could be found; he envisaged "a normal sexually reproducing strain in a warmer climate throwing off apomict strains along its northern fringe or in mountain ranges". For Pontania proxima, Benson (1950) stated that the normally reproducing parent stock was not known. In contrast,

Carleton (1939 citing Smith, 1876) suggested the lack of males may be due to their early emergence from the cocoon so, unless collected early, males would be missed. Working on Pontania pacifica, Caltagirone (1964) found that the males of this species passed through five instars before pupating, unlike the female who passed through six, giving the males a shorter development time of approximately 5.6 days.

White (1954: 360) stated that none of the thelytokous Hymenoptera was polyploid except for Diprion simile (=similis (Hartig)?) (Diprionidae). Benson (1950) suggested that the normal diploid number for Symphyta was 16.

Of the few examples of chromosomes studies, conducted on sawflies, Pristiphora pallipes which reproduces by thelytokous parthenogenesis, was noted by Comrie (1938) to have a diploid chromosome constitution of 16. This arose, after normal meiosis, by a fusion of the egg pronucleus and the second polar body. Another species of sawfly Priophorus tener Zaddach (=morio (Lepeletier)) (Tenthredinidae) had 2n = 12, but the mechanism for this formation was unknown. Other sawflies e.g. Thrinax macula (Klug) (= Strongylogaster macula (Klug), Tenthredinidae) have a diploid number of 14, which is formed by one non-reductional maturation division (Peacock and Sanderson, 1939).

Thelytoky has arisen repeatedly in a large number of phyla (except Vertebrata) and can be induced experimentally (White, 1954: 326), while arrhenotoky is much rarer with the normal sex-determining system being completely replaced. In Hymenoptera the whole order shows haplo-diploidy (White, 1954: 326) with thelytokous parthenogenesis arising secondarily. Various authors mention the lack of males in Pontania proxima (Carleton, 1939; Benson, 1943, 1950 and 1960b;

Hovanitz, 1959) and the rôle of parthenogenesis as a reproductive strategy.

The effects of thelytokous parthenogenesis would be expected to result in numerous closely-related, but genetically different biotypes, since each new female, through genetic mutation, has the protentiality to become the foundress of a unique strain. This form of reproduction would also be expected to lead to an increase in homozygosity as deleterious recessive genes are removed by selection. Benson (1950) stated that it was unclear as to whether the development of different strains actually occurred, as local forms did not appear to be prevalent.

It is interesting to note that Allard (1975) when analyzing the isozymes of the inbreeding wild oat Avena barbata Pott ex Link (Gramineae) found fewer homozygotes than would be expected, as different genotypes were being selected for by different microenvironments. Homozygous seedlings died early which led to an overall shift to heterozygosity, i.e. these were fitter and better buffered to withstand selection pressures. Whether similar effects are found in parthenogenetic insects has not been established.

Of the two types of thelytoky mentioned, that exemplified by Pristiphora pallipes involves meiosis followed by a doubling of the chromosome number, Chapman (1982: 442) described this as meiotic or automictic thelytoky. The second type (i.e. that shown by Thrinax macula), was called ameiotic or apomictic thelytoky and Chapman (1982: 442) suggested that this was of common occurrence in tenthredinids, involving the suppression of the normal process of meiosis with only mitosis taking place. White (1954: 342) suggested that automicts, by virtue of crossing over and segregation still taking place, would soon

eliminate deleterious recessive genes as they become exposed; in these organisms the drive would be to homozygosity. He suggested that the reverse would be true for apomicts. Here mutations and structural changes to chromosomes, unless immediately deleterious, would be retained, leading to an increase in heterozygosity, with the two sets of chromosomes eventually becoming almost completely dissimilar. Which of these two processes occur in Pontania proxima is not clear, but Benson (1950) described them as apomicts. Whether he used this term sensu stricto or not is not clear, but whatever the processes, White (1954: 343) believed that agamic complexes such as those that can be found in plant genera such as Hieracium L. (Compositae), Taraxacum Wiggers (Compositae) and Rubus L. (Rosaceae) do not occur in the majority of cases of thelytoky in animal genera, as thelytoky is of comparatively recent origin in terms of evolutionary time. Even where males do occur, although extremely rarely, as in Pontania proxima (Carleton, 1939; Benson, 1950), Benson made the point that it was unclear whether a haploid male could successfully fertilize a diploid female, to produce offspring that were not triploid. Gauld and Bolton (1988: 11) suggested that species that occasionally produce isolated males, could be considered as deuterotokous.

2.8 **The nature of the gall.**

In discussing the impact of gall-makers on plants, Abrahamson and McCrea (1986) considered that when measured on a per herbivore basis, these impacts would probably be greater for gall-makers than for other herbivores. The gall-maker is dependent on the plant, not only for nutrition, but also as a habitat and for protection against adverse

environmental factors and some predators (Weis et al., 1988). The gall itself is a product of two genomes. The insect coding for the stimulus, the plant for the response (Abrahamson and McCrea, 1986; Weis and Abrahamson, 1986), i.e. gall formation is an adaptation by the insect (Weis et al., 1988). Shorthouse and Lalonde (1988) demonstrated the intricate control that a gall-inducing insect has over its host's morphogenetic processes. Galls of Urophora cardui (L.) (Tephritidae = Trypetidae) on Cirsium arvense (L.) Scopoli (Compositae) were treated at various stages of development with systemic insecticide. From changes in gall development, after the death of the insect, they concluded that "the formation and retention of primary nutritive cells was under the direct control of the larvae. Similarly, the formation and maintenance of secondary nutritive tissue also required the larval presence, although this tissue degraded less readily than primary nutritive tissue. In contrast, other processes such as the formation of the zone of lignified tissue and the maturation of vascular tissues in the gall were due to developmental processes that were inherent to the plant".

The result of this interaction presents a paradox. The response of the plant is to produce a growth (often complex in structure), that seems to have no benefit, other than for the insect. This apparent altruism, on the part of the plant, has been the source of much discussion, at least since the latter part of the 19th century (Cockerell, 1890; Hollis, 1889 and 1890; McLachlan, 1889; Mivart, 1889a and 1889b; Romanes 1889a, 1889b and 1890; Wetterhan, 1889), it was considered by some to argue against Darwin's theories, "If it could be proved that any part of the structure of any one species had been formed for the exclusive good of another species, it would annihilate

my theory, for such could not have been produced through natural selection" (Darwin, 1906: 253-254). These questions were answered in part by Romanes (1889a) who suggested that natural selection was acting on the plant, but through the insect, by selecting those insects that will best cause a gall to form.

The purpose of this Section is to evaluate the relationships between the gall-maker, the host plant and the parasites and inquilines of the gall-maker, together with a discussion of the adaptive nature of galls, not only in terms of the organisms involved, but also as to whether theories of coevolution can be evoked. The arguments presented here relied heavily on the work of others, but I believe that they demonstrate and support the idea that amidst these complex relationships lies a simple but novel concept. This concept, while recognizing that to understand the nature of the gall it is essential to consider all the forces and participants involved, raises the gall above being merely an appendage of the plant, to being the result of a synergistic effect. This effect results in the gall being more than purely a product, it considers that a gall has a characteristic ontogeny of its own and should be considered as such. Perhaps it is within the concept of a greater patio ludens (van Steenis, 1976), that has allowed plants the scope to develop such specialized structures - "Such associations form, as it were, one compound organism" (Wetterhan, 1889). Waring (1986) acknowledged that it was difficult to draw general conclusions concerning galling insect-host relationships as they were very diverse. By concentrating in the main on sawflies, however, it is possible to understand some patterns of association. Projection of these to other pairs of species would call for caution.

2.8.1 The dynamics of interactions.

Benes (1968) listed a number of factors, both abiotic and biotic, that influenced the development of galls formed by species of Pontania and Phyllocolpa. These were the site of oviposition, the host plant species, the age and growth activity of the plant tissue, the number of galls per twig, the larvae, parasites, diseases and inquilines. However, the most extensive studies on population dynamics and community ecology of sawflies have been carried out by P. W. Price (Northern Arizona University) and colleagues. Much of their work concerns the relationships between Salix lasiolepis Bentham (Salicaceae), the arroyo willow, and the stem-galling sawfly, Euura lasiolepis Smith.

Salix lasiolepis has a shrubby habit, reaching a height of two to four metres and is abundant along streams throughout northern Arizona from a elevation of 1200 to 2300 metres. The species is dioecious, and can reproduce vegetatively via the layering of stems. The result of this is a single clone (genet), produced from many ramets, which eventually covers many square metres. Each clone can be distinguished from its neighbour by gaps in the canopy, sex, phenology of flowers and leaves as well as stem and bud colour. These clones are therefore easily distinguishable and are used as sampling units (Fritz et al., 1986; Price and Clancy, 1986b). Euura lasiolepis oviposits into the petioles of young leaves, initiating a cataplasmic gall in the portion of the young stem below the leaf (Price and Craig, 1984). Relationships between factors affecting the galling insect-host relationship are summarized in Figure 2.1.

Attacks by gall-forming sawflies cannot be described as random; my own observations confirm this. Each year certain trees can be relied

upon to be more heavily infested than neighbouring trees of the same species. As others have observed (Magnus, 1914:58; Shanahan with note by Benson, 1957; Smith, 1970) these are generally young or have been recently pollarded, so providing long fast growing shoots, but even here there is variation. At Northmoor Nature Reserve (see Appendix Two) gall infestation is concentrated on a few pollarded willows, whereas their neighbours, the same species (Salix fragilis L. var. russelliana (Smith) Koch), not more than a few metres away are much less heavily infested. At Sandford Lock (see Appendix Two), one tree which has never been pollarded is always preferentially attacked compared with neighbouring trees. Benson (1950) described sawflies as weak fliers, but the close proximity of less infested trees rules this out as the sole reason for the observed mosaic of successful attacks.

Investigations into the preferential attack strategies of sawflies have concentrated on aspects of the host plant rather than those of the insect.

Fritz et al. (1986) described the distribution of Euura as "spatially patchy" suggesting an underlying differential susceptibility between clones, which was genetic in basis. They also determined that the phenotype of the plant was more important in establishing community patterns than were the effects of interspecific competition. The shoots most favoured for attack were long ones, i.e. those usually associated with younger plants or ramets (Craig et al., 1986; Price et al., 1987a). This pattern of attack occurred regardless of habitat and resulted in a greater survivorship of the larvae. As willow branches aged and thus shoot length declined, so did the attack rates. This seems to be very similar for Pontania spp. (Fritz et al., 1987; personal observations). In brief, sawflies select for attack those

shoots that provide the best opportunity for survival. This drive results in positive correlations between similar species of sawflies, where shoot choice is more important, as a distribution factor, than interspecific competition (Fritz et al., 1987). Fritz et al. (1987) found their work supported the model of Bultman and Faeth (1985) which assumed that 1) leaves/shoots differed in chemical and physical properties, 2) larvae of herbivorous species required similar qualities in leaves or shoots, and 3) there was greater selection pressure on females to detect and oviposit on leaves and shoots of higher quality than to detect and oviposit in response to con- and heterospecifics.

Environmental factors affecting plant phenotype cannot be ignored and often have a more profound effect on galling than the genotype of the plant (Craig et al., 1988). Price and Clancy (1986b) found that relatively lower winter precipitation reduced the number of shoots per stem and shoot length. This reduction of fitness for oviposition by Euura lasiolepis was further enhanced by the retardation of the willow's phenology, so reducing the number of sites available for oviposition early in the spring. Willows are among the most water-demanding woody plants (Price and Clancy, 1986b) and easily become water-stressed. Price and Clancy (1986b) considered that not only was there a direct reaction to water-stress as seen in the plant's reduced fitness for oviposition, but there was also an indirect affect through the possible reduction of water available, to the egg, for absorption during embryogenesis (Price and Craig; 1984). Both these factors contributed to a higher rate of egg death (Price and Clancy, 1986b; Preszler and Price, 1988). What is evident from these studies is that Euura females are able to exercise discrimination in their choice of sites for oviposition. Waring (1986) acknowledged that water-stress

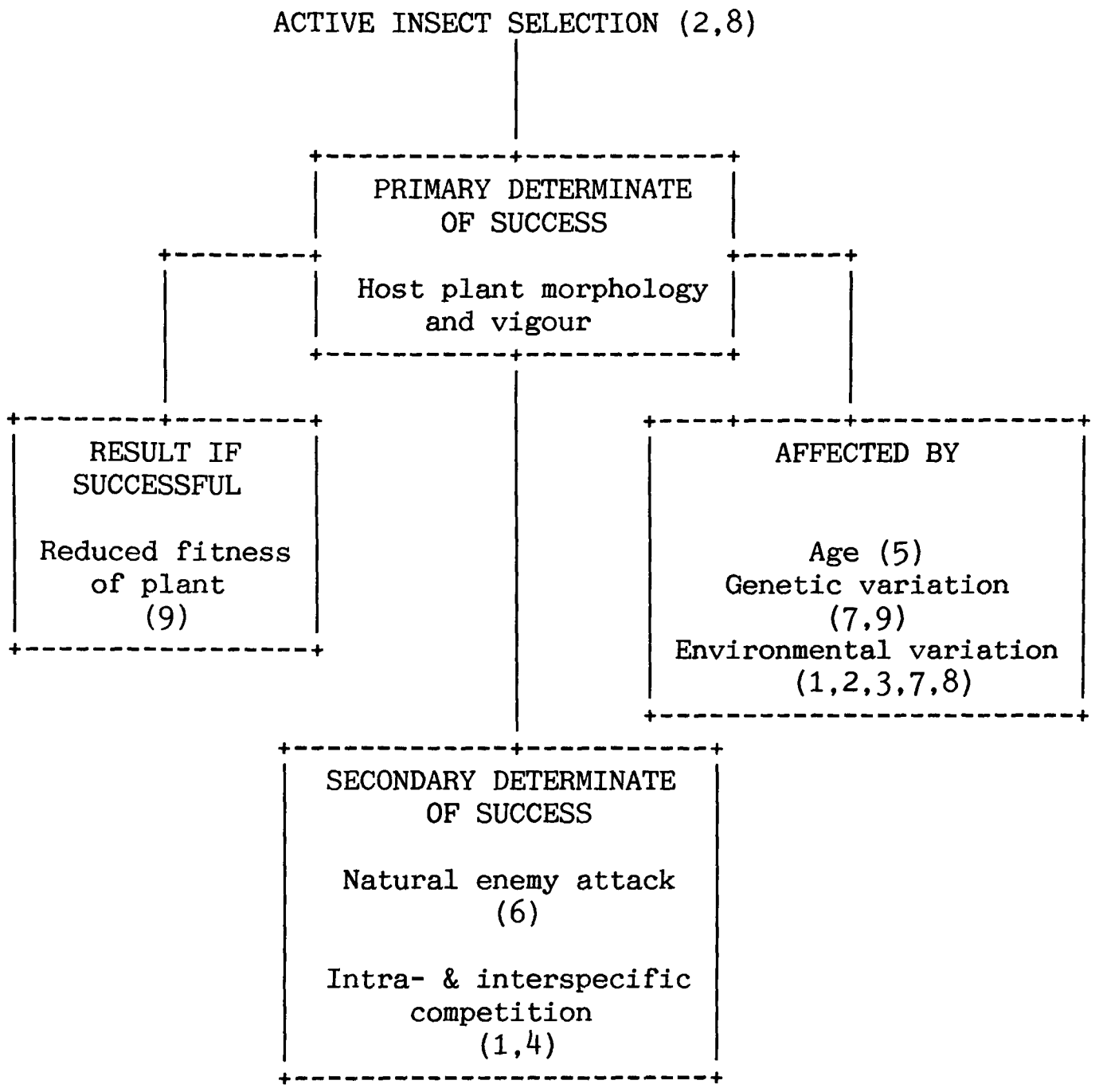
affected strongly the distribution of Euura, resulting in less colonization and a lowering of survivorship; however, this pattern did not apply to all gall-formers.

Of the natural enemies of Pontania species, Smith (1970) listed birds, which devour the gall, and, amongst the insects, examples include; species of Eurytomidae, Pteromalidae, Eulophidae, Mymaridae, Ichneumonidae, Braconidae, Sphecidae, Curculionidae and Cosmopterygidae as well as some species of mites of Anoetidae and Pyemotidae. Kopelke (1988) listed 29 species of parasite of European species of Pontania from the families Mymaridae, Ichneumonidae, Braconidae, Eulophidae and Pteromalidae, which affect all stages of the life-cycle of the insect from egg to pupa. As well as these, Kopelke (1988) detailed the aggressive inquilines that inhabit the gall.

Price et al. (1987b) asserted that natural enemies were less important, for a successful attack by sawflies, than the morphology and vigour of the host plant. Percentage parasitism varies enormously: at low host densities it may vary between 0 and 33% and exceptionally 100% (Price, 1988). For a species of Pontania on Salix lasiolepis, enemy attack by the ectoparasites Bracon angelesius Provancher (Braconidae) and a species of Pteromalus Swederus (Pteromalidae) was found to be independent of prolonged larval development, with the parasites more likely to attack rapidly developing sawflies, which tended to inhabited rapidly growing galls (Clancy and Price, 1987).

Gall size is also important for parasitoids which oviposit through the wall of the gall e.g. Lathrostizus lugens (Gravenhorst) (Ichneumonidae), rather than entering the gall e.g. Adelgnathus cubiceps Roman (Ichneumonidae), a parasite of the Pontania herbaceae Cameron group (= Pontania crassipes (C. G. Thomson)?). These parasites

will be limited by the length of their ovipositors (Weis et al., 1985; Price and Clancy, 1986a; Kopelke, 1988). A species of Pteromalus with a mean ovipositor length of 1.89 mm was found to have an attack range limited to the first two galls on a shoot, as these were generally the smallest. The gall diameter was determined both by the genotype of the plant and availability of water (Price and Clancy, 1986a). Price (1988) found that natural populations of the parasitoid (Pteromalus), were inversely density-dependent to host density i.e. concealing factors were important in defining responses to parasitoids. He stressed "that the three trophic levels of plants, herbivores and their parasitoids interact in complex ways and need to be understood as a unit, rather than in pairwise comparisons of only host and parasitoid" (see also Price and Clancy 1986a).



Key to Authors.

1. Fritz, Sacchi and Price, (1986).
2. Price and Clancy, (1986b).
3. Waring, (1986).
4. Fritz, Gaud, Sacchi and Price, (1987).
5. Price, Roininen and Tahvanainen, (1987a).
6. Price, Roininen and Tahvanainen, (1987b).
7. Fritz and Price, (1988).
8. Preszler and Price, (1988).
9. Sacchi, Price, Craig and Itami, (1988).

Figure 2.1. A summary of factors which affect the success or otherwise of sawfly attack and survival.

2.8.2 Impacts.

There have not been many studies of the impact of galling on a host plant, and the results of these studies do not present a generalized picture (Abrahamson and McCrea, 1986). For sawflies, Sacchi et al. (1988), provided evidence supporting the view that gall infestation resulted in a reduction of fitness in Salix lasiolepis. Shoots produced fewer reproductive buds and this occurred regardless of the sex of the plant. The extent of loss of reproductive potential was directly proportional to the level of infestation. Other factors, such as inflorescence size, seed mass, germination mass and pollen germination were not affected. Heavy infestations could also result in the stunting or death of galled shoots. When this occurred, dormant buds at the base of the damaged stem produced new shoots, which were ideal sites for further galling. The overall effect was to maintain the ramet in a juvenile state. Sawflies thus regulated the production of sites for oviposition. (Craig et al., 1986).

Negative effects of gall-makers can be manifested either through the reduction of reproductive potential or by the alteration of tissues that have a productive function. Abrahamson and McCrea (1986), in a review of this topic, reported that galls of Eurosta solidaginis (Fitch) (Tephritidae) on Solidago altissima L. (Compositae) acted as a sink (see also Slepyan and Gurevich, 1976; Shorthouse and Lalonde, 1988) for the mineral elements nitrogen and phosphorus. Other effects on the host included decreased leaf and inflorescence allocation, reduced new rhizome numbers and biomass. They concluded that seven percent of the ramet's energy went into supporting the gall and its inhabitant, but that these impacts were localized i.e. the gall intercepted materials, but did not mobilize them towards it. In the

galls of Pontania proxima on Salix alba significantly higher levels of carbohydrates have been found, than occur in non-galled leaves (Hartley, personal communication). Differences in the levels of nitrogen and phenolics were not significant. The insect manipulates the host's resources, but until there is a greater understanding of the nutritional requirements of gall-formers, and the levels of specific nutrients from specific areas of the gall are established, confusion will remain over whether they are active sinks (see also Bronner, 1983 for a similar discussion on cynipid galls, also Section 4.5.4b).

Hutchinson and Kearns (1930), in discussing the insect pests of Salix triandra, reported that Pontania species, even when many galls were formed, could not be considered a serious pest of basket willows, a view supported by Slepyan (1962). Lazareva et al. (1986), however, provided extensive details on the importance of its exclusion and eradication from willows. Examples of deleterious effects of gallers on plants of economic importance are given by Carter (1962: 194-201) and Abrahamson and McCrea (1986). From the lack of recent literature it is evident that the effect of galling and gall-formers, compared to the effects of other herbivorous insects, is dealt with sparsely. One area of interest is the use of gall-makers as biological control agents. Dennill (1988), was the first to use a gall-forming hymenopteran i.e. the gall wasp Trichilogaster acaciaelongifoliae (Froggatt) (Eurytomidae) to control the weed Acacia longifolia (Andr.) Willdenow, while the flies, Urophora affinis (Frauenfeld) and Urophora quadrifasciata (Meigen) (Tephritidae, Myers and Harris, 1980), have been introduced into British Columbia for the biological control of diffuse and spotted knapweed (Centaurea diffusa Lamarck and Centaurea

maculosa Lamarck, (Compositae). There are few other examples of this use of gall-makers.

2.9 The adaptive nature of the gall and evolution.

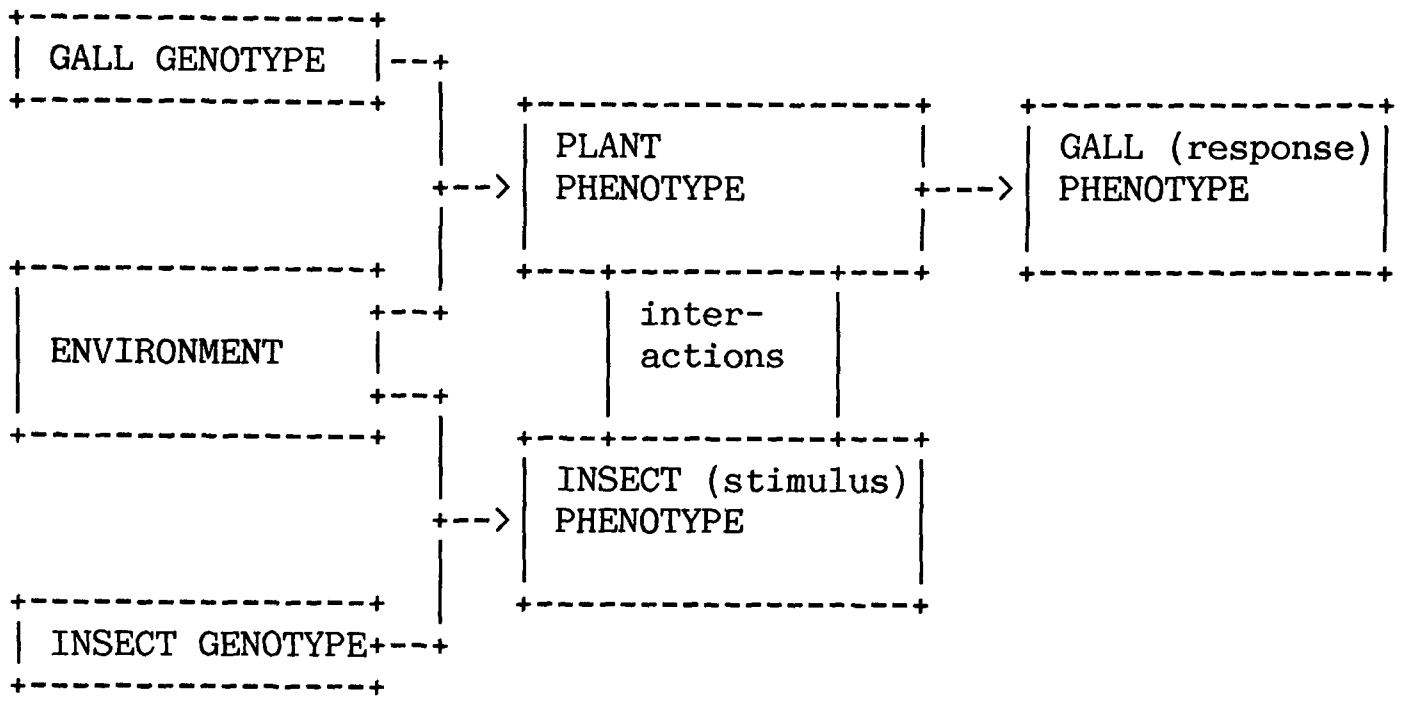


Figure 2.2. Relationships of factors affecting gall phenotype (from Weis and Abrahamson, 1986).

Weis et al. (1988) described gall-makers as excellent subjects for the study of ecological and evolutionary principles that apply to sedentary herbivores and host-plant selection. The previous section described some of the ecological aspects and Figure 2.2 summarizes the relationships between factors that affect the phenotype of the gall (Weis and Abrahamson, 1986).

Previous studies of the initiation of gall formation have, at various times, concentrated on either the plant, the insect or the gall, but with reference to the Pontania-Salix system, only one paper has been found that attempts to identify the cecidogen (McCalla, 1962). All the others concentrate on aspects of taxonomy, morphology or

ecology. With the exception of McCalla (1962) all physiological studies, few as they are, have been restricted to the plant and the gall. To an extent this is understandable as, from my own experience, it is easier to work with plant and gall material than it is to work with small insects. However, an argument can be presented that calls for this imbalance to be corrected.

A study of the evolution of the galling habit provides some interesting clues, that are germane to the identification of the stimulus which initiates gall-formation, or at least, to the direction of an investigation into this problem. Benson (1958) divided the British species of the tribe Nematini into 16 genera, of which only species of Euura and Pontania (= Pontania + Phyllocolpa) form galls. With the exception of Pseudodineura Konow which comprises two leaf-mining species, the other genera are free-living. Of the free-living insects, species of Stauronematus Benson, Pristiphora Latreille, Amauronematus Konow, Croesus Leach, Nematus Panzer and Pachynematus Konow are associated with members of Salicaceae. Gall-makers, however, are restricted to Salicaceae. Is this a consequence of the properties of this family of plants, or has evolution worked primarily through the insect? If evolution has worked primarily through the insect and galling species have evolved from free-living species (Togashi, 1970; Price et al., 1987), one would expect to find physiological differences between, for example, Pontania proxima and Nematus salicis (L.), both closely related species which inhabit Salix spp. but Nematus salicis is free-living. Unfortunately, comparative data concerning the physiology or biochemistry of the Nematini do not cover this area of study.

Even if the insect has played the dominant rôle in the evolution of the galling habit, it is doubtful whether the plant plays a purely passive rôle, For example, there is some evidence to suggest that in some plants premature leaf abscission occurs as an adaptive response to galling (Williams and Whitham, 1986). Thus, the rate and direction of gall formation must be an amalgam of the selection pressures on both the plant and the insect and of their respective genotypes (see Figure 2.2). The fundamental questions that arise from this postulation are:-

1. What were the selection pressures?
2. What effect did these pressures have on either the plant or the insect?
3. What are the relative contributions made by the insect and by the plant to the formation of the gall?

Answers to these questions would enable an investigation into gall induction to be more directed. But, with a lack of information, answers to these questions remain little more than hypotheses.

If no data are available, a review of the hypotheses on the adaptive value of galls may provide some direction to the discussion. Price et al. (1987) evaluated the hypotheses that galls were either:-

1. Non-adaptive to either the plant or the insect.
2. An adaptation by the plant only for its defence.
3. An adaptation by both the plant and the insect.
4. An adaptation by the insect, in terms of improved nutrition, microenvironment improvement and a greater protection from enemy attack.

They concluded, from predictions based on these hypotheses, that as the galler had, in general, a negative effect on the plant, the adaptive value was primarily to the insect itself. The benefits the insect

gained were through improved nutrition, microenvironment (see also Fernandes and Price, 1988) and, to a lesser extent, protection from enemies. Price and Pschorn-Walcher (1988) studied this last aspect more closely, as it related to tenthredinids, and concluded that the galling habit reduced the number of parasitoid species and percent mortality inflicted on sawflies. Further, they suggested that natural enemies may have been important, as a selective factor, in the evolution of the galling habit.

As the main beneficiary of the galling habit is the insect, it would be logical, therefore, to concentrate on the insect for the search of the cecidogen. This may seem axiomatic but, in the past, anomalies found in gall tissue, compared to that of the host plant, have been heralded as the cecidogen. To be that, they must be shown to be present in physiologically significant quantities in the insect itself. The following Chapter deals with this subject in more detail, demonstrating the lack of real evidence leading to the identification of a cecidogen in any gall-insect-plant association.

The rôle of the plant would be of greater importance if some evidence of coevolution between the insect and the host, that resulted in the evolution to the gall, could be presented. Coevolution has been suggested as playing some part in the dynamics of gall evolution (Ananthakrishnan, 1984), but no evidence was found to support its influence in the evolution of Euura galls (Craig et al., 1988). Within the Euura-Salix system, Craig et al. (1988) argued that the strongest forces affecting successful gall formation came from environmental factors and not from the parasite-host relationship. Although both had a high potential for reciprocal impact, the autecology of the plant means that selective pressures between the insect and the plant are not

driven by the genotype of the plant, but through its phenotype, which in turn is most profoundly affected by physical factors such as rainfall and soil disturbance.

The most effective defence against a gall-former would be to avoid producing a gall. In broad terms, galls benefit the insect, but reduce the fitness of the plant, or at best have a minimal impact on the plant. The insect is manipulating the plant and it is the insect which holds the key to gall-formation.

CHAPTER THREE

HYPOTHESES CONCERNING CECIDOGENESIS IN GALLS INITIATED BY INSECTS

"The production of the gall of Nematus [= Pontania] depends indisputably on the material which the venom gland produces".

(Translated from Beyerinck, 1887).

"There are no special reasons for believing that there are specific substances which cause the formation of a gall".

(Translated from Magnus, 1914: 88)

Both quotations refer to the gall of Pontania proxima.

3.1 Introduction.

By the beginning of this century, hypotheses concerning gall initiation by insect species fell into one of three groups:-

1. Hypotheses dependent on the belief that the physical injury sustained by the plant during oviposition, or the physical presence of an egg or larva, was sufficient to initiate gall formation.
2. Hypotheses based on the assumption that during oviposition the female insect injected, into the tissues of the plant, a gall-forming substance.

3. Hypotheses based on the active feeding of a larva, with secretions or excretions of a larva being the proximal cause of gall initiation.

The origins of these three groups can be found in the literature of the two preceding centuries. Both Swammerdam (1758: 81) and Réaumur (Plumb, 1953: 7) were early advocates of the first group of hypotheses, while Malpighi (Higton, 1989; Appendix Nine) suggested the second. The origins of the third group came later, the rôle of the larval secretion/excretions being discussed by Adler (1894: 100).

Gall initiation by Pontania proxima, unlike many other species of insect gall-formers, begins with the act of oviposition and does not require the presence of an egg or larva. The gall that forms before the egg hatches is termed a procecidium. For this study the relevant hypotheses concerning gall formation must acknowledge this difference.

The hypotheses in group one were refuted by the work of Rosen (1916), who used glass capillary tubes and glass knobs to mimic the physical damage caused by Viteus vitifolii (Fitch) (= Phylloxera vastatrix Planchon, Phylloxeridae) on the young leaves of Vitis vinifera L. (Vitaceae) (Plumb, 1953: 9).

Since gall production is over and above a response due to wounding, an hypothesis based on a "wound response" sensu stricto, is not considered here. This response, however, cannot be ignored, especially in the early stages of gall production. Taft and Bissing (1988) considered that the rôle of the wound response was that of the initial organizer of tissue patterns, from which the gall would develop, while Uritani and Asahi (1980) suggested that an increase in respiration during callus formation after wounding, played a rôle in the supply of precursors and cofactors for the biosynthesis of wound-healing layers.

In species of Arabidopsis Heynhold (Cruciferae), increased levels of mRNA have been shown to occur ten to 30 minutes after wounding (Braam and Davis, 1990). It would seem, therefore, that a wound response, by the plant, would play an important part in any hypothesis concerning gall initiation. However, the pertinent question still remains: does gall initiation occur through the influence of unique initiating chemicals provided by a cecidozoan, or are they products that are analogues of "plant growth regulators", with the insect controlling when and how the cecidogen is delivered?

The hypotheses developed over the latter half of this century have been based on the involvement, either directly or indirectly, of plant growth regulators. Predominant in this area are auxins and cytokinins, although gibberellins and abscisic acid have been mentioned. Ethylene, although it has been shown to have a significant impact on all stages of plant's development, has received scant attention and other growth regulators, such as polyamines and the brassinosteroids, have received none. In many ways, work on gall initiation has lagged behind the developments that have taken place in the study of plant growth regulators, both at experimental and theoretical levels.

3.2 **Hypotheses.**

To date there does not exist a body of information that favours one group of hypotheses above all others. Indeed, it may be that different insect galls are initiated by a variety of different mechanisms, and that the behavioural manipulations of the plant tissues, by the insect, play an important part in determining the resultant morphology of the gall.

3.2.1 Cytokinin hypothesis - Elzin (1983).

McCalla et al. (1962) identified two adenine derivatives, extracted from the colleterial fluid of Pontania pacifica, which were important for gall-growth. Elzin (1983) pointed out that cytokinins were adenine derivatives, but because McCalla et al. (1962) used growing galls for their work, this hypothesis may or may not be applicable for gall initiation.

Osborne (1972) considered that this rôle for cytokinins, was without justification, "kinetin equivalent of the insect in plant bioassays is of the order of 0.004 μg/Kg gland and since a concentration of kinetin in the order of 0.1 μg per injection applied seven times is the minimum required to maintain gall growth, it seems unlikely that kinins are the active, 260 nm absorbing substances in the Pontania gall-stimulating fluid". McCalla et al. (1962) favoured the view that substances derived from the insect, interacted with others from the plant. It is pertinent to note that Pontania oviposits through a lateral vein, with vascular tissue being a rich source of plant growth regulators (Itai and Vaadia, 1965). Direct evidence of high cytokinin levels in gall tissue, as opposed to normal tissue, has been provided by Lin and Lin (1990), working on the effects of the smut-fungus Ustilago esculenta Henning (Basidiomycetes) on Zizania latifolia Turczaninow (Gramineae). This work may provide a link with that of Braam and Davis (1990) as protein production in plants is characterized by higher levels of tRNA, ribosomes, cytokinins and mRNA complexes.

In insects, high levels of cytokinins have been found in the frass of the larva of the leaf-mining moth Stigmella argentipedella (Zeller) (Stigmellidae = Nepticulidae; Engelbrecht et al., 1969; Engelbrecht,

1971) as well as in the chalcid larvae which inhabit galls on Erythrina latissima E. Mey (Leguminosae; van Staden and Davey, 1978; van Staden et al., 1977). The source of the cytokinin was not established in these cases, but may have been derived from the plant, with the insect playing some rôle in its mobilization; the production de novo, by the insect, was not proved. There is evidence, however, that this might occur. Tsoupras and Luu (1983) identified a maternal ecdysone conjugate, found in the newly-laid eggs of locust, Locusta migratoria (L.) (Acrididae), as being the 22-N^6-(isopentenyl)-adenosine monophosphate of ecdysone. The function of this cytokinin in the development of the embryo was not established, but they suggested that it could be used in the construction of the carbon skeleton of juvenile hormone.

3.2.2 Auxin hypothesis - Miles (1968a and b).

Miles (1968a) and Livingstone (1978) reviewed the extensive literature that pointed towards a rôle for auxins in gall stimulation. An early advocate of the auxin hypothesis was Boysen Jensen (1948) and the presence of IAA in the saliva of insect larvae has been noted by numerous authors. Miles (1968b) demonstrated the endogenous production of IAA in Elasmolomus sordidus (Fabricius) (Lygaeidae) when injected with tryptophan, the proposed pathway for this production being shown in Figure 3.1. Miles was also able to change this normally non-cecidogenic insect into one that could, under certain conditions, produce intumescences in its food source. This change was effected by implantation, through an abdominal intersegmental membrane, of agar flakes containing tryptophan and phenylalanine.

Von Schäller (1965) demonstrated that although aphid saliva contained auxins, the levels found could be accounted for as being of plant origin. When aphids were artificially fed with auxin, von Schäller was able to detect it in their saliva. Auxins have also been found in the salivary glands of Wachtliella persicariae (L.) (Cecidomyiidae) which galls the leaves of Polygonum amphibium L. (Polygonaceae); Dieleman (1969) concluded that these were not synthesized by the larvae.

There is perhaps a synergistic effect between elements in the saliva of insects and plant IAA. Hori and Miles (1977) noticed this effect, in Avena tests, and suggested that some species of Heteroptera were capable of interfering in the IAA/IAA-oxidase balance of plant tissues. More recently, Mapes and Davies (1984) found that tissue cultures of the ball gall on Solidago altissima, caused by Eurosta solidaginis, grew independently of external sources of auxin, suggesting that the gall tissue was a source of auxin.

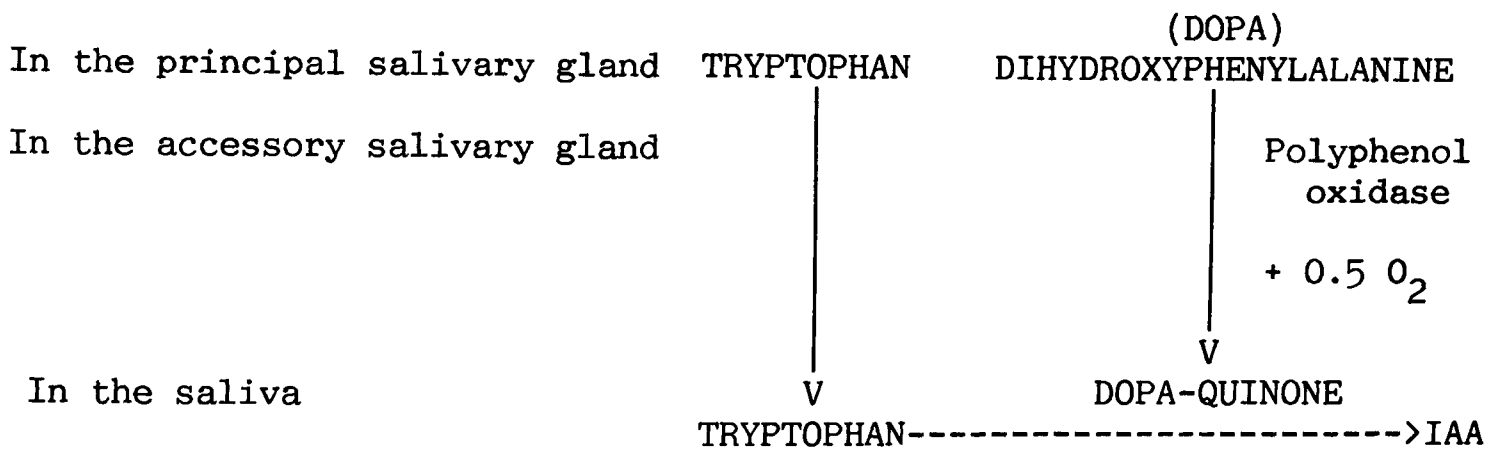


Figure 3.1. Hypothetical reactions leading to the production of IAA in the saliva of a plant-bug (from Miles, 1968b).

3.2.3 Amino acid hypothesis - Anders (1961).

Rather than the synthesis of auxins by the insect, this hypothesis suggests that free amino acids are transported through the insect's saliva and used for auxin production within the plant. High levels of amino acids have been detected in the saliva of the aphid Viteus vitifolii. Anders (1961) produced small swellings in the same plant by growing cuttings in aqueous mixtures of tryptophan, histidine and lysine. However, Miles (1968b) produced similar growths on vines by growing cuttings in potassium phosphate buffer only. Osborne (1972) considered that pure uncontaminated aphid saliva does not seem to contain amino acids and thus those that were found might well have been the result of the action of insect proteases upon the protein of the host plant.

3.2.4 Cynipine-virus mutualism hypothesis - Cornell (1983).

The notion of mutualistic associations between microorganisms and insects is not new. It has been shown (Stoltz and Vinson, 1979), that a large number of endoparasitic Hymenoptera, belonging to the families Braconidae and Ichneumonidae, contained virions which suppressed the cellular immune response system of their host, and so prevented the encapsulation of the parasitic egg (Edson et al., 1981). Cornell (1983) suggested that "DNA particles might act as regulator genes which supersede those in the normal DNA sequence and `switch on' structural sequences which lead to abnormal development".

This hypothesis would be dependent upon at least three criteria being met:

a) That gall formation is not purely a protective response by the plant, but more a manipulation by the insect of the plant's tissues.

Evidence for the adaptive value of the gall, for the insect, at the expense of the plant has been given by Price et al. (1987).

b) That the gall itself does not contain cells or structures unique to the plant. i.e. "Cecidogenesis does not involve processes unknown in normal ontogenesis" (Mani, 1964: 259).

c) That the virion is dependent on the continued presence of the larval secretions. Cornell (1983) suggests that this would be possible if the larval secretions altered the generative tissues of the plant, making a viral attack more successful.

Even if the presence of a virion cannot be found, Went (1970) stressed that the "extraneous stimulus, produced by the insect egg or developing larvae, has a similar effect as the DNA or RNA normally present inside the cell or nucleus". Thus some interference with the host nuclear material, by some means, may be occurring. This could also occur through horizontal gene transfer. This form of transfer occurs in the infection of plants by the Ti plasmid DNA of Agrobacterium tumefaciens, responsible for crown gall disease (Ream and Gordon, 1982).

The resultant hybrid DNA would have to play some part in the regulation of the expression of endogenous plant genes, so as to explain the unique morphological expression of galls. Bissett and Borkent (1988) suggested that "ambrosia galls", which are characterized by the interdependence of some species of Cecidomyiidae with fungi were originally attributable to these fungal symbionts, because some species of Botryosphaeria Cesati and de Notaris (Ascomycotina) cause canker-like growths in vascular plants. Also, from cladistic data, Bissett and Borkent (1988) hypothesized that the insect may have secondarily evolved or acquired the gall-inducing capacity.

However attractive this hypothesis may be, there are many arguments against. The introduction of foreign genetic material is one mechanism which might result in an increase of plant diversity (Juniper et al., 1989: 288) yet oaks for example with their myriad of gall-formers, have shown no sign of incorporating this possible source of an increase of diversity. In the course of evolution, galls are still dependent on the insect for their initiation and maintenance. Even if one accepts that gene expression will be dependent on the presence of the insect, Bronner (1981) has demonstrated that gall tissues remain malleable during all stages of development; in a study of the effect of inquilines on Neuroterus quercusbaccarum (L.) (Cynipidae) on one species of oak, Quercus pedunculata Ehrhart, he found that after the gall-former was killed by an aggressive inquiline, instead of the nutritive tissues of the gall degenerating, as is usually the case after the gall-former dies (Bronner, 1977), this layer was retained and, more importantly, they underwent slight changes in morphology. A gradation of influence over gall tissues has been noted by Shorthouse and Lalonde (1988), with the larvae and plant being responsible for the maintenance of different tissues within the gall (these arguments are continued in Chapter Five).

3.3 **Further considerations.**

As mentioned above, this author does not consider any one of the hypotheses, outlined above, as carrying a body of evidence that suggests that it alone can be favoured. This presents obvious difficulties for studies into gall-initiation, which in this instance is governed by limitations of time and resources. As an illustration of

the diversity of views that can be held on the question of gall-initiation, there follows four more hypotheses. These are not meant to detract from those already discussed, but serve to highlight other possibilities not previously considered.

3.3.1 Lignin formation as a response to chitin.

In a number of plant species, lignified cells appear to form a barrier to the invasion or spread of non-pathogenic fungi. Chitin was shown to be the inducer of lignification via the action of chitinases within plant cells (Pearce and Ride, 1982). Gall formation may, in some way, be linked to the presence of chitin of insect origin. This response would have to be specialized, as leaf miners and stem-boring insects do not, in general, effect a gall-forming response. The response may be one, as yet not known, that has as a parallel with the immune response initiated in animals when invaded by foreign bodies.

3.3.2 Anthocyanin affecting polar auxin transport (PAT).

Wounding can inhibit polar auxin transport. Jacobs and Rubery (1988) suggested an hypothesis whereby, after wounding, there was a rapid rise in flavonoids, which resulted in the retention of auxins near the wound. The red pigment characteristic of the gall of Pontania proxima is the flavonoid cyanadin-3-monoglucoside (Blunden and Challen, 1965). This could be involved in holding auxins, thus encouraging hypertrophy and hyperplasy. Leach and Morris (1990) demonstrated the presence of a form of the enzyme phenylalanine: ammonium lyase (PAL), in oak marble galls formed by Andricus kollari (Hartig) (Cynipidae), not present in normal leaf tissue. The function of PAL was to catalyze the reaction which converts phenylalanine to cinnamic acid - a precursor to the

synthesis of phenolics, a group of compounds which includes the flavonoids. As far as those authors were aware this was the first demonstration of a gall-specific enzyme in cynipid galls.

3.3.3 Abscisic acid.

Higher levels of abscisic acid have been found in the bark of Abies balsamea (L.) Miller, when attacked by Adelges piceae (Ratzeburg) (Adelgidae; Bonga and Clark, 1965). Similar responses of hyperplasy and hypertrophy were found in explants of Abies balsamea when grown in vitro with a higher than usual concentration of abscisic acid.

3.3.4 Ecdysone and gibberellins.

Byers et al. (1976) assayed for auxin- and gibberellin-like activity in galls on Pinus edulis Engelmann (Pinaceae) formed by the larvae of a midge species of Janetiella Kieffer near Janetiella coloradensis Felt (Cecidomyiidae). For both, they found increased levels, especially during the early stages of gall development. Extracts of the insect larvae did not show the presence of an auxin, but they did contain trace amounts of a gibberellin-like substance which could have been ecdysone.

Ecdysteroids are not only found in insects, they also occur in other arthropods, as well as nematodes, mussels, corals and plants. They have been identified in 27 families of the Pteridophyta, 10 families of the Gymnospermae and 74 mainly dicotyledonous families of the Angiospermae. Phytoecdysteroids are thought to function as a defence mechanism (Horn and Bergamasco, 1985), although this view is not universally accepted (Geuns, 1978). In animal groups other than the arthropods, there is no evidence that ecdysteroids have an endogenous

origin, or an hormonal rôle; they may be derived from the animal's food (Lafont et al., 1986).

In insects, ecdysteroids are present at all stages of the animal's development e.g. in Locusta migratoria migratoriodes (Reiche and Fairmaire) (Acrididae) they not only control cuticulogenesis during embryonic and post-embryonic development, but also the maturation of the ovaries. In the adult they are usually restricted to the female, but they have also been found in the testis of the male blowfly (Horn and Bergamasco, 1985). The rôle of ecdysteroids in vitellogenesis was discussed by Hagedorn (1974) with respect to the yellow fever mosquito, Aedes aegypti (L.) (Culicidae), where it appeared that ecdysone triggered a series of events, including vitellogenesis and the appearence of DOPA decarboxylase activity. In insects, these steroids are produced by the prothoracic glands from cholesterol or a plant sterol precursor (insects cannot synthesize the sterol ring system) (Riddiford and Truman, 1978).

Gibberellins affect many aspects of plant growth and development. Juntilla (1982) found that shoot growth in Salix pentandra L. stops in June-July and is accompanied by tip abscission. Application, in the spring, of exogenous GA enhanced shoot elongation and prevented tip abscission. Analysis of endogenous levels of gibberellin-like substances showed a marked drop which preceded growth cessation and formation of the shoot tip abscission zone. Barros and Neil (1987), working on Salix viminalis L., linked senescence and abortion of shoot tips to low water potential and transient increases in ABA. Growth of rooted cuttings was inhibited by exogenous ABA, the effect of which was relieved by the addition of GA. Gibberellins are thought to mediate their effects by influencing cell wall extendability and their action

with auxins are synergistic (Galton and Davies, 1970: 102-106) and that between gibberellin and abscisic acid antagonistic. Weiler (1981) found that in cultures of crown gall on Beta vulgaris L. (Chenopodiaceae), gibberellins seemed to function in the initiation of growth of the culture.

In this brief review of ecdysone and gibberellins it is apparent that an hypothesis concerning ecdysone as a stimulus for gall formation is an attractive option, and is certainly worth consideration.

Points in favour:

1) Ecdysone is an important and universal hormone in insects. It is involved in many processes from moulting to egg production. As such, a supply would be continuous throughout gall occupation.

2) U/V absorption of the unsaturated ketones in ecdysteroids is strong at 243 nm (Lafont et al., 1982). This is within the area of active constituents found by McCalla et al., (1962), from an analysis of the colleterial fluid from the Pontania pacifica.

3) Carlisle et al. (1963) linked ecdysone with gibberellin-like properties. Reciprocal applications of GA and homogenates of prothoracic glands from locusts had similar effects on fourth instar locust larvae. Also, both had similar effects on internode elongation of dwarf pea seedlings.

4) Osborne (1972) reported that in the insect GA acted indirectly, through the brain, by enhancing the production or release of brain hormones. These, in turn, stimulated the prothoracic gland to produce ecdysone. The hormone involved in the stimulation of the prothoracic gland is prothoracicotropic hormone, produced by the neurosecretory cells of the brain and released from the neurohaemal organ (corpora cardiaca) of these cells (Riddiford and Truman 1978).

Points against:

1) Although both are terpenoids, they are structurally very different: GA is based on the gibbane skeleton and is a diterpenoid, while ecdysone is a triterpenoid or steroid.

2) Jacobs and Suthers (1971) and Hendrix and Jones (1972) all provided evidence against the link between ecdysone and gibberellin-like activity. They stressed that Carlisle et al. (1963) used a crude insect extract, of unknown composition. Hendrix and Jones (1972) pointed out that as ecdysone and gibberellin share the isoprenoid pathway of terpene biosynthesis, a crude extract may contain a diterpenoid compound, closely related to, or converted into gibberellin.

3) Also among terpenoid compounds are abscisic acid (a sequiterpenoid) and carotene (a tetraterpenoid); both share the same isoprenoid pathway (Harborne, 1984: 100-103). Czeczuga (1977), analyzing a range of galls, found that the galls of Pontania capreae L. (=Pontania proxima?) on Salix alba not only contained lower levels of carotenoids, than either leaves with galls or leaves without galls, but that the range of the carotenoids was also different, with cryptoxanthin being common for all the galls tested. He concluded that the insect had enhanced the synthesis of some carotenoids and inhibited the synthesis of others. If this is true, then the regulation of one class of terpenoid by the galling insect may well suggest that other classes e.g. gibberellins, might also be modified.

3.4 Conclusions.

From this brief résumé it is evident that there are numerous strands in common between the various hypotheses e.g. interaction of hormones,

DOPA metabolism, common metabolic pathways etc. Unfortunately, there are no strong leads in any particular direction. Within the aims stated in the Introduction to this thesis, it is hoped that the experimental work that follows will help to narrow the options for future study and provide a basis on which the initiation of at least one insect-induced gall may be fully understood.

CHAPTER FOUR

REARING AND CULTURING

> "I cut some of these Knots off the Leaves, and opening one a little, I saw there was a Worm in it, and shut it together again; I put several of these Knots into a great glass Tube, to the end that the Worms might attain their full growth, but I could not find any of them did so".
>
> (Leuwenhoeck, 1701)

4.1 Introduction.

Pontania proxima demonstrates a high degree of host specificity. It also has a bivoltine life cycle, with each cycle lasting approximately 36-56 days (Carleton, 1939), during a flight period which extends from May to September (Magnus, 1914: 57-58). The seasonal nature of gall-inducers represents one limitation to work on gall-induction. To overcome or reduce this limitation the aims of the work covered in this Chapter were as follows:-

1. Obtain and grow, under controlled conditions, cuttings of Salix fragilis.
2. Obtain and maintain, under controlled conditions, populations of Pontania proxima.

3. To provide suitable conditions which would encourage galling by Pontania proxima.

4. To extend the seasons of both organisms beyond their normal ranges.

Hovanitz (1959) succeeded in producing a continuous supply of both host and parasite by the use of "phytotrons", but he supplied few details of the methods he adopted. Moreover, he used a different Salix/Pontania pairing from the one used in this study, but it is unclear as to the exact identification of the species used (see Section 2.2).

4.2 **Propagation of the host.**

4.2.1 Introduction.

Species of Salix are noted for their ease of propagation by cuttings. In a trial of over 300 species of willow, Chmelar (1974) found that the majority rooted easily in water, the only limitation being if they were planted upside down. Interest in the propagation of Salix species arises from their use in wickerwork and as ornamentals (Schluter, 1985), as a source of pollen for bees (Rudnyanskaya, 1987), in the stabilization of riverbanks (Dethioux and Damme, 1987), as fuel stands (Wan et al., 1986), pulping (Mitchell, 1988) and, increasingly, since the energy crisis of the 1970s as a source of biomass production through the afforestation of unproductive areas. Sweden, for example, aims to plant by the end of this century 200 000 ha of species of Salix, Populus and Alnus, specifically as an energy resource (Dawson, 1988; Matthews, 1989: 198-201, Mitchell, 1988).

In Oxfordshire, Salix fragilis and its varieties are common lowland

trees, frequently found by ditches and riversides. They are easily distinguished, in their leafless state, from Salix alba with which they are sometimes confused, by their deeply fissured bark, the ridges of which form an open network (Clapham et al., 1981: 226), their spreading branches and twigs that are brittle at their point of attachment (Meikle, 1984: 27). When removed they come away from the branch with a sharp snapping sound, hence the common name for this group of trees, Crack Willow. Much has been written about the many hybrids of Salix (Linton, 1913; Meikle, 1984: 5-6) and problems of identification. Added to this, our perception of the "species" is probably different from that of Pontania proxima.

To overcome these difficulties, both cuttings and galls were collected from individually marked trees. This allowed sawflies to carry out oviposition on cuttings from their "parent" tree. All dates and areas of collection are recorded in Appendix Two and, except where stated otherwise, all the trees were Salix fragilis var. russelliana, the Bedford Willow, which is by far the most common variety of Crack Willow found in this area. Voucher specimens of both host and insects are held, respectively, in the Fielding-Druce Herbarium, Oxford and the University Museum, Oxford (Hope Entomological Collections).

Plate 4.1. Pollarded trees by flowing water, the type of habitat favoured by Pontania proxima. Northmoor Nature Reserve, Little Wittenham (22.5.89 see Appendix Two).

4.2.2 Establishment of cuttings.

McCalla et al. (1962) established cuttings of Salix alba in a quartz-vermiculite mixture and watered them daily with a nutrient solution. This degree of culturing was felt unnecessary, as one of the main aims of this work was to create as natural an environment as possible.

Initially, cuttings were collected in January, allowed to root in water and then potted up in a peat/course sand (50:50) mixture containing a slow release nitrogen fertilizer. Other cuttings were wrapped in black plastic sheeting, to prevent desiccation, and placed in a cold room (Cram and Lindquist, 1982) at a temperature of 5° C to be used later in the year. These cutting were to provide a continuous supply of newly shooting material available for oviposition by Pontania proxima. Unfortunately, those in storage were found to be susceptible to fungal infection. Even dipping in a Benlate solution did not prevent these cuttings from being destroyed. It is highly probable that the temperature in the cold room was not low enough, a more ideal temperature being 1-2° C (Durell, personal communication). At this lower temperature and using larger cuttings (two to five centimetres thick and 50 cm in length), storage was more successful, with cuttings originally collected in February being removed and propagated the following August/September, allowing for growth throughout the winter in a heated greenhouse fitted with supplementary lighting.

Newly potted cuttings were placed in either a heated greenhouse with additional lighting, or a plant growth cabinet maintained at 22° C with a photoperiod of 16 hours. All cuttings were susceptible to infection by the red spider mite (species of Tetranychus Dufour:

Tetranychidae). To control these outbreaks, the use of chemical pesticides was felt to be out of the question, since they may have affected adversely the Pontania adults and/or their larvae. The use of a biological agent was adopted and periodic infestations by Tetranychus sp. were controlled using the predator mite Phytoseiulus persimilis Athias-Henriot (Phytoseiidae) (Koppert [U.K.] Ltd., Tunbridge Wells, Kent. TN2 5BX). Unfortunately, not only were the growth cabinets too small, but their thermostatic control mechanism was unreliable and they lacked humidity controls. It was finally decided to abandon their use, relying instead on the availability of space in greenhouses. This also obviated the possibility of ethylene production caused by fluorescent lighting (Wills and Paterson, 1970).

The first cuttings to be established were one-year-old twigs; these produced only short shoots and had a brief growing period of up to approximately three months. Each shoot had, on average ten leaves, the largest of which were approximately 110 mm in length (see Plate 4.2). In an effort to gain a longer growing season, larger two- to three-year-old hardwood "truncheons" were obtained from pollarded trees. The shoots of these larger cuttings maintained a longer growing period, with average shoots containing 20+ leaves, the longest of which were approximately 160 mm in length (see Plate 4.3). These approximated, more closely, to the fast growing shoots preferred by Pontania (see Plate 4.1).

Advice on the propagation of larger cuttings was supplied by Good and Parfitt (both in litt., 1988). The final methods adopted were as follows:

a) A collection of hardwood cuttings, preferably of 2-year-old stems,

with a length of 50 cm was made in February/March. Half were dipped in a 0.05% Benlate solution (ICI, Farnham, Surrey. GU9 7UB), wrapped in black plastic sheeting and stored at 2° C.

b) When required, the cuttings were planted to 2/3 their length, in a rooting medium such as sterilized sand or peat and sand (50:50), rather than rooting in water. This obviated the danger of breaking fragile roots.

c) Ideally these should have been rooted for 10-15 days with a bottom heat of 22° C, thus ensuring a gradient between "soil" and air temperature. This arrangement would discourage the premature emergence of shoots, before the root system is properly established. Unfortunately, facilities that would make this arrangement possible were not available and the cuttings were therefore maintained in a heated greenhouse.

d) Once rooted the cuttings were finally potting up in a free-draining peat-based compost.

This procedure resulted in fast growing shoots being available throughout the year. It was found, however, that cuttings planted in the autumn did not grow as fast as those established in the spring. After completing their growth, spring cuttings were cut back and overwintered in the open air, after which they were repotted in fresh compost. Specimens, for early use, were moved into a heated greenhouse in February. Cuttings potted in the autumn were prepared fresh each year.

Plate 4.2. Original cuttings of Salix fragilis. Note the small size of the leaves compared to those of the larger cutting shown in Plate 4.3.

Plate 4.3. Later cutting of Salix fragilis using a "truncheon". Note that the leaves are larger and more numerous than those shown in Plate 4.2.

4.3 **Rearing** Pontania proxima.

4.3.1 Introduction.

Heslop Harrison (1927), Carleton (1939) and Benson (1954) all provided details of the methods they used to rear sawflies, but, in each case, there was no attempt to extend the fight period of the insect, or create a self-perpetuating population.

4.3.2 Collection and rearing.

During the course of this study, young galls were observed on Salix leaves by the second week of May and were ready for collection from mid-June. The latter stages of development of the larva were usually signalled by the appearance of an aperture at one end of the gall. This was always on the abaxial surface of the gall and usually facing the leaf-tip. It was easily distinguished from holes made by predators e.g. birds, and possibly inquilines e.g. Curculio crux Fabricius (Curculionidae, see Kopelke, 1985a) which are larger and usually found on the uppermost surface of the gall. The aperture was formed during the fourth, third or exceptionally second instar stage of larval development and its function has been linked with either (a) aeration of the gall, (b) more feeding on areas of higher nutritional quality leading to the puncturing of the gall, or (c) as an aperture through which frass can be ejected (Carleton, 1939; Lazareva et al., 1986). Whatever its function, a larva could sometimes be seen half-emerged from this hole and will, as a prepupa, use it to escape from the gall. If galls were collected before the appearance of this aperture, the larva will be insufficiently advanced to be able to complete its development. If collected too late, the occupant will have

Plate 4.4. Pontania proxima (Lepeletier) (Hymenoptera: Tenthredinidae) (scale divisions mm).

already left the gall. Galls were collected, therefore, if there was evidence of this aperture, or if the gall was soft to the touch i.e. most of the nutritional layer had been consumed.

Sites used for the collection of galls are recorded in Appendix Two. Although limitations of time prevented detailed quantitative comparisons, there were obvious differences in the frequency of galling, in terms of both the numbers of galls observed and the number of galls per leaf. These differences occurred between the spring and summer broods, between sites and between years.

Differences between broods are understandable: there were always far fewer galls formed in the spring, by over-wintering adults, than in the summer when galls would be produced by newly emerged adults. The differences between sites may not only have been due to environmental differences, but there may also have been a genetically-based difference in susceptibility between trees (see Section 2.8.1). Efforts to encourage Pontania adults, reared from galls on Salix fragilis var. russelliana, to oviposit into leaves of the hybrid between Salix alba and Salix fragilis (Salix x rubens Schrank nothovar. basfordiana (Scaling ex Salter) Meikle forma basfordiana Meikle) failed.

There were also differences from one year to the next, an observation also noted by others (Leitch, personal communication). Long-term patterns or predictions of abundance were impossible to make from such a short period of observation, even collecting the galls must have had some impact on future numbers. Some tentative suggestions, however, could be made when observations were combined with local weather data supplied by the Radcliffe Meteorological Station (Appendix Three). The past three winters have been uncommonly warm (see Appendix

Three, Figures A3.1, A3.3 and A3.4) with both air temperatures and grass temperatures above their long-term means and this might have had some impact on the numbers of Pontania proxima, its parasites and inquilines. The winter of 1989-1990 was the warmest on record at the Radcliffe Meteorological Station and, for central England, taken together the months of January and February were the mildest in the 330-year record (Ratcliffe, 1990). At the Radcliffe Meteorological Station the winter of 1988-1989 was the fourth warmest in the record. In central England it was the mildest since 1869 (Ratcliffe, 1989) and 1987-1988 was the 30th warmest, as measured at the Radcliffe Meteorological Station. Miller and Werner (1987) studied extreme supercooling in a species of Euura and found that its larvae, together with the larvae of two other species of willow gall-formers, had the lowest supercooling point recorded for any insect. None of these insects are freeze-tolerant, winter survival depending on their ability to depress the temperature at which spontaneous freezing occurs (i.e. the supercooling point). For the majority of freeze-intolerant insects this point lies between -7° C and -12° C; for the galling insects studied by Miller and Werner, these temperatures were much lower. During summer months, supercooling points were determined experimentally and had a range of -25.5° C to -29.4° C, these fell during the winter months to -56° C to -58° C with an absolute minimum for some insects being -60° C to -64° C. This freezing avoidance was accomplished by a build-up of carbohydrates, primarily glycerol. If these same observations can be applied to the related species, Pontania proxima, cold winters might function as one mechanism in the control of parasitoid numbers. However, in April 1990 there were some unusually

severe frosts, these would have coincided with the beginning of pupation for some sawflies and may have been adversely affected their development. These late frosts have been suggested as one possible cause for the near extinction of spangle galls in central England during 1990 (Leach and Manson, 1990).

Although not directly related to these suggestions, personal observations did suggest that there were differences in the response to cold between the sawfly and its parasites. Sample adult insects, when collected for preservation and storage in gelatin capsules (size 3, Agar Scientific Ltd. Essex CM24 8DA), were placed in a domestic freezer for approximately five minutes to immobilize them without killing them, since this made handling easier. One observation from this procedure was that adults of Pontania proxima were immobilized much faster (approximately two minutes), than either their parasites or inquilines. Leuwenhoeck (1701) made reference to the presence of cocoons in the gall itself. This was not noted for collections made during 1989 or 1990, but in October 1988 galls collected did contain cocoons, the time of these collections coinciding with the only period over the past three years when mean autumn air temperatures fell below their long-term means (Appendix Three, Figure A3.1).

As well as being the warmest winter since records began, 1990 was also the wettest since 1769 and many of the best collection sites, used in this study, were affected by flooding. Sites that usually produced good numbers of galls were always near to flowing water. Whether this was related to greater water availability for the tree growth, or whether it was related to a higher localized level of air humidity, to which Pontania proxima is very sensitive (Smith, 1970), is unclear. What is clear, however, is that the viability of the prepupa in the

cocoon is adversely affected by flooding (Carleton, 1939) and this may have reduced the number of adults in the spring of 1990.

Unlike that of Pontania triandrae, the prepupa of Pontania proxima undergo obligatory diapause (Carleton, 1939). The stimulus for this event is not known, so attempts were also made to extend the flight period of the sawfly beyond its usual range.

Once collected, galls were cut from the leaves and placed in clear plastic boxes (230 mm x 120 mm x 80 mm), each with a fine gauze-covered hole (dia. 40 mm) in the lid to aid ventilation. To prevent fungal infection, the galls were sprayed with a 0.05% solution of Benlate.

Different substrates were tested, as were a variety of environmental conditions. The substrates used included a damp peat/sand mixture (50/50), heat sterilized John Innes potting compost No. 2, expanded polystyrene granules, shredded paper towelling and perlite. To some extent all were effective, but the most successful seemed to be the peat/sand mixture and the paper towelling, with the sawfly prepupa readily producing cocoons in both. Caution was required when using the paper towelling substrate as it may have contained chemicals able to affect the growth and behaviour of insects (McGavin, personal communication). Because of this, the peat/sand mixture was used to the greater extent. The use of expanded polystyrene and perlite were not continued as they dried out too easily and, conversely, the John Innes compost remained too damp, with many cocoons becoming flooded. One other observation on the use of John Innes compost, whether this was a product of its dampness is unclear, but when this was used many prepupae did not form cocoons, but remained below the surface of the compost, lying on top of the base of the container.

The boxes containing the galls were kept in either a plant growth cabinet, though here temperature regulation was unreliable, or in an insect-breeding room, which had the benefit of a high humidity, but there was no method of monitoring the temperature inside the plastic containers (which tended to be higher than the ambient temperature of the room). To overcome these problems an incubator was purpose-built. This had the advantages that humidity could be maintained at a high level and temperature could not only be measured from inside the plastic containers but also regulated from there using a thermistor probe; illumination was controlled using overhead fluorescent lighting on a timing mechanism. The final conditions used were: temperature 25° C, relative humidity 80%+ and a 16 hour photoperiod.

The extension of the flight period involved two aspects. Firstly, the continuation of emergence to produce a third brood, rather than the usual two; secondly, to encourage early emergence in the spring. In the time available the complete abolition of diapause was not accomplished.

If kept under conditions of high humidity Pontania proxima could be encouraged to pupate and produce a third brood (see Plates 4.6 and 4.7). One hundred galls collected from Long Wittenham in October 1988 were kept under the same conditions of temperature and light as shown above. However, 50 were maintained in a relative humidity of 30%-40%, while the rest in a relative humidity of 80%-90%. Although cocoons were observed in both trials no sawflies emerged from those maintained in the more arid conditions. It was found that these had entered diapause, because after a period of exposure to lower temperatures, adults emerged the following spring. Those maintained in condition of high humidity did not enter diapause, but began to emerge after 12 days:

within the next 39 days a total of 14 had emerged, thus extending the flight period.

Early emergence from diapause is also possible. Cocoons were overwintered in a cold room at 5° C. Care was taken to ensure that the peat/sand was not too damp, since at the lower temperature of the cold room the humid atmosphere of the container condensed and flooded the cocoons. They were placed in the cold room during November and, periodically, a few were removed and placed in an incubator. The earliest emergence was mid-April, 26 days after removal from the cold room.

The numbers of cocoons produced from collections of galls made from a sample of sites in 1989 are shown in Table 4.1. The reasons for these low figures were not clear. They could reflect poor larval development at the time of collection, or the effect of parasites (except those that pupate in the sawfly's cocoon e.g. Angitia vestigialis (Ratzeburg) (Ichneumonidae, Carleton, 1939), or unfavourable conditions for development either before or after collection.

The cocoons (see Plate 4.5) of Pontania proxima were found to be cylindrical in shape with rounded ends, one of which was slightly more obtuse than the other. It was this end that carried the anterior part of the prepupa, either its head or anterior dorsal surface, because sometimes the prepupa was flexed with its head held against the ventral surface of its body. The wall of the cocoon was composed of a lattice work of fibres, the inner surface being a light brown colour, slightly shiny and smooth. The outer surface was rougher and had a veined appearence; it darkened with age and become a matt brown or black colour. The mean dimensions were, length 4.4 mm and breadth 1.8 mm (see

Site of collection	Number of galls collected	Number of cocoons formed	%
Abingdon Lock	861	15	2
Long Wittenham	1219	56	5
Meadow Lane	2559	49	2
Park Farm	150	9	6
Radley tow path	1276	59	5
Sandford Lock	2879	127	4
Shabbington	540	43	8

Table 4.1. Number and percentage of cocoons formed from sample collections of galls. Details of sites are given in Appendix Two.

Plate 4.5. Cocoon of Pontania proxima (scale divisions mm).

Appendix Five); these are slightly smaller than those give by Carleton (1939) and Lazareva et al. (1986).

Using the methods described in this Chapter, it was possible to extend the flight season for these insects by 60%, from five to eight months; future work may be able to extend this further, since in this study only two winters were available for experimentation.

4.4 Observations of oviposition.

Oviposition has been described by numerous authors (see Section 2.6); the comments in this Section refer to observations made during the course of this study.

Adult wasps exhibited both positive phototaxis and positive geotaxis. If they were placed in a container, half-covered to exclude the light, they moved towards the illuminated area. When this area was covered and the previously dark area illuminated, they reverse direction and again moved towards the light. If they were placed in a container, either in the dark or equally illuminated from all sides, they move upwards. This pattern of behaviour would be advantageous for sawflies in finding the leaf buds they require for oviposition.

Within 24 hours of emergence, sawflies were placed in a muslin covered frame, which contained a willow cutting, and observations were made on their behaviour. Generally, movement was in an upward direction. If a leaf-tip was reached the wasp would move down the leaf, up along the stem and up the next leaf, and so on. At all times their antennae moved and probed the surface of the leaf, but at an apical bud these movements became more intense and in many cases the wasp began, almost immediately, to saw into the lamina of the leaf. Behaviour was

more random than others have mentioned, with the female sawing into the leaf facing either towards the leaf-tip or the petiole e.g. Murphy (1929) noted that oviposition took place from the leaf-tip downwards, then up the other side. At times the wasp was observed to leave the leaf completely and push her saw into the stem, then return to the leaf. This behaviour may have been part of a saw cleaning process. Other movements observed included preening, extending and reflexing the saw, biting the waxy covering of the leaf or resting.

Oviposition took between 30 seconds and two minutes per event. However, if the ovipositor passed through a leaf, as it did on numerous occasions, it was immediately withdrawn. Successful attempts were all through a lateral vein. Oviposition began with the wasp arching her abdomen and bringing the saw into perpendicular contact with the leaf. The two lancets (Ross, 1945) oscillated backwards and forwards, against each other, and the saw was driven through a lateral vein and into the leaf. Once the epidermis was pierced, the saw then twisted and bent through 90° so that its usual dorsal ventral axis was now horizontal. The oscillating movement of the lancets continued until the saw was fully extended into the leaf; after a short pause, the ovipositor was withdrawn, leaving a sickle-shaped wound. Within 24 to 36 hours the first external evidence of gall-formation was visible with the naked eye; small swellings were evident, the areas affected being paler in colour than the rest of the leaf.

Plate 4.6. Third brood galls produced by Pontania proxima and maintained under the conditions detailed in this Chapter. Photograph taken six weeks after oviposition. (scale divisions mm).

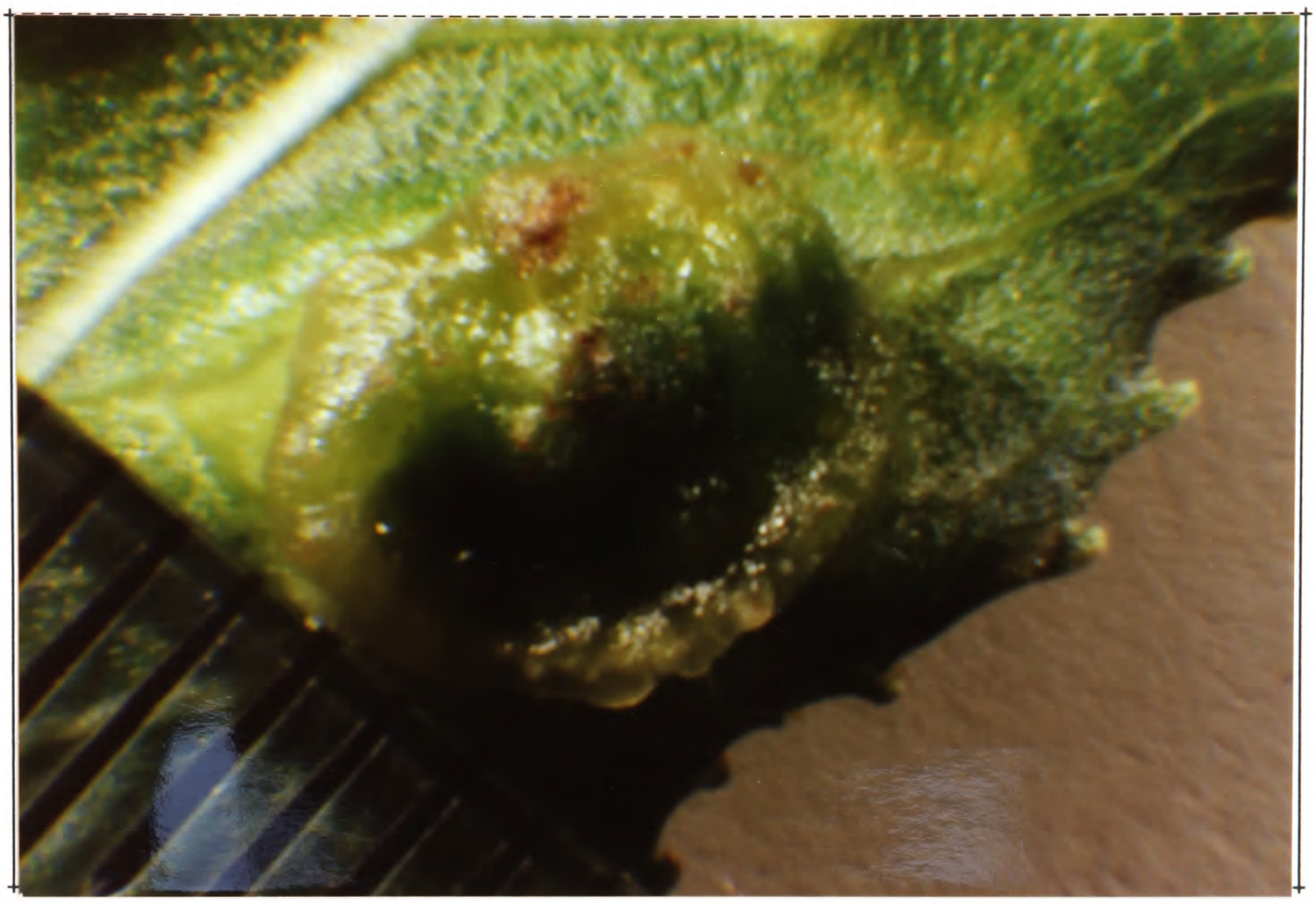


Plate 4.7. Third brood gall produced by Pontania proxima and maintained under the conditions detailed in this Chapter. Photograph taken six weeks after oviposition (scale divisions mm).

4.5 Topological specificity.

Plate 4.8. Galls of Pontania proxima on the leaves of Salix fragilis.

4.5.1 Introduction.

Benes (1968) maintained that the effect on the plant of the number of galls on a leaf was not very important, as only young stout twigs were usually infected. He observed that at high densities, galls were smaller and the larvae took longer to develop. However, the analysis of the distribution of galls on a leaf is necessary for two reasons. The first concerns insect behaviour; although an analysis will not identify the mechanisms governing distribution, it will reveal aspects of an insect's behaviour that deserve further study. Secondly and more directly related to this study, is that choice of oviposition site will

be important in the development of a bioassay, using the injection of material into Salix leaves. If the insect demonstrates discrimination in the choice of site then this should be reflected in the bioassay. Slepyan (1962) provided a series of graphs to support the suggestion that oviposition by Pontania proxima was topologically specific., However, he did not account for differences in leaf area, neither did he submit his results to any statistical analysis.

The aims of this Section were to analyse the distribution of galls on Salix leaves (see Plate 4.8) and to establish whether their distribution exhibited topological specificity or whether the distribution of galls could be regarded as random. As well as that of distribution, an analysis was also made of gall mass as a ratio of leaf mass.

4.5.2 Methods.

a) Galled leaves were collected at random from trees that had been pollarded the previous year (see Appendix Two, Little Wittenham). For this analysis only galls of a reasonable size were used. It was noted that some galls remained small (Beyerinck, 1887; Magnus, 1914: 73; see Section 2.2), reflecting an attempt at oviposition, rather than a successful operation, for the purposes of this study these galls were ignored and only fully grown galls were recorded.

The total number of galls on each leaf was noted together with the position of each gall. This was achieved by dividing the leaf into 16 areas (see Figure 4.1) and plotting the position of the mid-point of each gall using the co-ordinated 1 to 4 and A to D.

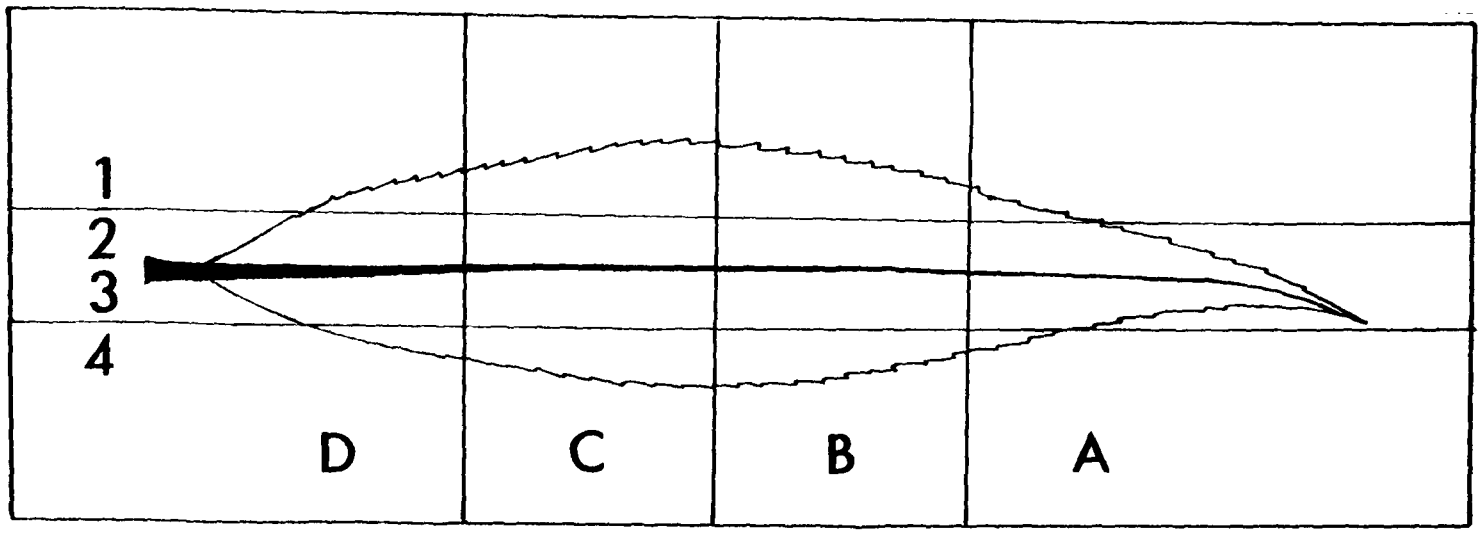


Figure 4.1. Position of areas 1A to 4D on leaves of Salix fragilis, used to plot the position of galls.

These data were displayed using graphical methods, after the removal of possible bias caused by differences in size between the areas (see Appendix Four: Figures A4.3 to Figure A4.5) and analysed using a chi-square test (see Appendix Four). The observed numbers of galls at each position were tested against an expected number, which was calculated from the size of each area (1A to 4D) from ten leaves, expressed as the mean percentage of the total area of these leaves. Probabilities were obtained from Bailey (1981: 209), the Null Hypothesis being that there was no difference between the observed pattern of distribution and that of a random distribution based on the size of each area.

b) The ratio of the mass of gall tissue to the mass of remaining leaf material, was calculated from the mass of 3361 galls cut from leaves and divided by the mass of the remaining leaf material.

4.5.3 Results.

Area of leaf	Number of galls per leaf.											
	1	2	3	4	5	6	7	8	9	10	11	12
1A	0	2	2	12	4	11	7	6	4	1	0	0
2A	1	6	8	5	29	13	16	6	14	4	5	2
3A	0	5	4	7	25	14	11	15	13	4	5	0
4A	0	1	1	4	9	11	7	2	2	1	1	0
2A	3	9	6	23	18	16	7	16	6	4	0	0
2B	14	33	55	60	84	50	31	35	29	10	5	3
2C	14	27	46	51	69	48	31	27	26	8	2	0
2D	2	3	7	8	15	10	14	4	6	5	3	0
3A	9	13	11	12	20	17	9	16	5	5	0	0
3B	15	31	48	87	82	45	34	26	17	8	3	2
3C	14	31	58	63	60	50	33	21	18	13	2	2
3D	5	5	8	9	8	12	5	3	1	3	0	0
4A	9	7	5	11	10	10	8	7	7	4	2	0
4B	5	11	18	19	26	19	11	9	11	4	3	2
4C	5	10	19	24	30	15	8	7	8	3	1	1
4D	4	6	4	5	11	13	6	8	4	3	1	0
Total number of leaves in each group												
	100	100	100	100	100	59	34	26	19	8	3	1
Total number of galls in each group												
	100	200	300	400	500	354	238	208	171	80	33	12

Table 4.2. Results of counts of galls in each area of the leaf, for each of one to 12 galls per leaf.

Area of leaf	Number of galls per leaf.			
	13	14	Total	10-14 Total
1A	1	0	50	2
2A	2	0	111	13
3A	4	2	109	15
4A	0	0	39	2
1B	1	0	109	5
2B	2	1	412	21
3B	6	4	359	20
4B	1	0	78	9
1C	5	1	123	11
2C	4	2	404	19
3C	4	1	370	22
4C	3	1	63	7
1D	2	0	82	8
2D	0	1	139	10
3D	2	1	134	8
4D	2	0	67	6
Total number of leaves in each group				
	3	1	654	16
Total number of galls in each group				
	39	14	2649	178

Table 4.3. Results of counts of galls in each area of the leaf, for 13 and 14 galls per leaf, the total of all galls and the total of 10 to 14 galls per leaf.

a) Tables 4.2 and 4.3 show the number of galls counted in each area, for leaves with one to 14 galls per leaf. As only 16 leaves had 10 or more galls, the data from these leaves were combined in the 10-14T column. Tables A4.6, A4.7 and A4.8 (Appendix Four) give the number of galls in each area corrected to remove possible bias caused by the different size of each positional area and expressed as a percentage (details of calculation methods are given in each Table of Appendix Four). The information contained in Tables A4.6 to A4.8 are displayed using graphical methods (see Figures 4.2 to 4.3).

Results of each chi-square test and the probabilities obtained are shown in Table A4.15. Given a Null Hypothesis that there is no difference between the observed and expected results, i.e. that the distribution of galls on the leaves is random and therefore based on the relative sizes of each area (1A to 4D), the probabilities calculated suggest that this hypothesis should be rejected. Therefore, this analysis provides strong evidence that the distribution of the galls of Pontania proxima on the leaves of Salix fragilis is not random, but that some areas are favoured by the sawfly more than others. The Null Hypothesis could be accepted for leaves containing 10, 12, 13 and 14 galls, but the numbers of leaves used for each of these calculations were small, too small for valid comparisons to be made when compared to the total of ten to 14 galls per leaf (10-14 TOTAL).

b) Ratio of mass of gall tissue to mass of remaining leaf tissue.

Number of galls removed from leaves = 3361

Mass of galls removed from leaves = 391.98 g

Mean mass of 1 gall = 0.1166 g

Mass of leaf remains = 486.07 g

Ratio of mass of gall tissue to mass of leaf remains = 0.81

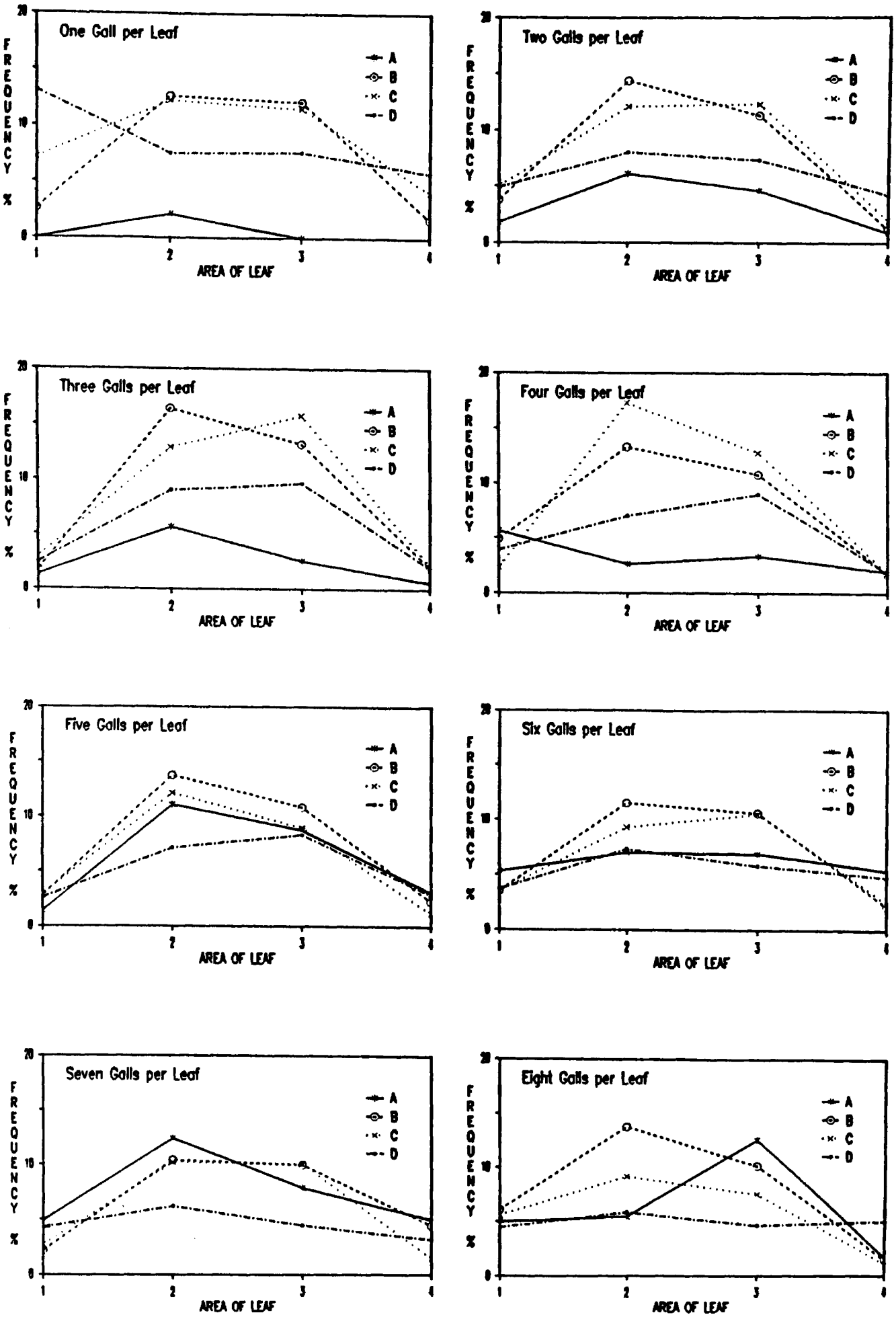
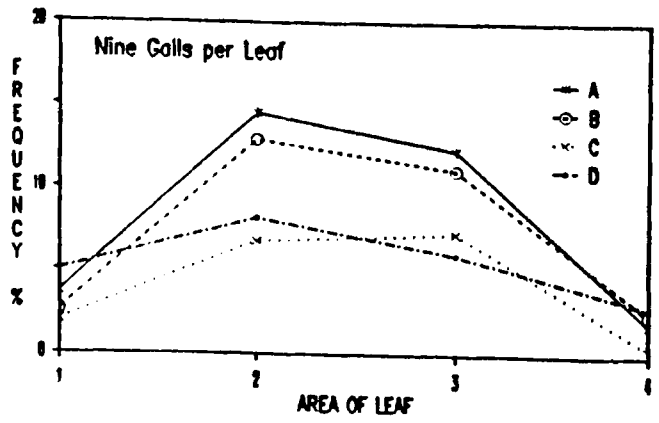
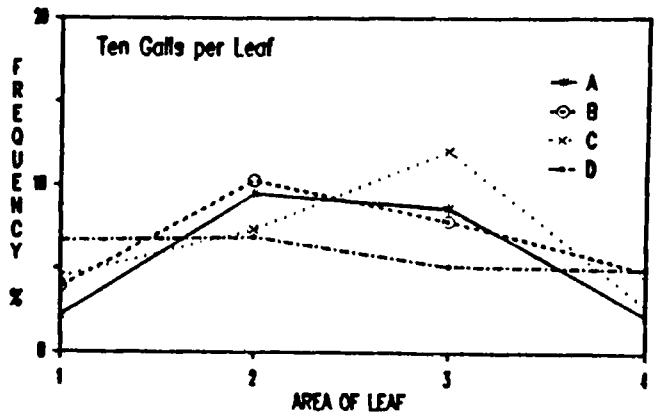
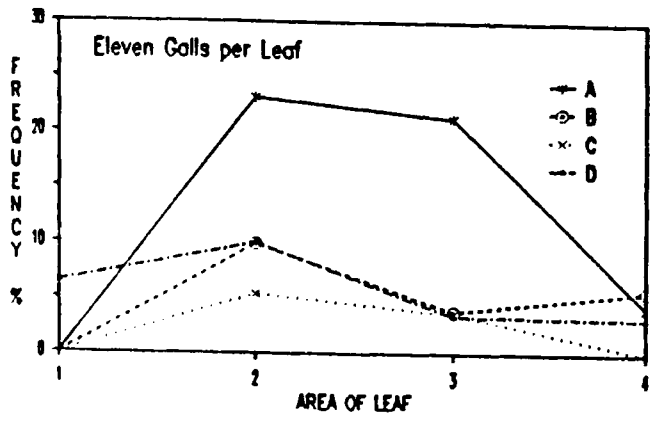
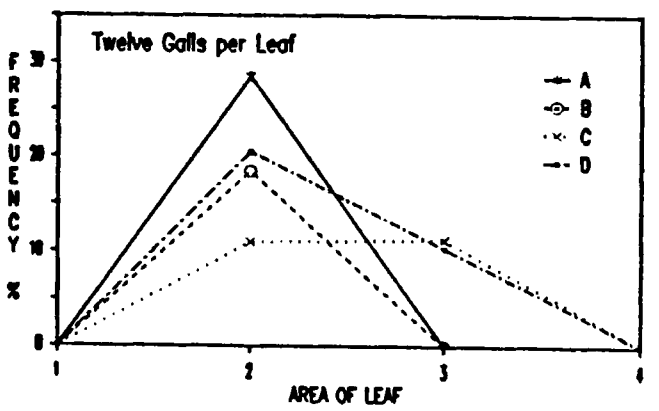


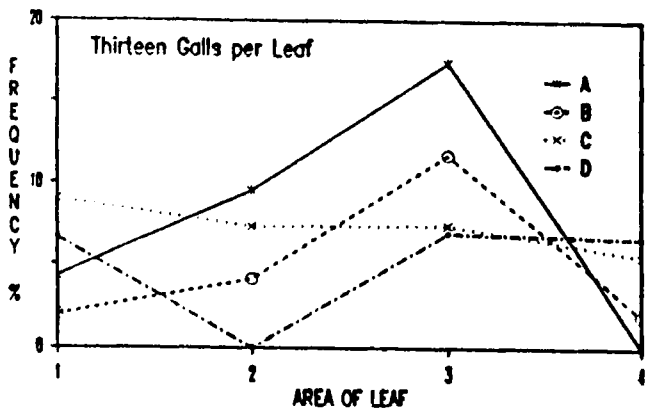
Figure 4.2. Number of galls expressed as a percentage frequency and corrected to remove possible bias caused by the difference in size of positional areas for leaves with one to eight galls per leaf.

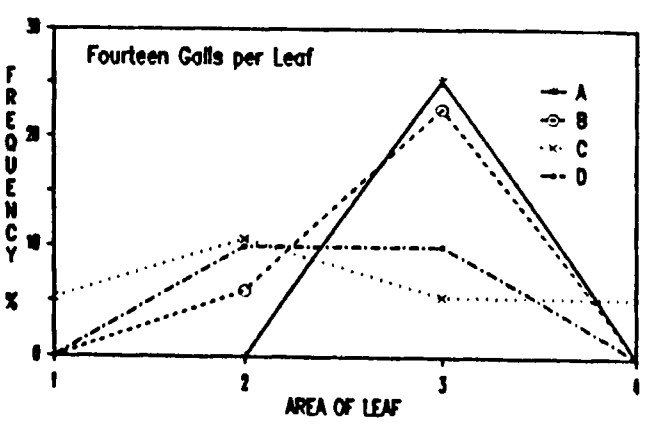


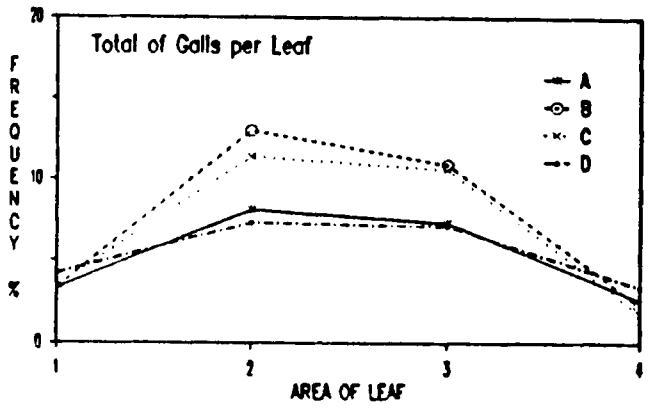












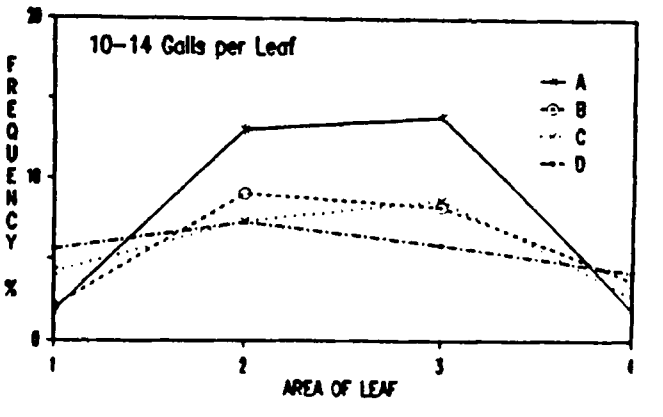


Figure 4.3. Number of galls expressed as a percentage frequency and corrected to remove possible bias caused by the difference in size of positional areas for leaves with nine to 13 galls per leaf, the total number of all galls and the total number of galls for 10-14 galls per leaf.

4.5.4 Discussion.

Plate 4.9. Terminal bud of Salix fragilis showing the outer leaves just separating (Mag. x2.0).

The results obtained for this Section demonstrated that:-

a) Oviposition occurred on a terminal bud of the type shown in Plate 4.9. and mainly in the central part of the leaf lamina, on either side of the mid-rib as shown in Figures 4.2 to 4.3. The results also

indicated that all areas (1A to 4D) were able to support and sustain gall formation, suggesting that position is governed by oviposition selection and not by preferential suitability of tissue within the leaf. These results suggested, also, that the differences in distribution were not a result of the differences in size of the 16 areas. Distribution followed a regular pattern, regardless of the number of galls on a leaf.

The central areas of the lamina were used first for oviposition and, when these sites were full, the tip areas of the leaf were used in preference to the basal areas (see Figure 4.3, 10-14 Galls per Leaf). These results suggested that gall position was dependent upon the behaviour of the sawfly, with Pontania proxima preferring to oviposit in certain areas. The reasons for this are not clear; indeed, published knowledge of the behaviour patterns of this sawfly is sparse. Methods by which the insect found the correct tree, leaf and area within the leaf have not been studied.

The differences in oviposition patterns illustrated here were acknowledged in all experiments, concerned with artificial gall production (see Chapter 5). For these experiments areas 2B, 3B, 2C and 3C were used.

b) Slepyan (1962) analysed the photosynthetic pigments of galls, leaves with galls removed and non-infected leaves and concluded that the pathogenicity of Pontania proxima was low. Water content of the undamaged leaves was measured at 67.5%, while that of the galls was 85.7%. This difference may go some way to explain the very high gall mass ratio calculated in this study. Galls of Pontania proxima are able to photosynthesize to some extent, but rates are low (Slepyan, 1962). In order to maintain these potential "sinks", the products of

photosynthesis formed in the non-galled areas must be sufficient to supply the needs of the gall.

Pontania proxima is noted for preferring new fast-growing shoots (Magnus, 1914: 58; Carleton, 1939). This is not a unique choice because other insects show a similar preference (Price et al., 1987a). Perhaps during the course of evolution, selection has ensured that the sawfly lays its eggs on trees with large reserves of food and rapid spring growth i.e. pollarded willows (personal observation; Magnus, 1914: 58; Carleton, 1939; Shanahan, 1957). The impact of galling and gall-formers on host plants was discussed more fully in Section 2.8.2.

CHAPTER FIVE

MORPHOLOGY AND ULTRASTRUCTURE

"By over-emphasis on originality within a narrow field of enquiry an elitist body tends to ignore how much one person's achievements are rooted in the work of his predecessors and supported by traditional disciplines."

(Stearn, 1976)

5.1 Introduction.

5.1.1 Aims.

The quotation at the start of this Chapter serves to illustrate two aspects of past work on the Pontania-Salix system. Firstly, the anatomy and morphology of the gall has generated much interest, Beyerinck (1887), Magnus (1914), Rey (1967, 1968, 1972, 1973 and 1974) and Slepyan and Gurevich (1981) all described, in varying detail, the structure of the gall, its ontogeny and ultrastructure. The information supplied by each produced a coherent and thorough body of knowledge. In comparison, work at this level on the anatomy and morphology of the reproductive organs and associated glands of Pontania proxima was limited, with little being added since the work of Beyerinck (1887) and Magnus (1914). Although the work of Magnus (1914) was more accurate,

the description by Beyerinck (1887) has gained general acceptance, perhaps, because he included a diagram in his description (see Plate 2.1.). This has led to many assumptions, based on Beyerinck's description of the insect, which the work described in this Chapter has shown to be incorrect.

As well as providing a description of the structure of the internal reproductive system and associated glands of Pontania proxima, the aim of the work in this Chapter was to screen, using optical techniques, for the presence of microorganisms within the insect and the gall. There are many examples of associations between insects and microorganisms, these may not only be pathogenic (Evans and Entwistle, 1987), but they may also be beneficial, such as the symbionts that inhabit the gut lumina of wood-eating cockroaches and termites, or the mycetocytes common to many species of insect. Mutualistic associations have also been suggested as one possible method whereby galls may be induced. This hypothesis has been more fully explained in Section 3.2.4, where one example of a mutualistic association was described, i.e. the rôle polydnaviruses play in the suppression of a host's immune response system (Edson et al., 1981). Polydnaviruses were produced in the calyx tissues of the oviducts of some endoparasitic Hymenoptera (Stoltz and Vinson, 1979) and recently, they have also been identified in non-ovarian tissue of both male and females of the parasitoid species, Hyposoter fugitivus (Say) (Ichneumonidae, Stoltz et al., 1986).

Because of these examples, the suggestion that bacteria or viral particles might play a part in gall production (Magnus, 1914: 63; Cornell, 1983) deserved investigation. The mere presence of microorganisms would not, of course, provide evidence of their putative

rôle in cecidogenesis, but information providing identification, position and abundance would be of interest and have provided the basis for future investigations into this hypothesis. Within this area there have been a few reports which were of interest; for example, Lewis and Walton (1964) considered that the cecidogen injected by species of Pachypsylla (Psyllidae) into the leaves of Celtis occidentalis L. (Ulmaceae) was a virus; while Westphal and Michler (1975) identified rhabdovirus-like particles in "Wirrzoph" galls of the catkins of willows. Matrenina and Gusteleva (1984) studied the epiphytic microflora of the larch bud gall caused by Dasineura laricis (F. Löw) (Cecidomyiidae) and found that the majority of the microorganisms identified produced auxins, but the best producers were those found associated with the gall and the larvae. However, none of these authors established a direct link between the microorganisms they observed and the formation of a gall.

5.1.2 The insect.

The structure of the internal reproductive organs of insects has been described by Chapman (1982: 330-349), while Baird (1921), Richards and Davies (1977: 1194-1195) and Gauld and Bolton (1988: 8-9) described that of female Hymenoptera. Iwata (1958) and Togashi (1970) detailed the comparative morphology and anatomy of the ovaries and internal reproductive system of Symphyta.

The general plan of the female reproductive system of Symphyta (see Figure 5.1) is similar to that of other Hymenoptera. The reproductive organs occupy the greater part of the abdominal cavity and consist of two ovaries, each composed of a number of polytrophic ovarioles (i.e. the trophocytes are enclosed in follicles with each oocyte) arranged in

a parallel manner. The number of ovarioles varies, but is usually fairly constant within each species. From a survey of 330 species of Symphyta, Iwata (1958) found that four per ovary was the minimum number, as found in Orussus japonicus Tozawa (Orussidae), the highest number being 62 to 82 per ovary, which were found in Urocerus japonicus Smith (Siricidae). The two ovaries lie above the alimentary canal and are attached to each other at their distal ends, through a twisting together of the ovary ligaments. The proximal ends of the ovarioles empty into the lateral oviducts through a region called the calyx. Each lateral oviduct circles either side of the alimentary canal, then join in a forked manner, to form the median oviduct which lies directly under the alimentary canal. The median oviduct of Hymenoptera is comparatively short compared to other insects and is often hidden, in dorsal view, by the spermatheca, accessory glands and associated colleterial sac. The median oviduct empties into the vaginal pouch and from there to the outside via the saw. Opening into the median oviduct is the spermatheca, which serves for the storage of sperm. Some species of Tenthredinoidea lack the spermathecal gland (Baird, 1921), the secretions of which, in those species that possess it, provide nutrients for sperm. In Hymenoptera the accessory glands have a variety of functions, e.g. in apocritans they produce venoms which are capable of incapacitating their host organism. In Pontania, the accessory gland produces colleterial fluid, which is stored in a large colleterial sac.

It has been generally assumed that the colleterial fluid injected along with the egg during oviposition is responsible for gall formation (Beyerinck, 1887; McCalla et al., 1962). However, Magnus (1914: 87) disputed this, maintaining that its only function was to seal the wound, and that it played no part in gall formation. Subsequent work

(McCalla et al., 1962), suggested that the colleterial fluid was, at least, effective in encouraging gall growth. In Symphyta the accessory glands and their associated reservoir (colleterial sac) form a variety of shapes and configurations, these were used by Togashi (1970) as part of his consideration of the phylogeny of this group.

Studies of the reproductive anatomy of Tenthredinoidea have been limited to Cimbex americana Leach (Cimbicidae; Severin and Severin, 1908) and Perga affinis affinis Kirby (Pergidae; Tait, 1962). Both Beyerinck (1887) and Magnus (1914) mentioned briefly the gross morphology of the internal reproductive system of Pontania triandrae. From a personal search and a survey of the data bases AGRICOLA 1970-1990, CAB ABSTRACTS 1972-1990, BIOSIS PREVIEWS 1969-1990 and LIFE SCIENCES COLLECTION 1978-1990, it would appear that no-one has studied the internal reproductive anatomy of Pontania proxima, or of any other species of Tenthredinidae in detail. It was a further aim of this work to fill this omission.

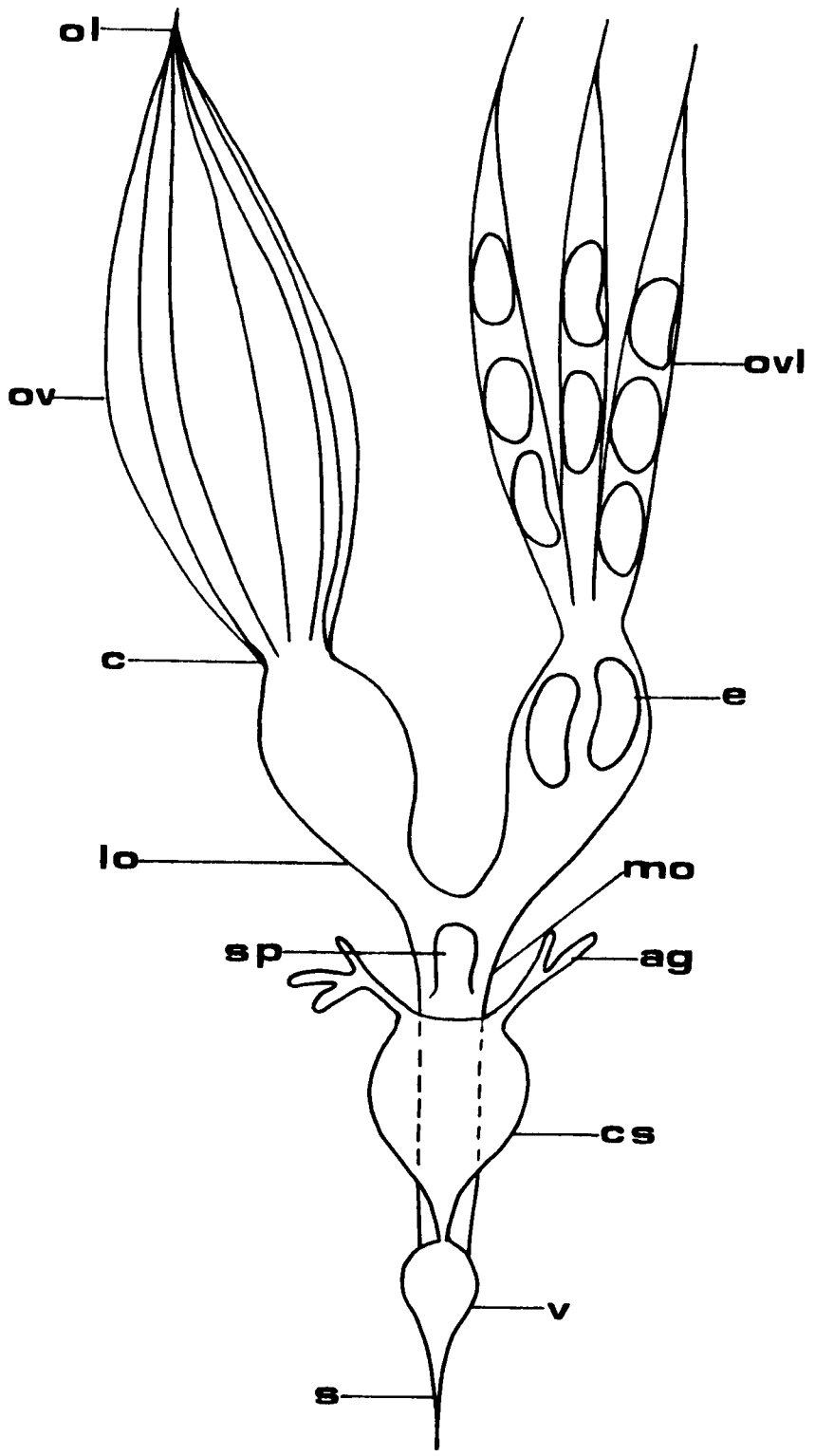


Figure 5.1. Generalized diagram of the female reproductive system of Symphyta (dorsal view, not to scale). ag. accessory gland; c. calyx; cs. colleterial sac; e. egg; lo. lateral oviduct; mo. median oviduct; ol. ovary ligament; ov. ovary; ovl. ovariole; s. saw; sp. spermatheca; v. vaginal pouch.

5.1.3 The gall.

The galls of Pontania proxima are of the simple cataplasmic type, with little internal differentiation compared to those, for example, of Cynipinae. In the Oxfordshire area, galls of Pontania proxima were found predominantly on the leaves of Salix fragilis, where they were positioned between the midrib and the leaf-edge of the central part of the leaf lamina (see Section 4.5). They were characterized by being fleshy hollow outgrowths, oval in shape and extending almost equally from either side of the leaf. When fully grown, they were approximately 12 mm in length, 6 mm in width and 5 mm thick. The uppermost (adaxial) surface was usually smoother than the lower (abaxial) surface and when not shaded from light it was a bright red colour which darkened with age. The cause of this colour has been identified by Blunden and Challen (1965) as the anthocyanin cyanidin-3-monoglucoside. Raven (1978) suggested that one possible rôle for anthocyanins was to protect plant tissues from the harmful effects of ultra-violet radiation; perhaps in the case of the gall of Pontania proxima, cyanidin-3-monoglucoside plays a part in protecting the sedentary larvae from possible damage cause by U/V light. The abaxial surface of the gall was, when fully developed, a green or yellow or pink colour. Sometimes the surface was smooth, at other times ridged or almost pointed. When the surface was ridged it extended further than the adaxial surface. In young galls, the lower surface was also red, but usually this colouration was lost, after a period of time. It should be noted that when the galls were young, the leaf buds with the developing galls were fully exposed, and orientated so that both surfaces of the leaf were equally exposed to the sun.

The reasons for the observed differences in shape of the abaxial surface of the gall were unclear, they have, at various times, been linked to the presence of parasites and inquilines (Benes, 1968) but personal observation suggested these influences were not the dominant force. Indeed, as Magnus (1914, 60) observed, it would seem that these variations were determined more by the patterns of growth of the trees themselves. Although quantitative work needs to be done in this area, it appeared that galls of the first brood, especially when grown under controlled conditions, were smoother than those of the second brood. Generally, it was observed that fast spring growth produced smoother galls than later in the year; in July to August the lower surface of galls were more ridged.

The final size of the adult gall presented an interesting paradox. Healthy, fully developed galls were all of a comparable size, regardless of the final size of the leaf they inhabited i.e. small leaves did not produce proportionally smaller galls. In this way, the growth of the gall was seen as being independent of the growth of the leaf. However, if this were to be so, then distortions of the leaf would have been evident e.g. if the gall infested the right-hand-side of the leaf lamina and encouraged greater growth in this area, compared to the left-hand-side, then the resulting leaf shape would have been falcate. This was not observed to occur to any great extent. A possible reason for this observed pattern of growth is given in the Discussion in this Chapter.

Histological details of gall development have been given by Beyerinck (1887), Magnus (1914), Rey (1967, 1968, 1972, 1973 and 1974) and Slepyan and Gabarayeva (1981). For the purpose of this thesis a

study of the ontogeny of the gall was made, the results of which agreed with those obtained by the authors listed above. For this reason, they are not reproduced in this thesis. Only those aspects of gall development that related to the aims of this Chapter are included.

The most detailed description of the ontogeny of the gall was supplied by Rey (1967, 1968, 1972, 1973 and 1974). His observations like those of Carleton (1939) confirmed, at least for Pontania galls on Salix triandra, that the growth of the gall was completed before eclosion. In this study hundreds of newly full grown galls were opened, all contained either no egg, an egg or a small larva. These personal observations, together with measurements on the growth of the gall (see Appendix 7), agreed with those of Rey (1968), and Carleton (1939), i.e. by 15 days the adult state of the gall had been reached. Also, as observed by Rey (1968) the ontogeny of the gall was fixed very early on in its development (i.e. within 60 hours of oviposition). Thus, the egg and its larva played no part in gall development. This point, as illustrated earlier (see Section 2.2) has caused confusion, the dominant view being that galls produced by all species of Pontania require the presence of a larva to complete development (Murphy, 1929; Hovanitz, 1959; McCalla, 1962; Lazareva et al., 1986; Gauld and Bolton, 1988: 118). For the majority of gall-formers the presence of an actively feeding larva is essential for gall-formation e.g. Cynipinae, where gall-formation does not occur until after eclosion and is under the influence of the feeding larva. However, although this may occur in some species of Pontania (Benes, 1968; Clancy et al., 1986), for galls of Pontania proxima the presence of the larva was not necessary (Beyerinck, 1887; Magnus, 1914; Benes, 1968; personal observation) and

the suggestion that it was, has arisen by repetition of generalisations with respect to the genus Pontania.

Both Beyerinck (1887) and Magnus (1914) agreed that neither an egg nor a larva were necessary for gall-formation, both executed experiments to confirm their observations. They disagreed, however, on the reason for the variation in the size of galls, especially the abnormally small ones, which were a few millimetres in diameter and were the result of abortive attempts at oviposition. Beyerinck (1887) maintained that variations in size were due to differences in the volume of colleterial fluid injected into the leaf at the time of oviposition. Magnus (1914: 62, 73) contended that these differences were due to both the age of the leaf at the time of oviposition and the amount of wounding inflicted upon the leaf.

Oviposition by Pontania proxima (see Sections 2.6 and 4.4) occurred in young leaves of Salix fragilis (see Plate 4.9) that did not yet contain fully differentiated tissues (see Plate 5.23). For Salix triandra (Rey, 1967 and 1968) the leaves consisted of seven to eight layers of cells i.e. one layer, upper epidermis; one layer, palisade; three to four layers, central area (provascular tissue, with 2nd and 3rd order veins consisting only of protoxylem; Slepyan and Gabarayeva, 1981); one layer, sub-epidermal tissue; one layer, lower epidermis. The wound caused by the ovipositor was observed by Rey (1967) to pass through the central layers of the leaf, parallel to the leaf-surface, with one, perhaps two layers of cells being destroyed. These observations demonstrated less precision, by the ovipositor, than those given by Magnus (1914: 66), but in general there were always fewer cell layers above the wound than below it. The egg was observed by Rey (1967) to be placed at the distal end of the wound, in the part nearest

to the midrib. Because the egg was large in comparison to the thickness of the leaf, it pushed the upper layers away from those under the egg. The egg itself was surrounded by colleterial fluid and, at the distal end of the wound, there was a collection of cellular debris which had been pushed there be the action of the ovipositor. Within a short space of time (12 hours at the maximum) the colleterial fluid was observed, by Rey (1967), to have disappeared; with cecidogenesis being evident six to 12 hours after oviposition. In a normal leaf at a similar stage of development, the majority of cellular divisions were predominantly anticlinal, those of the procecidium (a gall produced before eclosion) were mainly periclinal, and although they occurred throughout the tissues surrounding the wound area, they were concentrated near the ends of the egg. Hypertrophy occurred in all surrounding areas and was orientated in directions of least resistance. These observations by Rey (1967) of the initial stages of gall development present a less organized sequence of events than those suggested by either Beyerinck (1887) or Magnus (1914: 67). Instead, the observation of Rey (1967) suggested that the cecidogenic action occurred in all the tissues of the leaf, but not in a uniform manner, the only consistent features being the change in the polarity of division and the concentration of divisions near the tips of the egg. Rey (1967) observed that by the end of 24 hours each epidermal layer had doubled, while the palisade and sub-epidermal layers had more than doubled. The central layers had demonstrated the maximum number of divisions. These had resulted in bulges round the edge of the cavity so reducing its free space. Thus, all the cells of the gall had divided at least once, but without a corresponding increase in size of the gall, because cell enlargement had not yet taken place. Rey (1967) also observed a clear boundary to

the effect of the cecidogen, which in the early stages of gall development, he calculated to be approximately 2.6 mm in length and 1.9 mm in breadth, these figures were very close to those given by Magnus (1914: 86) and they agreed that this area remained proportionally constant throughout the growth of the leaf. Thus, during the development of the gall, the cecidogenic action remained within the environs of the initial wound site and did not spread to other areas of the leaf, i.e. it was a localized reaction throughout all stages of its development.

Rey (1967 and 1968) maintained that, after 60 hours of development, the pattern of the gall was fixed and that development continued without any great changes in structure. All the cells remained in their respective layers, in regular rows perpendicular to the surface of the leaf, with the number of cells in each layer being proportional to the number in the initial layers. Divisions were still more numerous in the central layer; here they were at a maximum either side of the poles of the egg and weakest opposite the centre of the egg. Away from the egg the number of divisions occurring decreased towards the edges of the gall. The differences in the number of observed divisions were, in part, compensated for by a greater hypertrophy of cells occurring in the regions of fewest divisions.

From 60 hours to 15 days, by which time the galls of Pontania proxima on Salix triandra had reached adult size, there had just been, essentially, a modification in size, although there was a slight merging between the zones of cells in the tissues of the leaf. The overall growth of the gall paralleled that of the leaf (Rey, 1968) and was completed before the hatching of the egg. By this time the cells of the central layers had remained small, practically the same size as

normal mesophyll cells of the central layers. They had a rounded isodiametric shape and contained little tannin, but they did contain numerous chloroplasts. Hypertrophy of cells increased with distance from the cavity and was especially prevalent near vascular tissue. The midrib exerted a limiting effect on the gall growth, whereby, unlike first order veins, the gall was unable to cross its boundary. Slepyan and Gabarayeva (1981) observed that second and third order vascular tissue, within the gall, remained underdeveloped and was displaced from its usual position. Within the central areas of the gall no normal vascular tissue developed, provascular tissue formed randomly located annulate and spiral tracheid-like elements. Cell lines that originated from the palisade and sub-epidermal layers were rich in tannins and contained fewer and smaller chloroplasts compared to normal cells of this type. Unlike tissues from normal leaves, gall tissues contained no starch or lipids, but the central layers contained a higher than usual concentration of proteins, especially near the cavity (Rey, 1968).

Rey (1972, 1973 and 1974) studied the ultrastructure of the developing chloroplasts in the central tissues of the gall. He interpreted electron micrographs of developing chloroplasts as containing a smaller number of lamellae than usual and he also observed the development of electron-dense vesicles at the extremities of the lamellae.

5.2 Methods

5.2.1 Introduction.

A variety of methods was adopted for the study of specimens of both insect and plant origin and these are listed below. All specimens were freshly obtained, the plant material collected on the same day as fixation, the insect material collected within 24 hours of emergence, except where stated otherwise.

5.2.2 Negative staining.

For this technique the methods of Roland (1978, 23-24) were used. These were applied to insect material extracted from the colleterial sac, oviduct and ovarioles.

The material to be observed was dissected out into cold distilled water and homogenized with a glass rod. Distilled water was used in preference to insect Ringer (see Appendix Six), since it avoided contamination by precipitated solutes. A small drop of the homogenate was placed on a 3.05 mm diameter square 300 mesh copper grid (Agar Scientific), previously coated with a carbon support film. To stain the specimens, one drop of 2% uranyl acetate was added and the two liquids mixed with a pipette. The mixture was allowed to settle for ten minutes and then the excess liquid was drawn off using a piece of filter paper. A clean pipette was used each time to prevent contamination.

5.2.3 Embedding schedule.

Two protocols were used: the first, except where stated otherwise, was used for the embedding of plant specimens; the second, except were

stated otherwise, was used for the embedding of insect-derived specimens.

a) Reagents used.

1. 0.1 M phosphate buffer at pH 7.2. This was prepared in accordance to the schedule of Roland (1978: 54-55). Fourteen millilitres of stock 0.2 M monobasic phosphate (2.67 g $Na_2H_2PO_3.H_2O$ made up to 100 ml in distilled water) solution, was added to 36 ml of stock dibasic phosphate (3.56 g $Na_2HPO_4.2H_2O$ made up to 100 ml in distilled water). The pH of the resulting 0.2 M phosphate solution was checked with a newly calibrated Philips PW 9410 digital pH meter using a Pye Unichem Ingold combined pH probe (Pye Unicam, Cambridge. CB1 2PX). This solution was then diluted, as required, with an equal volume of distilled water to give a 0.1 M solution.

2. 0.05 M cacodylate buffer at pH 7.4. This was prepared by adding 2.7 ml of 0.2 M HCl (BDH Chemicals, Dagenham, Essex. RM8 1RY) to 50 ml of 0.2 M sodium cacodylate (42.8 g/l, BDH Chemicals) and the resulting solution was made up to 200 ml using distilled water. The pH was adjusted to 7.4, after which five grams of sucrose was added (Sigma Chemical Company, Poole, Dorset. BH17 7TG).

3. Fixation using a 3% solution of glutaraldehyde in 0.1 M phosphate buffer. This was prepared by adding 1.2 ml of a 25% stock solution of EM grade glutaraldehyde (TAAB Laboratories Equipment, Aldermaston, Berkshire. RG7 4QW) to 5.0 ml of the 0.2 M phosphate buffer and diluting the mixture to 10 ml with distilled water.

4. Fixation using a 2.5% solution of glutaraldehyde in cacodylate buffer. A 25% stock solution of glutaraldehyde was diluted to the required concentration with 0.05 M cacodylate buffer.

5. Postfixation in 1% aqueous solution of osmium tetroxide in 0.1 M phosphate buffer. One millilitre of a 2% aqueous solution of osmium tetroxide prepared from a 0.1 g vial (Johnson Matthey Chemicals, Royston, Hertfordshire. SG8 5HE), was added to one millilitre of 0.2 M phosphate buffer.

6. Staining in an aqueous solution of uranyl acetate, as prepared by Pharmacia LKB (Milton Keynes, Buckinghamshire. MK9 3HP).

7. TAAB embedding resins were used. These were either made as detailed in Table 5.1, or alternatively, a TAAB T027 TER premix kit (hard) was used and the instructions supplied with the kit were followed.

TAAB embedding resin	25.0 ml.
DDSA hardener 964 (dodecenyl succinic anhydride)	22.5 ml.
MNA hardener (methyl nadic anhydride)	2.5 ml.
accelerator DMP-30 (2.4.6-tri [dimethlyaminomethyl] phenol)	0.5 ml

Table 5.1. Constituents of embedding resin. These were combined and shaken until the mixture was fully homogeneous. The container holding the resin was then placed in a sonic water bath for 30 minutes until all air bubbles had been removed and the resin was clear.

b) Protocol One.

Small pieces (approximately 2 mm^3) of leaf or gall were fixed, for two hours in a 3% solution of glutaraldehyde made up in 0.1 M phosphate buffer, to establish a rapid stabilization of proteins. After this the glutaraldehyde solution was removed and the specimens were washed in

0.1 M phosphate buffer (three x 20 minutes using a TAAB Rotator type N, turning at two rotations per minute).

After washing, postfixation of previously stabilized proteins and those compounds, e.g. lipids, not affected by aldehydes was achieved in 2 ml of a 1% osmium tetroxide solution in 0.1 M phosphate buffer for one hour. The specimens were then washed as before but using distilled water. Staining took place overnight in aqueous uranyl acetate.

The specimens were then dehydrated by passage through an alcohol series (30%, 60%, 90%, 95% and 100%), and finally through two changes of dry acetone, 15 minutes each change. To minimize mechanical and osmotic stresses, infiltration of resin was achieved using the method described by Marchese-Ragona and Johnson (1982). For this technique an acetone/resin gradient was prepared: firstly by mixing together equal parts of acetone and resin, then adding pure resin which sank to the bottom of the container. Finally dry acetone was added which formed the uppermost layer. Careful shaking of the container broke the boundaries between these liquids and ensured that a gradient was formed, from dry acetone at the top surface to 100% resin at the bottom. The dehydrated specimen was transferred directly from dry acetone to the dry acetone layer of the gradient and as acetone/resin exchange took place the specimen gradually sunk through the gradient until it reached 100% resin. After approximately two hours the acetone/resin gradient was decanted off and 100% resin added. The specimens were then removed and placed in fresh resin. After 24 hours the specimens were again transferred to fresh resin, then after one hour transferred into a A0527 mini mould (Bio-Rad, Hemel Hempstead. HP2 7TD) containing fresh resin and heated in an oven, at 60° C for 24 hours.

c) Protocol Two.

The tissue was fixed overnight in cold 2.5% glutaraldehyde made up 0.05 M sodium cacodylate buffer and washed (three x 30 minutes) in buffer without glutaraldehyde, after which the specimens were left in a fridge overnight. It was found that these times resulted in a good penetration of fixative into the insect. Postfixation took place in a 1% aqueous osmium tetroxide solution for one hour. The tissue was then dehydrated and embedded as detailed in Protocol One.

d) Sectioning.

Sections were cut using a Reichert "Om U2" Ultramicrotome (Cambridge Instrument Products, Bar Hill, Cambridge. CB3 8El) or a Sorvall MT5000 Ultra Microtome (DuPont (U.K.), Stevenage, Hertfordshire. SG1 4ON). Knives were prepared using a LKB Knifemaker Type 7801B (Pharmacia LKB), from freshly washed 25 mm x 6.5 mm x 400 mm glass strips, using the methods of Reid (1975: 255-257). These were set at a cutting angle of three to five degrees. Sections for light microscopy were cut between 0.5 and ten microns thick, and, if required they were mounted in DPX Neutral Mounting Medium (Aldrich Chemicals, Gillingham, Dorset. SP8 4JL) and covered with a glass cover slip. Ten-micron-thick sections were also used to scan specimens and then those that were of special interest were re-embedded in preparation for ultramicrotomy and electron microscopy. Beem capsules (size 00, Agar Scientific) were filled with embedding resin so that it lay in a dome over the rim of the capsule, thus preventing the collection of air bubbles. The section, still on the slide, was placed on the capsule so that it was in contact with the dome of resin. The slide and capsule were then inverted and placed in an oven, set at 60° C, to harden. To remove the newly embedded section, a rubber bung was cooled in liquid nitrogen and

the slide placed on the bung. This cooled the slide, contracting the glass, so separating it from the section (O'Brien and McCully, 1981: A-xv).

On large sections, to increase efficiency and to allow scanning for particular areas of interest, the mesa technique was adopted (Reid 1975: 285-187). This involved using large surfaces from which thick survey sections could be cut, then using an ultramicrotome to pare the block back to a depth of approximately 20-30 microns, but leaving a ridge containing the selected feature. It was from this area that ultrathin sections were obtained. Sections for electron microscopy were cut to an approximate thickness of 90 nm using the methods described by Reid (1975). Ribbons of gold-silver were obtained and collected on water, and retained using LKB Trufs (Pharmacia LKB). Before collection on the dull side of a specimen grid (pre-washed in acetone), the sections were treated with chloroform vapour. For electron microscopy several types of specimens grids, including 2.0 x 0.5 mm slot grids with a supporting pioloform film, were used. The pioloform film was prepared by dipping a glass slide into 3.0% (w/v) pioloform (Agar Scientific) in chloroform, then allowing the slide to drain and dry. The edges of the pioloform film were then scored and the film floated on distilled water. The slot grids were then placed dull side down on the film. The whole preparation was then lifted out with freshly peeled parafilm (O'Brien and McCully, 1981: A-xx to A-xxii).

e) Staining.

A LKB Multiplate 2208 (Pharmacia LKB) was used during the staining of sections for light microscopy. Sections for light microscopy were stained with either 1% methylene blue in 1% borax, or 1% toluidine blue in 1% borax. Sections were also stained using freshly prepared 1%

aqueous p-phenylenediamine, which enhanced osmium staining. A large drop was applied and the slide warmed for approximately three minutes. The excess was washed off using distilled water and the slide dried (Estable-Puig et al., 1965, Ledingham and Simpson, 1970 and 1972) and viewed using a Zeiss Standard 18 Microscope (Carl Zeiss Jena, Borehamwood, Hertfordshire. WD6 1NH) adapted for phase contrast microscopy. The use of p-phenylenediamine was especially effective on the insect specimens, and was also used exclusively on specimens that were to be re-embedded.

The enhancement of staining of sections for electron microscopy was achieved by floating specimen grids on a drop of 2% uranyl acetate for 20 minutes; after washing this was followed by flotation on a drop of preprepared stabilized solution of lead citrate (Pharmacia LKB) for five minutes in a carbon dioxide free environment, to prevent the formation of lead carbonate (Roland, 1978: 16-17). After washing in boiled and cooled distilled water the specimen grids were allowed to dry on a filter paper.

If the stain precipitates were present in the section, excess uranyl acetate was removed by washing. Lead citrate was removed by washing with 10% glacial acetic acid for one minute, followed by washing with distilled water.

All sections for electron microscopy were viewed using a JOEL JEM 2000 EX electron microscope (Joel (U.K.), Colindale, London. NW9 0JN). The acceleration voltage was 120 kV and the size of the aperture was 100 microns.

f) Developing plates from the TEM.

The plates used were Ilford E.M. film (Ilford Photo, Tottenham, London. W1P 0AN). These were developed using Ilfotech H C (1+15 for four

minutes at 20° C; Ilford Photo). Prints were obtained on Ilford Multigrade III R.C. Deluxe (Ilford Photo) paper, using a De Vere 504 enlarger (De Vere [Kensington], Beckenham, Kent. BR5 4NB) with condenser element positions of 180 mm and 180 mm and an enlarger objective lens with a focal length of 135 mm. Ilford Multigrade developer (1+9) was used; the stop bath was a weak solution of acetic acid, and finally, the paper was fixed using Ilford Ilfospeed fixative.

5.3 **Results.**

5.3.1 The structure of the internal reproductive system and associated glands of Pontania proxima.

a) Gross anatomy.

The internal reproductive organs and associated glands of Pontania proxima (see Plate 5.1) filled most of the abdominal cavity. They extended from the tergal side of the second abdominal segment to the eighth and ninth sterna, which were modified to form the ovipositor. The two ovaries were held together, at their distal ends, by filamentous threads that also anchored them to the wall of the abdomen. Each ovary consisted of a variable number of ovarioles, usually 11, each approximately one millimetre in length. These opened, through a calyx region, into a lateral oviduct of similar length to the ovarioles. Often the calyx and lateral oviducts were expanded by the presence of eggs. If not, then the calyx region was marked by a constriction between the ovarioles and lateral oviduct (see Plate 5.2). Fully developed eggs were present in all the adults dissected. Proximally the two lateral oviducts united to form the median oviduct on the sternum side of the sixth abdominal segment. The alimentary canal ran through a ring formed by the ovaries and lateral oviducts. Previous authors have mentioned that Nematinae associated with Salix feed from catkins in the spring (see Section 2.5); debris was often observed in the lumen of the alimentary canal, even though the sawflies observed had been raised in artificial surroundings.

The median oviduct was approximately half the length of the lateral oviducts and at its proximal end it was much compressed where it passed into the vaginal pouch (see Figure 5.3, p. 132).

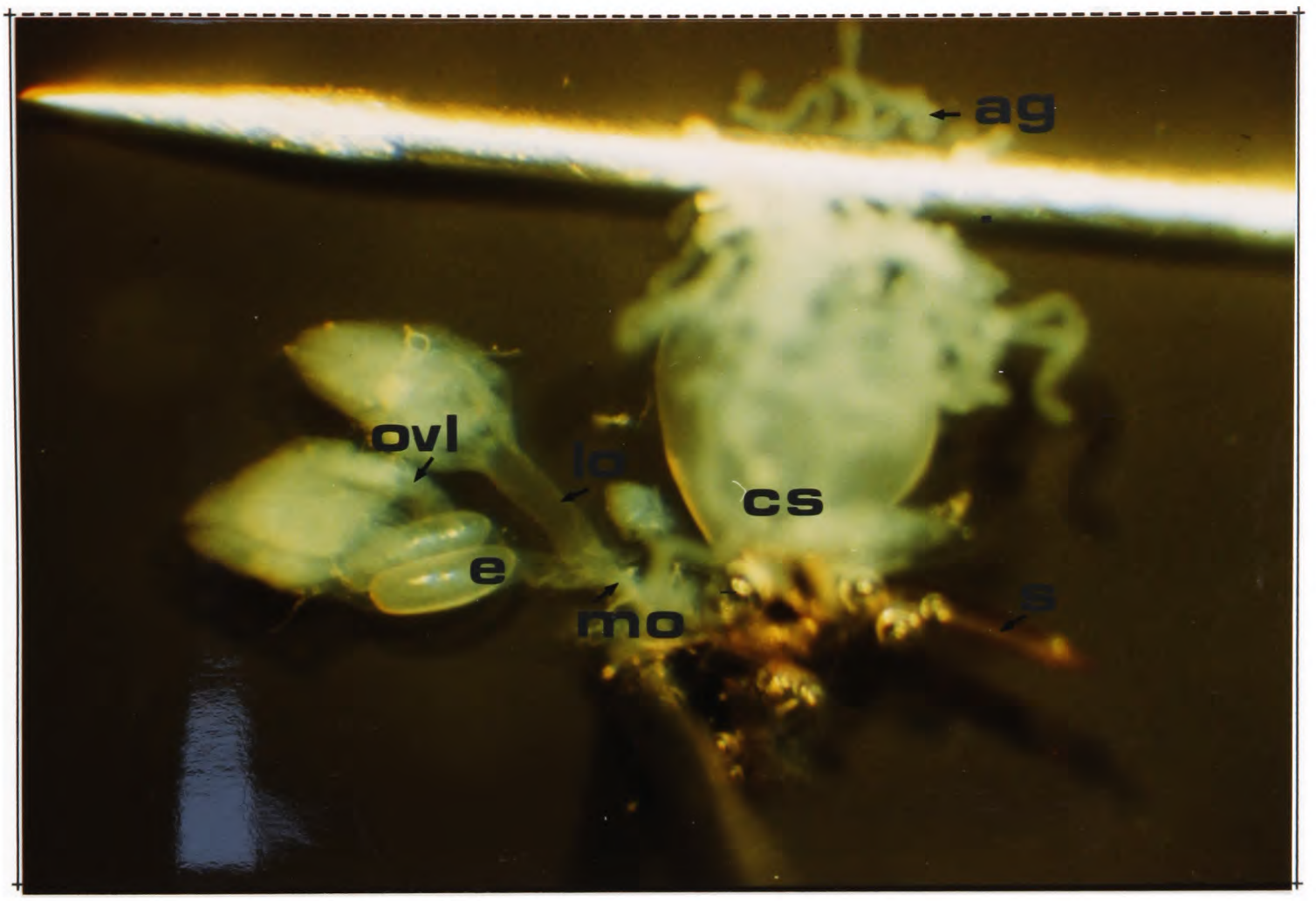


Plate 5.1. Internal reproductive organs and associated glands of Pontania proxima; ag. accessory gland; cs. colleterial sac; e. egg; lo. lateral oviduct; mo. median oviduct; ovl. ovarioles; s. saw (Mag. x40).

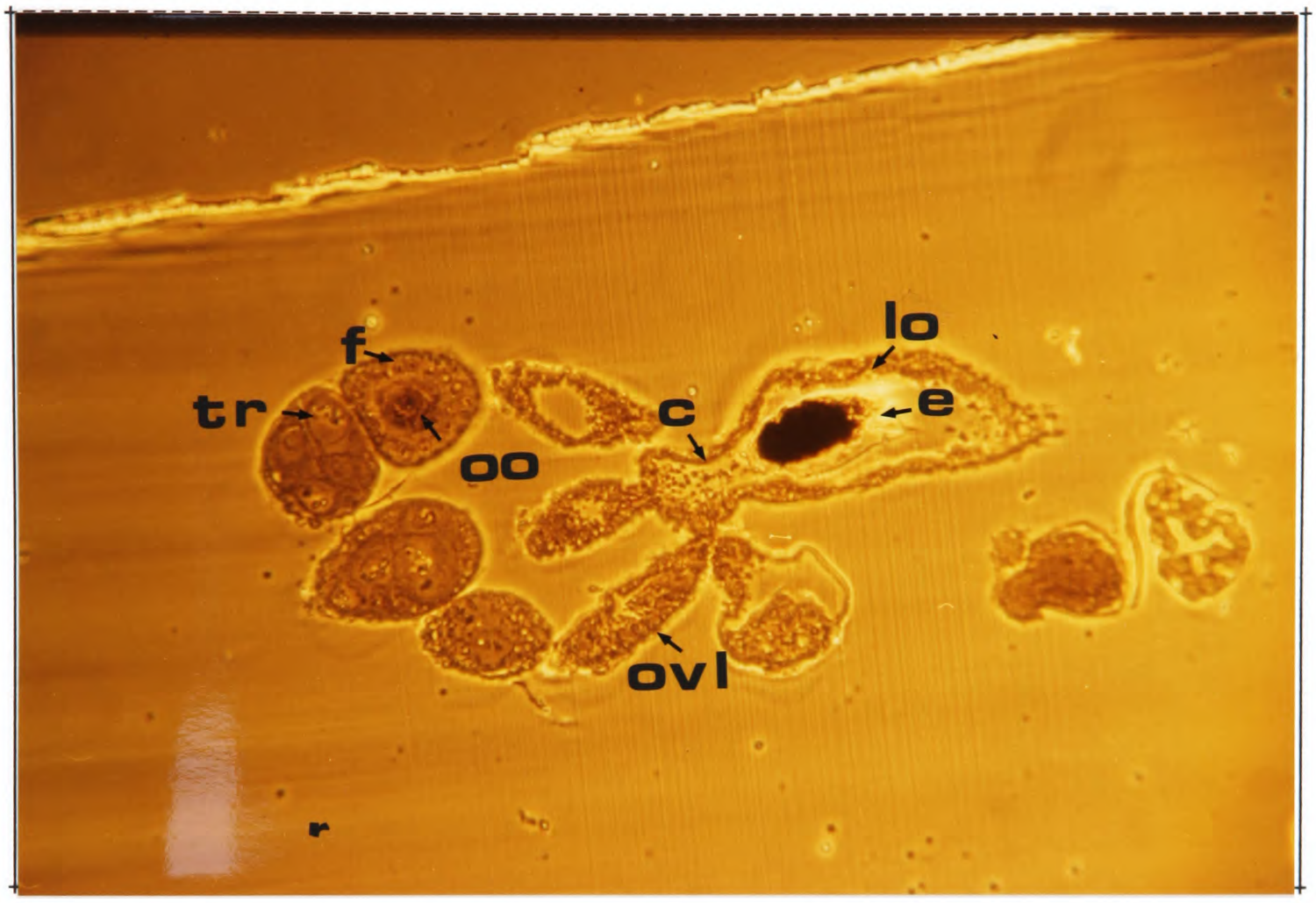


Plate 5.2. Section through lateral oviduct and ovarioles of Pontania proxima. Thickness five microns. Fixation using protocol one. Stain p-phenylenediamine; c. calyx; e. egg; f. follicular epithelium; lo. lateral oviduct; oo. developing oocyte; ovl. ovariole; tr. trophocytes (Mag. x140).

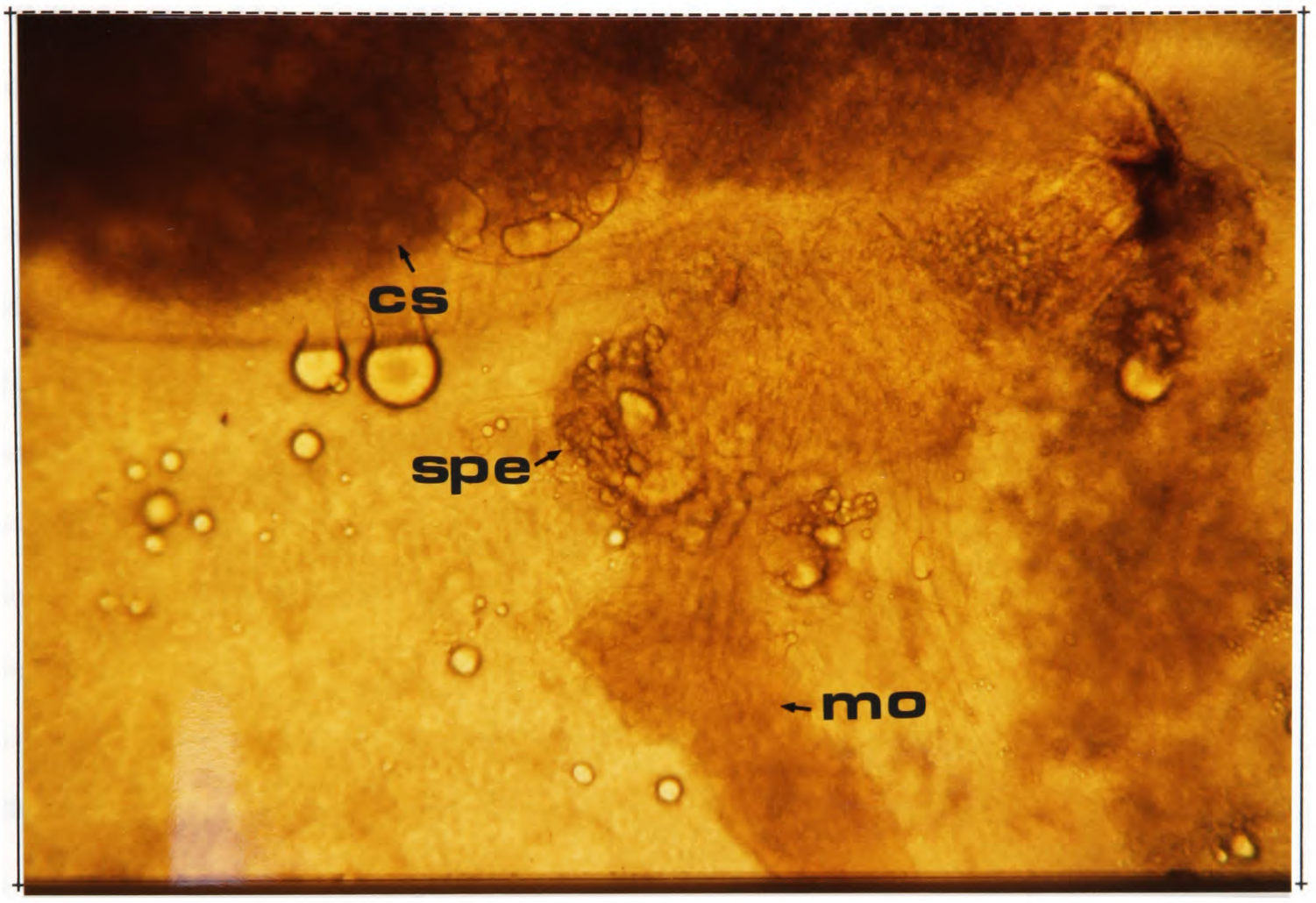


Plate 5.3. Unstained squash preparation of the colleterial sac, spermatheca and median oviduct of Pontania proxima, mounted in water; cs. colleterial sac; mo. median oviduct; spe. spermatheca (Mag. x134).

Lying dorsal to the median oviduct were the terminal abdominal ganglia (see Plate 5.10 and Figure 5.3). The end of the median oviduct was marked first by a muscular structure, the vaginal clamp, then by the spermathecal duct (see Plate 5.20 and Figure 5.3). Here the median oviduct and the spermatheca combined and emptied into the vaginal pouch. In dorsal view the median oviduct, spermatheca and lateral oviducts were obscured from view by a large colleterial sac (mean diameter = 0.98 mm, n = 93 see Appendix Eight) and its contents. At the distal end of the colleterial sac was a highly-branched accessory gland, which emptied through a single short tube into the colleterial sac. The duct that passed from the colleterial sac into the vaginal pouch was much compressed. At no time did the median oviduct or the duct from the colleterial sac empty into each other: both emptied by separate routes into the vaginal pouch.

b) Structure of the accessory gland and colleterial sac.

The accessory gland was formed from a mass of fine tubules (see Plate 5.1) each approximately 0.075 mm in diameter (n = 20, see Appendix Eight). Throughout its length, each tubule was composed of two layers of cells (see Figure 5.2 and Plate 5.5). The innermost layer, which lined the lumen of the central duct of the tubule, were small epidermal cells which secreted cuticle. These epidermal cells also produced a smaller cuticle lined duct which passed down into the underlying, larger secretory cells (see Figure 5.2 and Plate 5.4). The cuticle produced on these small ducts seemed to be of a different structure from that lining the lumen of the tubule, with an electron-dense region lying immediately under the cuticle layer (see Plate 5.6). This region was absent from the central ducts of the accessory gland tubules.

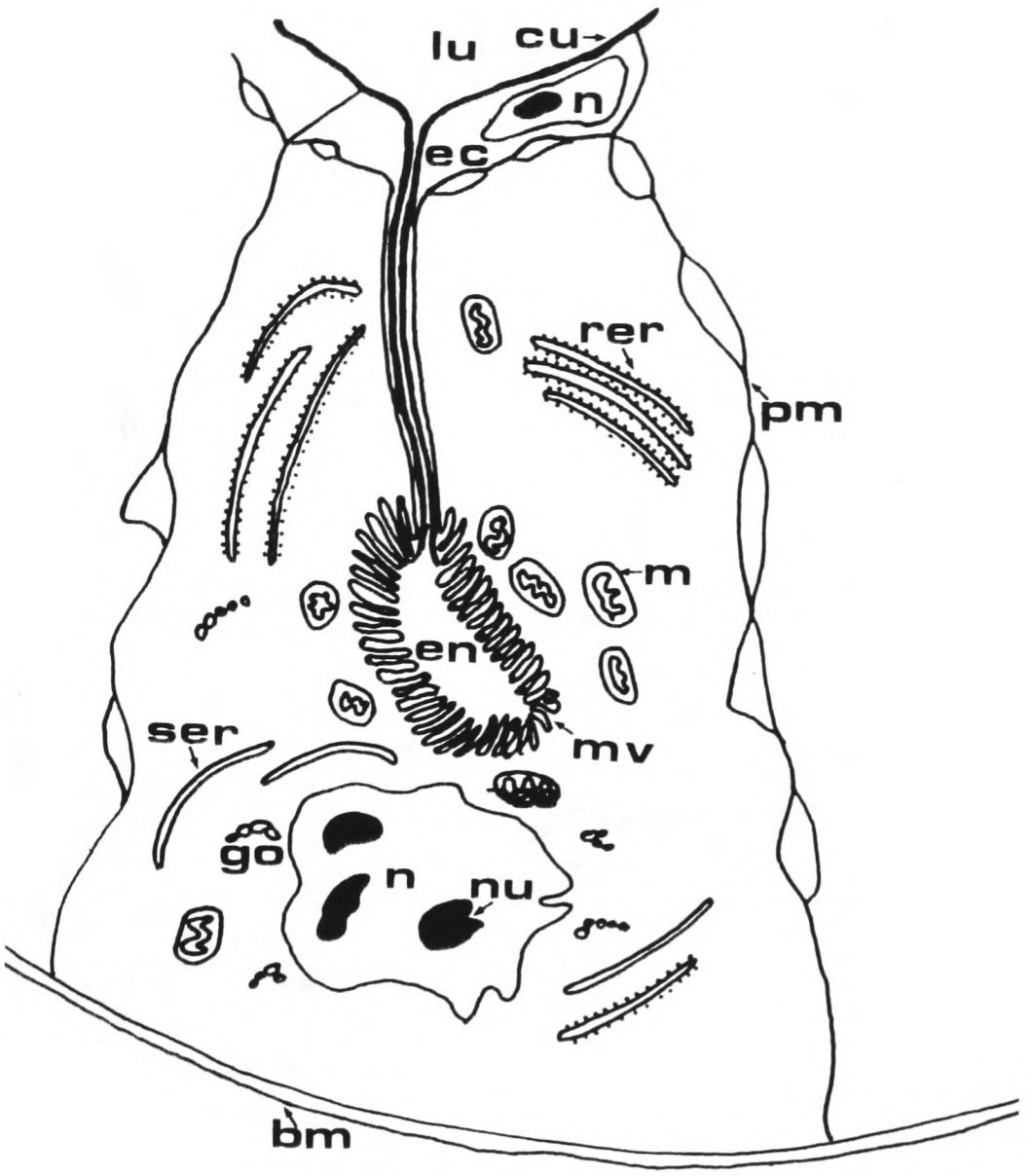


Figure 5.2. Simplified diagram of a transverse section of an accessory gland tubule of Pontania proxima, taken from the distal portion of the gland and showing an inner epidermal cell and a single secretory cell; bm. basement membrane; cu. cuticle; ec. epidermal cell; en. end-apparatus; go. golgi complex; lu. lumen (of tubule); m. mitochondrion; mv. microvilli; n. nucleus; nu. nucleolus; pm. plasma membrane; rer. rough endoplasmic reticulum; ser. smooth endoplasmic reticulum.

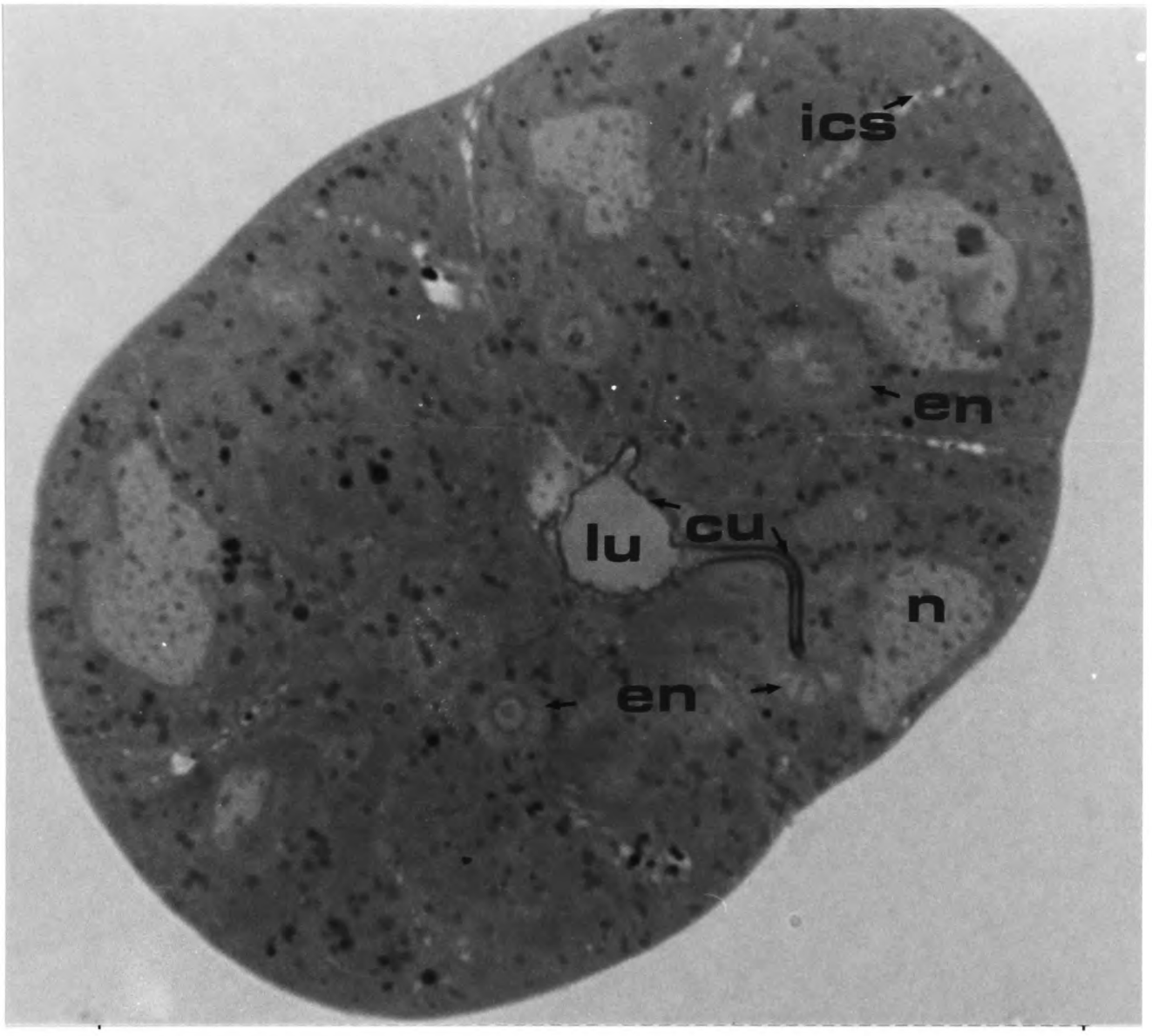


Plate 5.4. Section through the distal portion of the accessory gland of Pontania proxima. Thickness two microns. Fixation using protocol two. Stain p-phenylenediamine; cu. cuticle (duct to secretory cell); en. end-apparatus (of secretory cell); ics. intercellular spaces; lu. lumen (of accessory gland tubule); n. nucleus (of secretory cell) (Mag. x168).

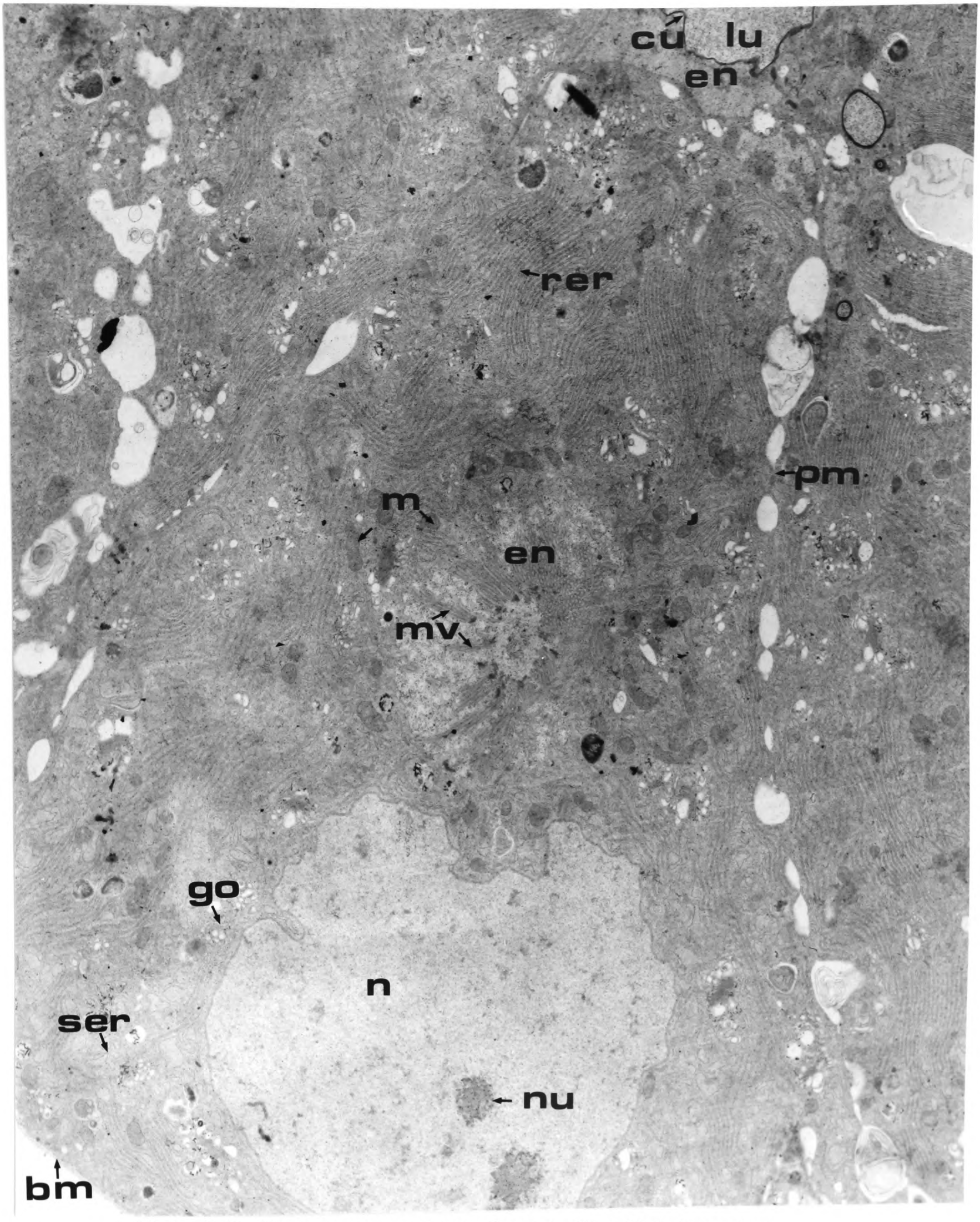


Plate 5.5. Transverse section through the distal region of the accessory gland of Pontania proxima, similar to Figure 5.2. Fixation using protocol two; bm. basement membrane; cu. cuticle; ec. epidermal cell; en. end-apparatus; go. golgi complex; lu. lumen (of tubule); m. mitochondrion; mv. microvilli; n. nucleus; nu. nucleolus; pm. plasma membrane; rer. rough endoplasmic reticulum; ser. smooth endoplasmic reticulum (Mag. x6280).

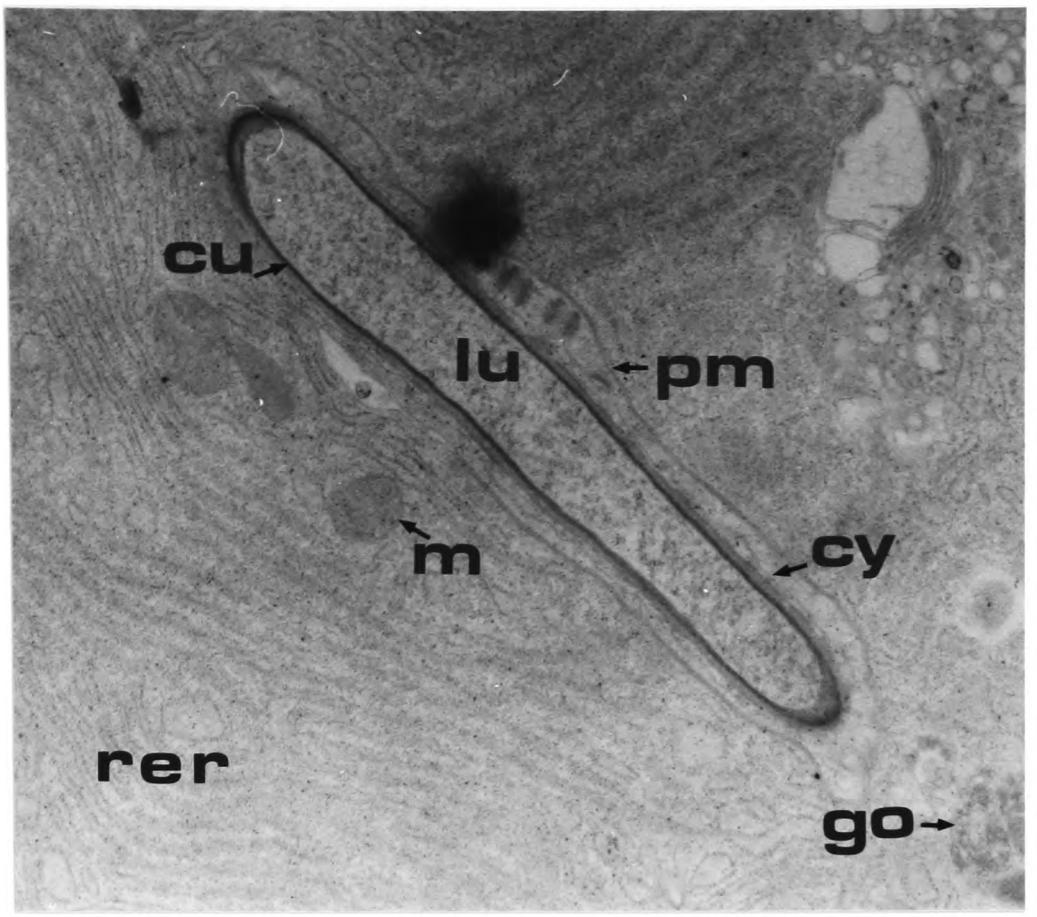


Plate 5.6. Section through part of the cuticle-lined duct that passes from the epidermal cells into the invaginated apical portion of the underlying secretory cells of the accessory gland of Pontania proxima. Fixation using Protocol two; cu. cuticle; cy. cytoplasm (of epidermal cell); go. golgi complex; lu. lumen (of duct); m. mitochondrion; pm. plasma membrane; rer. rough endoplasmic reticulum (Mag. x15000).

Throughout most of the accessory gland the secretory cells were loosely adherent to each other and to the epidermal cells. In all the sections with this feature, vesicles were present in the intercellular spaces. However, in the proximal portion of the gland, just before it entered the colleterial sac, the cells were more tightly connected (see Plate 5.8). The secretory cells were bounded at their basal surface by a basement membrane, which was in direct contact with the haemolymph. The basal portion of each secretory cell contained a large, irregularly shaped nucleus with several nucleoli. This basal area of the cell

contained rough-surfaced endoplasmic reticulum, mitochondria, golgi complexes (especially adjacent to the nucleus) and smooth-surfaced endoplasmic reticulum, the latter was more concentrated here than in any other region of the cell.

Within the central area of the secretory cell was the end-apparatus (sensu Mercer and Brunet, 1959), which was formed from an invagination of the apical membrane of the cell and consisted of a blind-ending tube which terminated in numerous microvilli, surrounding a central lumen full of secretory products (see Plate 5.5). Some of the microvilli were observed to have electron-dense tips. Surrounding the end-apparatus were numerous mitochondria and abundant sheet-like rough-surfaced endoplasmic reticulum, which suggested that this area of the cell was producing protein-based materials.

The secretions produced by these cells, together with the contents of the lumen of the accessory gland, and the contents of the colleterial sac were all examined (see Plate 5.7). Throughout all these regions, the secretion was heterogeneous in appearence, with no evidence of microorganisms or virus-like particles. The fluid contained within lumen of the distal part of the accessory gland contained electron-dense areas, but these were smaller and less numerous than those observed in the fluid from the colleterial sac.

The most common shape of the end-apparatus was circular, but in some preparations it appeared cylindrical, often curved within the cell. It was not clear, whether these differences were due to the plane of the section examined, or whether they indicated different types of end-apparatus (see Plate 5.8a and b). These differences were most marked in the proximal region of the gland, where it entered the colleterial sac. In serial sections, there appeared to be two types of secretory cell in this region.

As the lumen of the accessory tubule enlarged, cells with a circular end-apparatus were visible lying adjacent to the lumen of the tubule, these needed only a short duct. In some of these type of cells, the contents of the end-apparatus was deeply stained (see Plate 5.8b). Other cells contained the usual type of cylindrical end-apparatus, these were positioned further from the lumen and connected to it by a longer secretory duct.

Throughout the accessory gland a few cells, that might have been undergoing lysis, were observed (see Plate 5.9). As far as could be established their outer cell membrane was intact and in some sections, led directly to the lumen of the tubule. However, the contents of these cells could not be observed in the lumen. Internally the cell contents appeared to be completely disrupted, containing large membrane-bound vesicles, some of which were probably the remains of mitochondria. In other vesicles, the contents and outer membranes contained electron-dense material, a type of structure which was not observed in intact cells. In other areas spiral masses of rough-surfaced endoplasmic reticulum were observed.

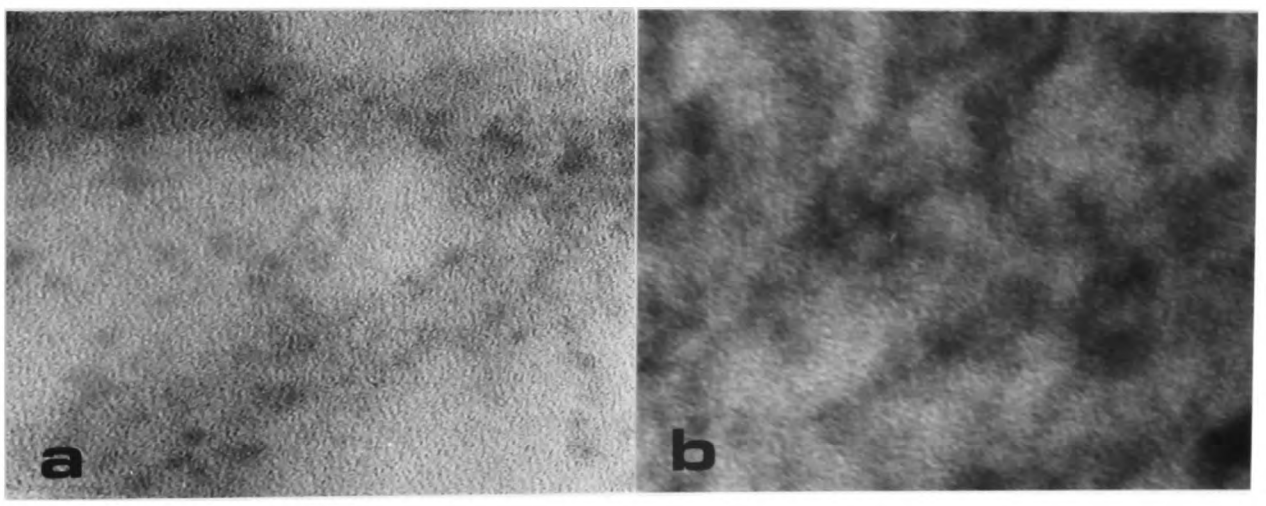


Plate 5.7. (a) Secretion from the tubule of the distal portion of the accessory gland of Pontania proxima. (b) Secretion contained within the colleterial sac. Fixation using Protocol two (Mag. x450K).

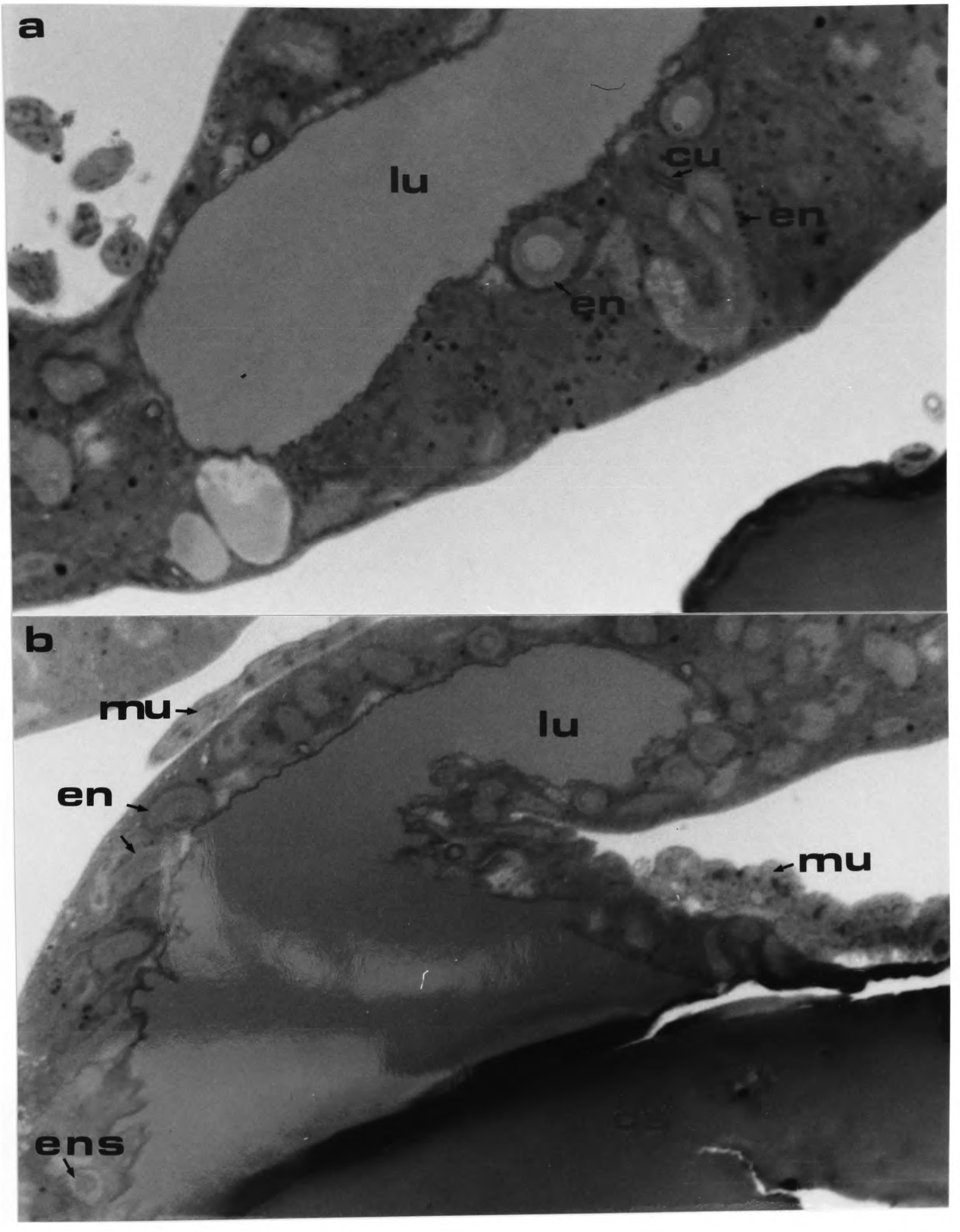


Plate 5.8. Sections through the accessory gland of Pontania proxima, just before it enters the colleterial sac. Thickness two microns. Fixation using protocol two. Stain p-phenylenediamine; cs. colleterial sac; cu. cuticle (lining duct); en. end-apparatus; ens. densely staining end-apparatus; lu. lumen (of accessory gland tubule); mu. muscle (Mag. (a) x1600, (b) x1066). Light microscopy.

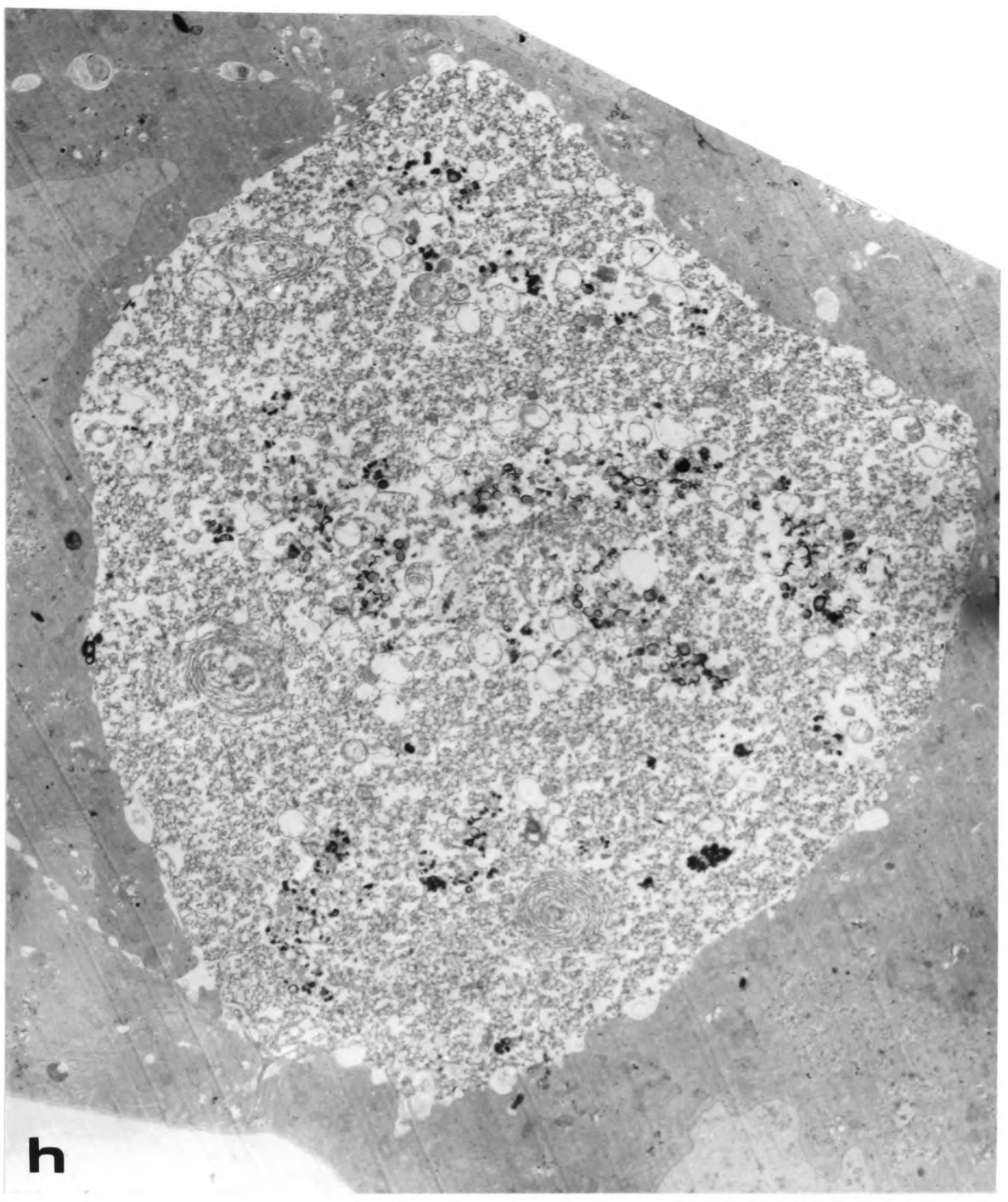


Plate 5.9. A cell from the accessory gland of Pontania proxima, seeming to be undergoing lysis. Fixation using Protocol two; h. haemolymph (Mag. x3750).

The colleterial sac contained no secretory cells, except in the region of the junction between it and the accessory gland (see Plate

5.8b). The wall of the sac was lined with cuticle throughout and at its entrance and exit this intima was folded and ridged. The wall consisted of a layer of epidermal cells, bounded externally by a thin layer of striated muscle, that covered over the entire sac. This muscle layer increased in thickness where the accessory gland entered the sac and where the colleterial duct led into the vaginal pouch.

c) Structure of the ovary and oviducts.

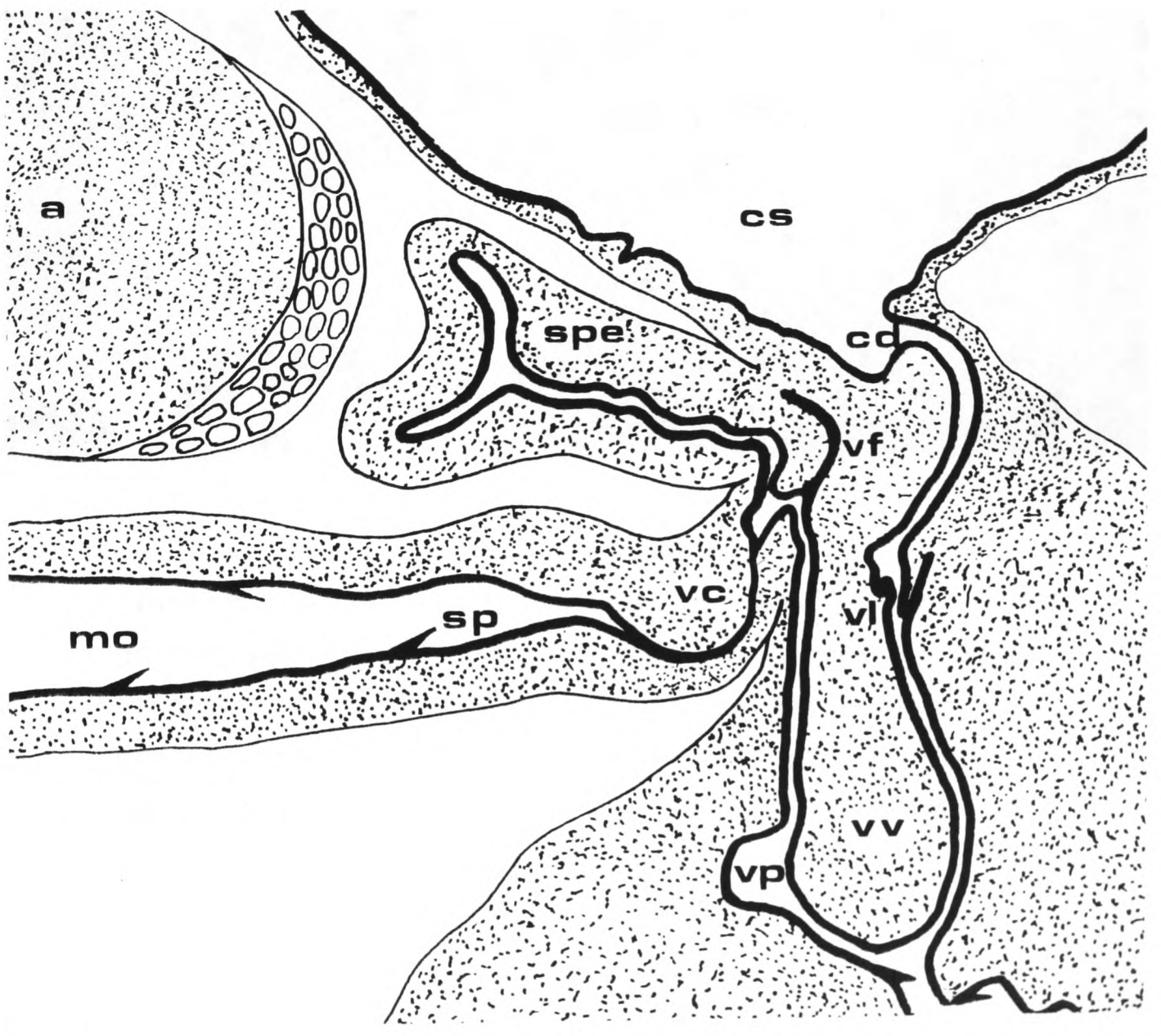


Figure 5.3. Simplified diagram showing the relative positions of the median oviduct, spermatheca, exit of the colleterial sac and vaginal pouch of Pontania proxima. a. abdominal ganglion; cd. colleterial duct; cs. colleterial sac; mo. median oviduct; sp. spine; spe. spermatheca; vl. vaginal lip; vc. vaginal clamp; vf. vaginal fold; vp. vaginal pouch (leading to the saw); vv. vaginal valve.

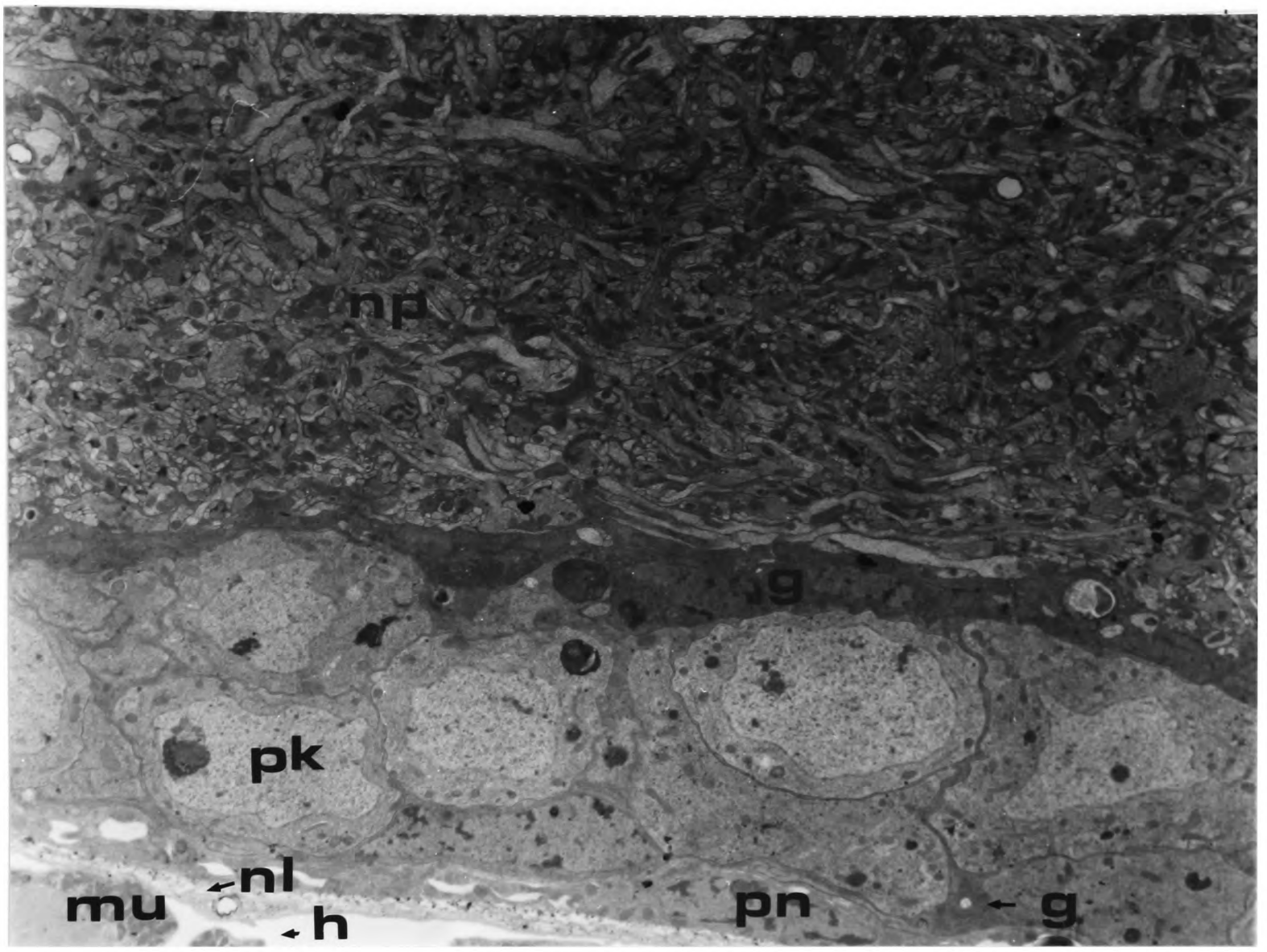


Plate 5.10. Wall of an abdominal ganglion lying on dorsal surface of median oviduct of Pontania proxima. Fixation using Protocol two; g. glial cells (with processes passing between cell bodies of neurons); h. haemolymph; mu. muscle; nl. neural lamella; np. neuropile; pk. perikaryon; pn. perineurium (Mag. x4630).

The ovaries of Pontania proxima were of the polytrophic type, with the trophocytes enclosed in a follicle, together with the developing oocyte. The trophocytes occupied the distal portion of the follicle and were connected to the oocyte by a nutritive pore (see Plate 5.11). The number of trophocytes associated with this species was not ascertained, but from casual observations the maximum number observed was 11.

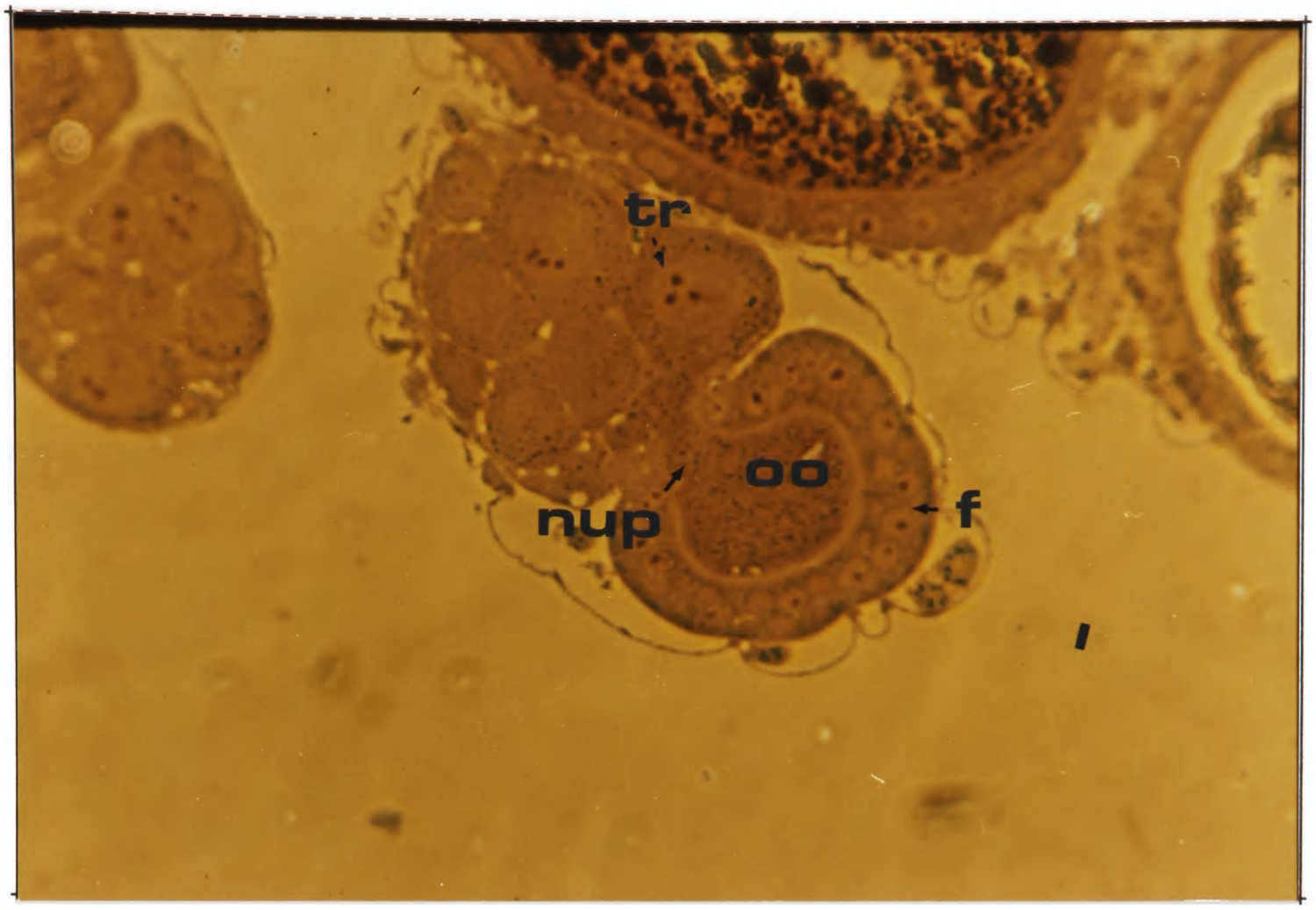


Plate 5.11. Developing oocyte with associated trophocytes of Pontania proxima. Thickness two microns. Fixation using protocol two. Stain p-phenylenediamine; f. follicular epithelium; nup. nutritive pore; oo. oocyte; tr. trophocytes (Mag. x570).

The fluids within the ovarioles, oviducts and vaginal pouch were all examined, using an electron microscope, for the presence of microorganisms, but none was found. However, there was evidence of a secretory rôle for the wall of the lateral oviducts.

The wall of each lateral oviduct was bounded, on its external surface, by a convoluted peritoneal membrane, under which were layers of striated muscle. The most apparent of these layers consisted of longitudinal muscle, but bands of muscle that encircled the oviduct, were periodically observed, and these were positioned exterior to the

longitudinal muscle. Both layers of muscle became thicker in the median oviduct, but again the longitudinal muscle formed the dominant layer.

Under the muscle layers was a single layer of cells that lined the lumina of the oviducts. Throughout the oviducts these cells were of a similar type. The basal portion of each was highly folded, presenting a large surface area to the overlying layers (see Plate 5.12), to which they were loosely connected. Their nuclei were varied in shape, with those in the distal portion of the lateral oviducts being more regular in shape than the others in the rest of the lateral oviduct and median oviduct, but all possessed numerous nucleoli, some of which were associated with the nuclear envelope. The cells themselves were tightly bound to each other; both septate desmosomes and junctions of the macula adhaerens type were observed together with tight junctions and these were especially frequent in the apical regions of the lateral cell membranes. Internally the cells of the lateral and median oviducts contained smooth-surfaced endoplasmic reticulum, golgi complexes, mitochondria and numerous vesicles (see Plate 5.13). On their apical surface were numerous microvilli, which in the median oviduct contained mitochondria (see Plate 5.15). These may have also been present in the microvilli of the lateral oviduct but were only observed in the microvilli near the junction between the cuticle-lined and non-lined areas. Mitochondria were more numerous in the cells of the common oviduct, than in the lateral oviducts, as was the amount of rough-surfaced endoplasmic reticulum.

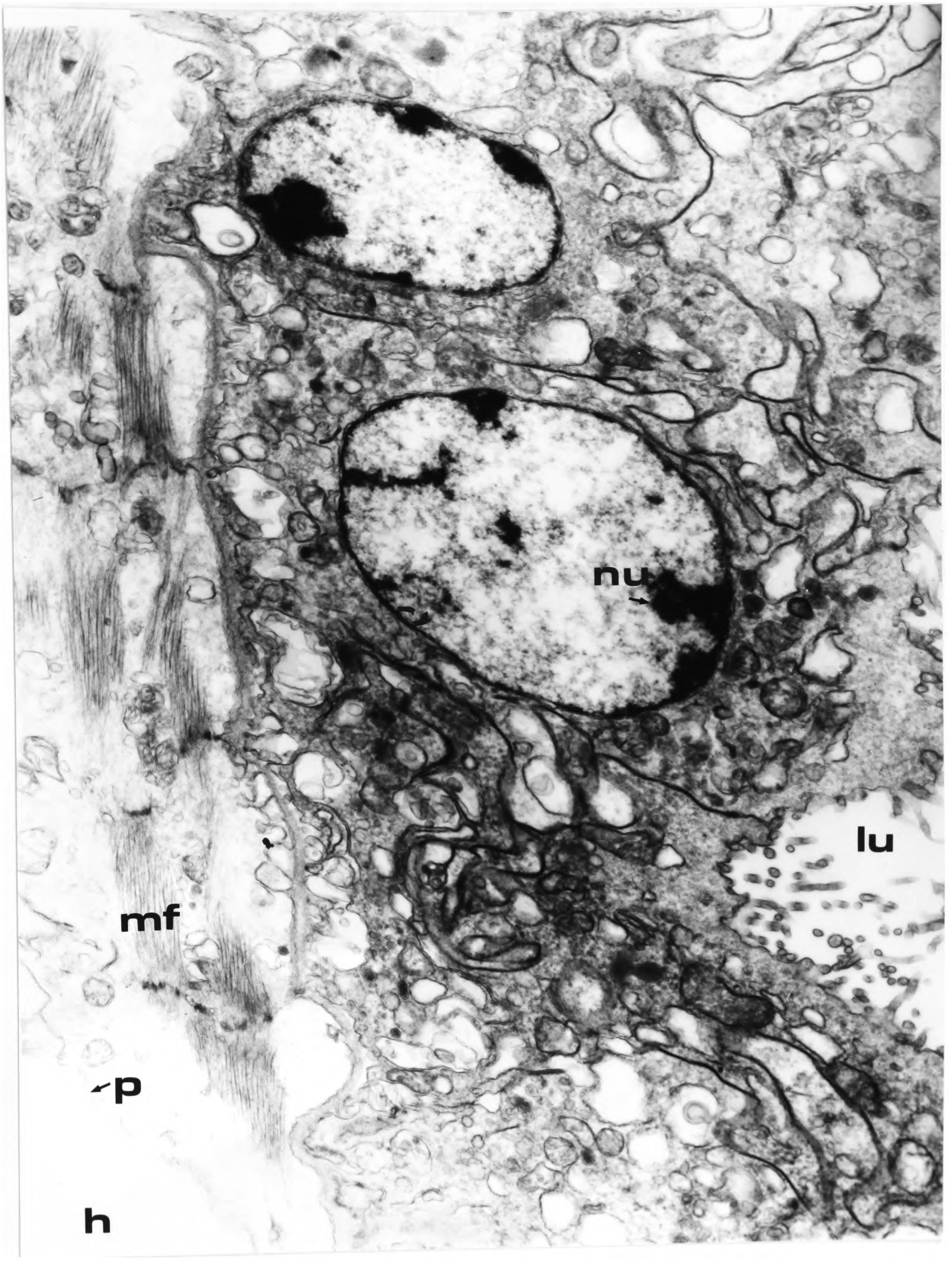


Plate 5.12. Longitudinal section through the wall of the distal portion of the lateral oviduct of Pontania proxima. Fixation using Protocol one; mf. muscle fibrils; h. haemolymph; lu. lumen (of oviduct); nu. nucleolus; p. peritoneal membrane (Mag. x12500).

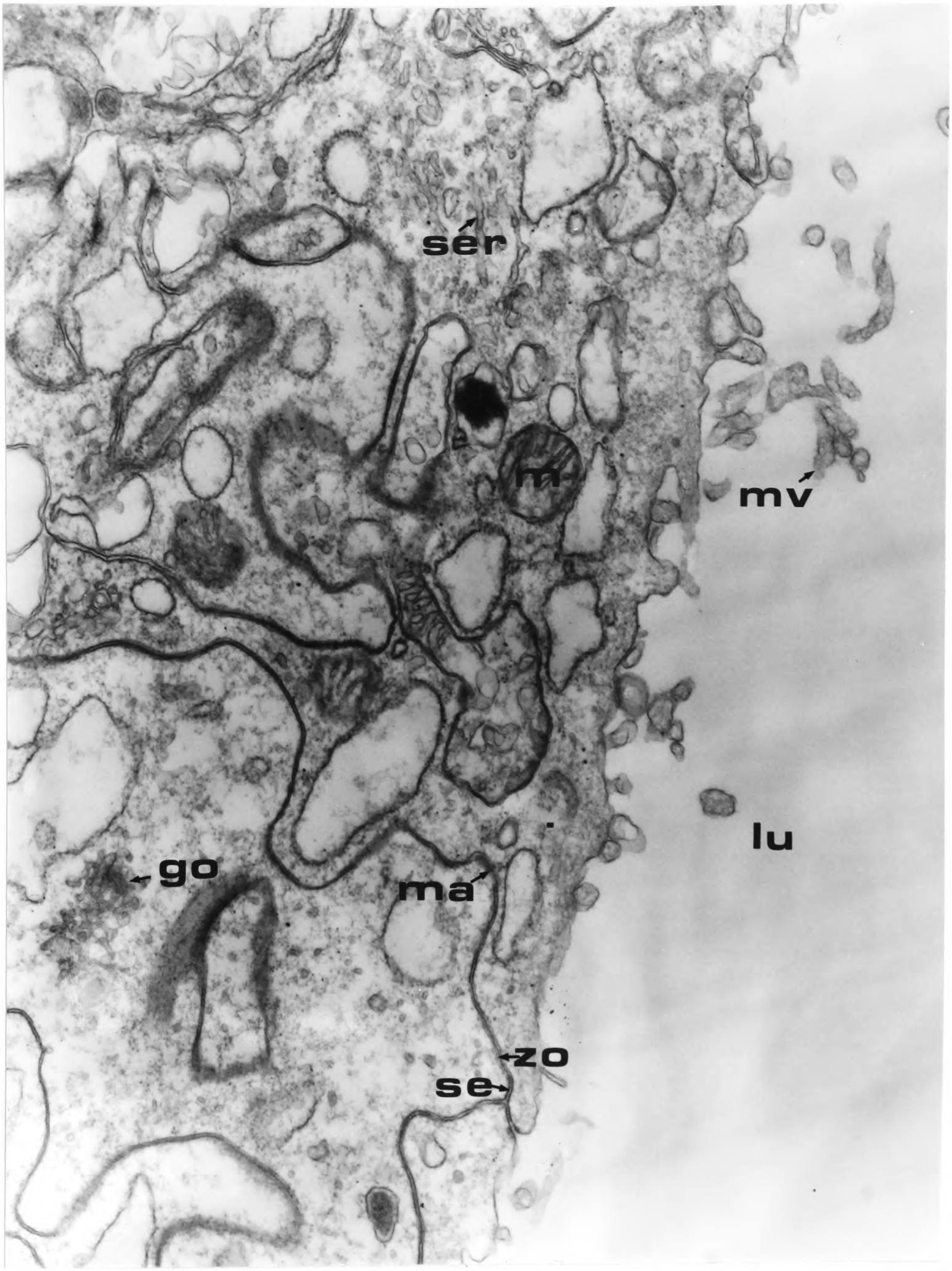


Plate 5.13. Apical surface of cells lining the distal portion of the lateral oviducts of Pontania proxima. Fixation using Protocol one; go. golgi complex; lu. lumen (of oviduct); m. mitochondrion; ma. desmosome (macula adhaerens); mv. microvilli; se. desmosome (septate); ser. smooth endoplasmic reticulum; zo. zonula occludens (Mag. x25K).

The apical surfaces of the cells of the median oviduct were bounded by a layer of cuticle, which contained occasional pores surrounded by thickened cuticle (see Plate 5.14). The cuticle intima began as a thin layer approximately half way along the lateral oviduct (see Plates 5.16 and 5.17) and was modified throughout the remaining regions of the reproductive tracts to form spines (see Plates 5.18 and 5.19), which increased in number, reaching a maximum in the proximal portion of the median oviduct.

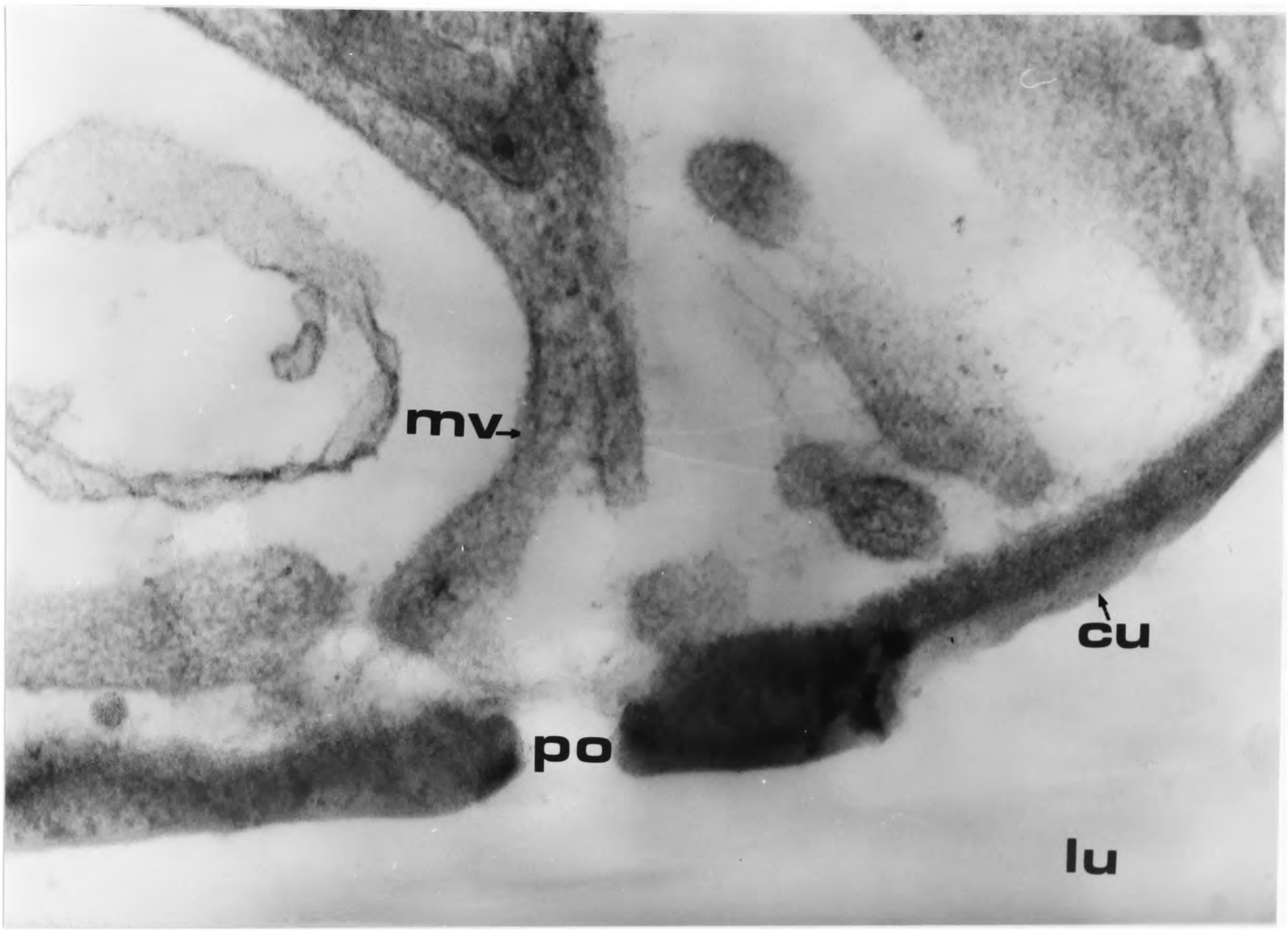


Plate 5.14. Pore in the cuticle lining of the lumen of the median oviduct of Pontania proxima. Fixation using Protocol two; cu. cuticle; lu. lumen (of oviduct); mv. microvilli; po. pore (Mag. x70K).

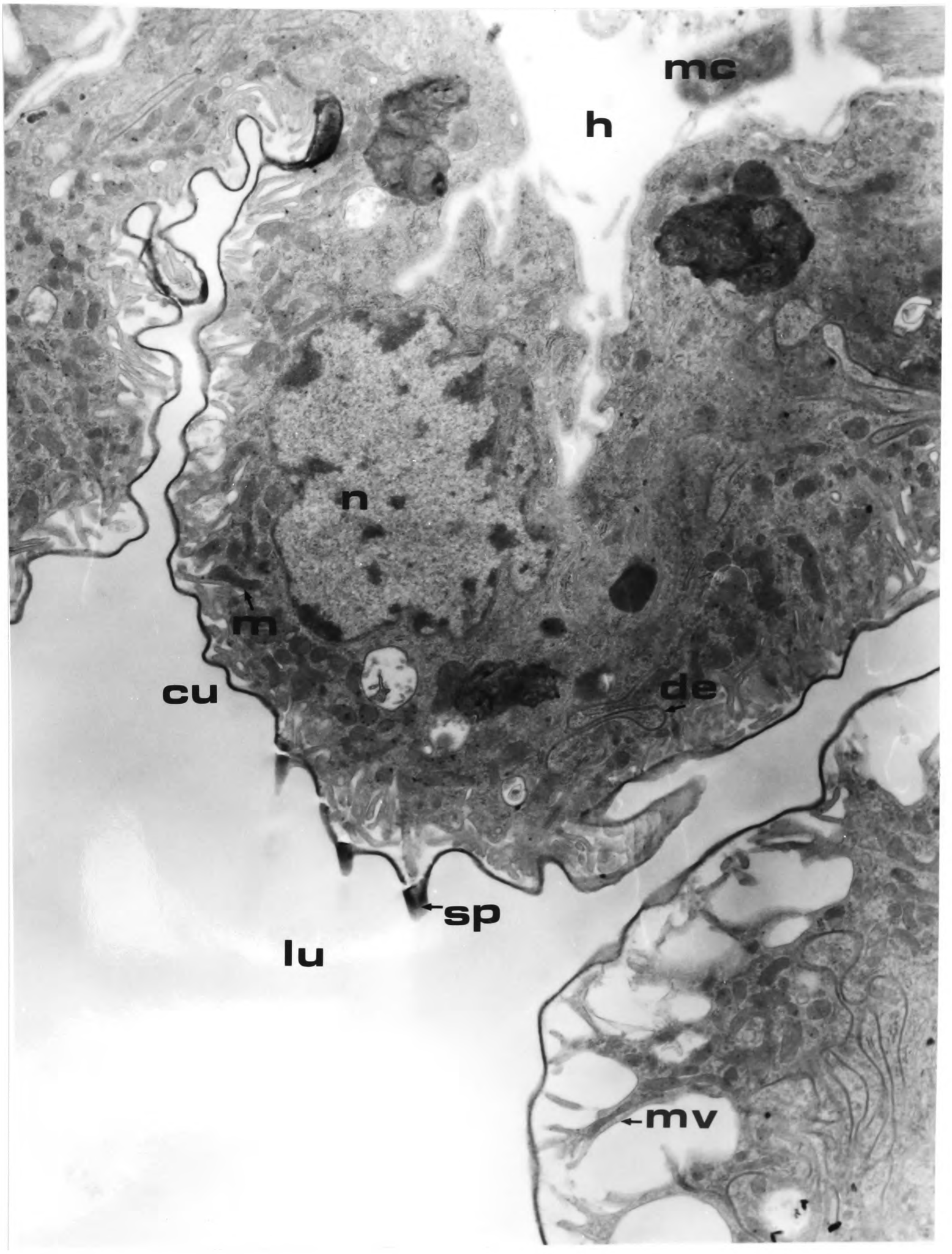


Plate 5.15. Wall of the median oviduct of Pontania proxima. Fixation using Protocol two; cu. cuticle; de. desmosome; h. haemolymph; mc. muscle cell; lu. lumen (of oviduct); m. mitochondrion; n. nucleus; sp. spine; mv. microvilli (containing mitochondria) (Mag. x11200).

Plate 5.16. Folded wall of the lateral oviduct of Pontania proxima, showing the start of the cuticle lining at(*). The micrograph shows a single portion of the oviduct folded back on itself. A comparison of the distal wall, without a cuticle, shows fewer mitochondria, more smooth endoplasmic reticulum and numerous vacuoles. The nucleus labelled n in the Plate was situated in the proximal region of lateral oviduct where the cuticle layer has thickened, and mitochondria are more numerous. Fixation using Protocol two; cu. cuticle; de. desmosome; h. haemolymph; lu. lumen (of lateral oviduct); m. mitochondrion; mf. muscle fibrils; mv. microvilli; n. nucleus; sp. spine (Mag. X11200).

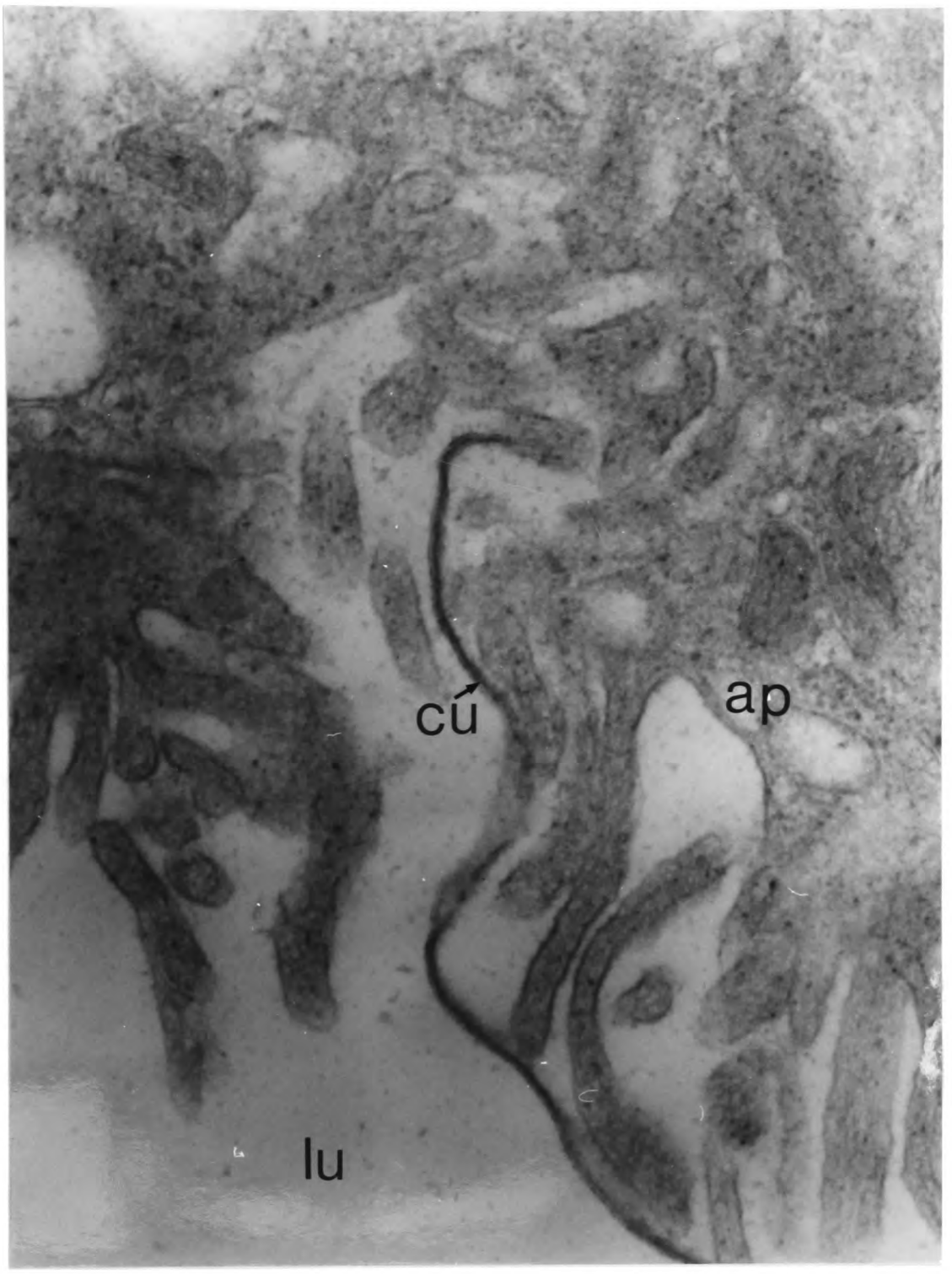


Plate 5.17. Junction at start of cuticle lining of lateral oviduct of Pontania proxima. Fixation using Protocol two; ap. apical surface (of cells lining the lateral oviduct); cu. cuticle; lu. lumen (of lateral oviduct) (Mag. x62.5K).

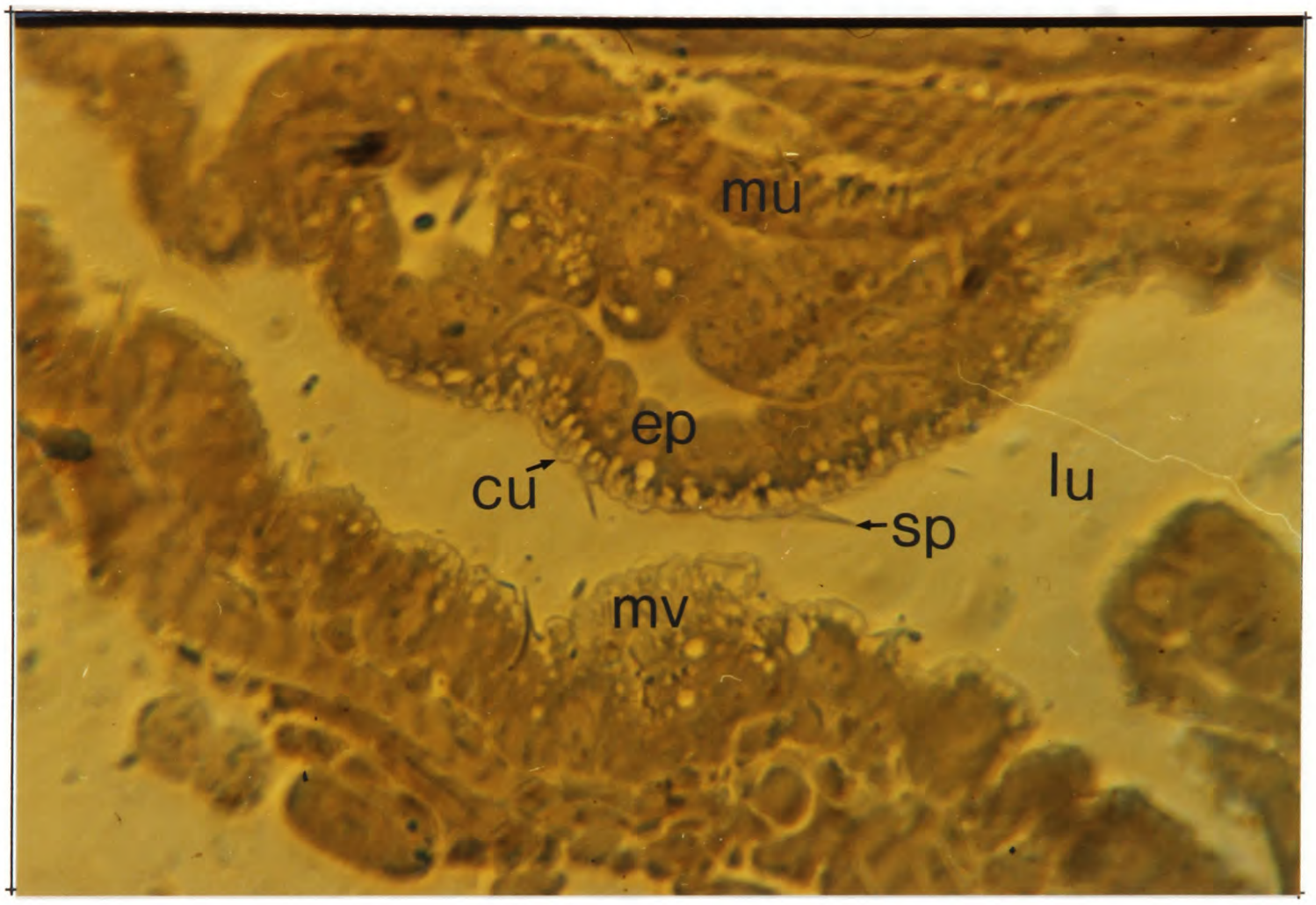


Plate 5.18. Section through the median oviduct of Pontania proxima. Thickness five microns. Fixation using Protocol two. Stain p-phenylenediamine; cu. cuticle; ep. epithelium (with microvilli at apical surface); lu. lumen of median oviduct; mu. muscle; mv. microvilli; sp. cuticular spine (Mag. x1400).

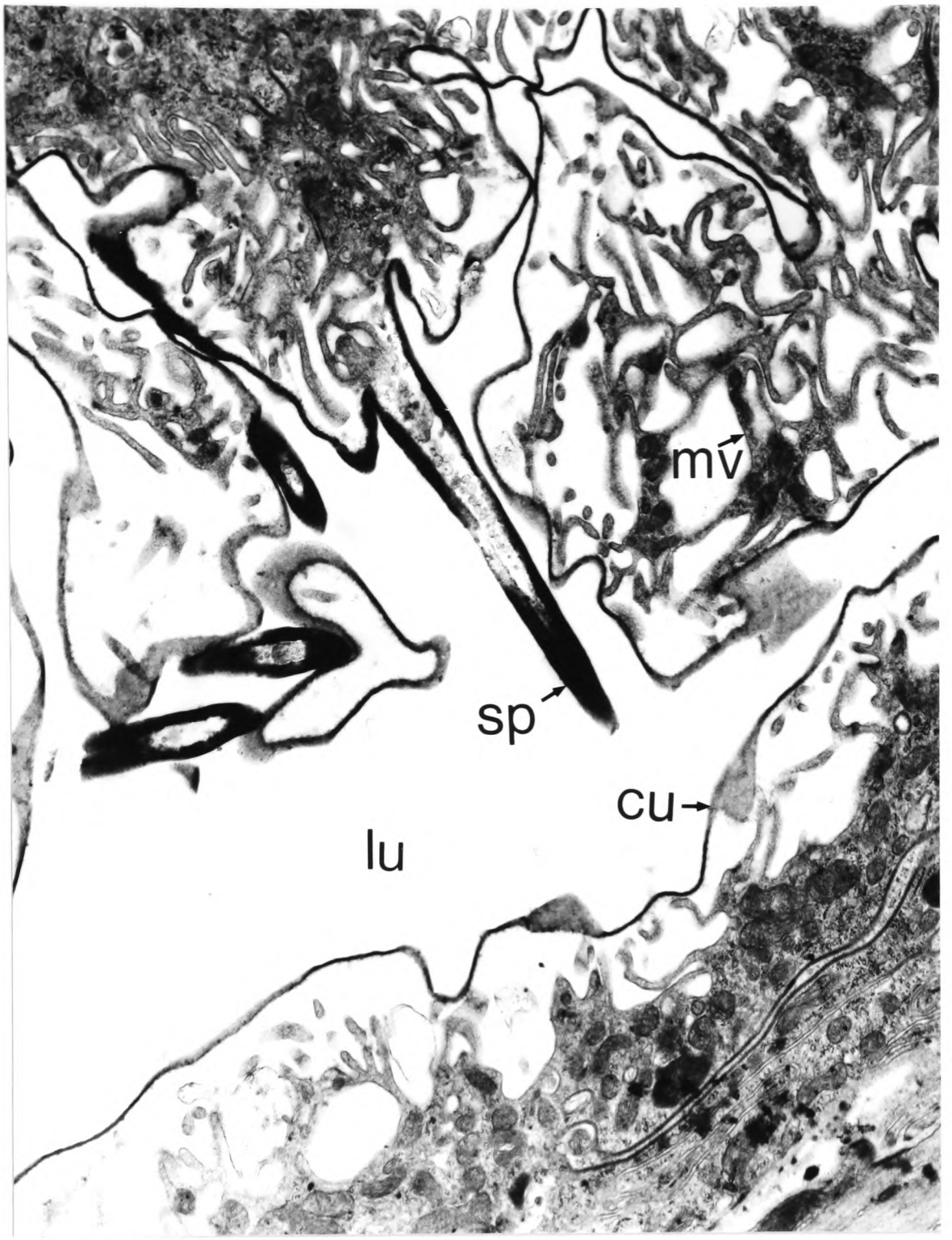


Plate 5.19. Cuticular spine in the median oviduct of Pontania proxima. Fixation using Protocol two; ap. apical surface (of cells lining the median oviduct); cu. cuticle; lu. lumen (of lateral oviduct); mv. microvilli; sp. spine (containing processes from microvilli) (Mag. x14.5K).

d) Vaginal pouch.

The exits from the oviduct, spermatheca and colleterial sac emptied into the vaginal pouch or genital chamber. The spermatheca (see Figure 5.3 and Plate 5.20) was small and lined with cuticle on its internal surface. In mid-longitudinal section the body of the spermatheca was crescent-shaped and followed the curve of its internal wall. However, at the start and end of a series of longitudinal sections through the organ it was circular in shape, suggesting that overall the spermatheca was bell-shaped. Transverse sections were not obtained to confirm this. No spermathecal gland was observed, but the epidermal cells adjacent to the cuticular intima were glandular in appearence and the lumen of the spermatheca stained more deeply than the interior of the oviducts (see Plate 5.20).

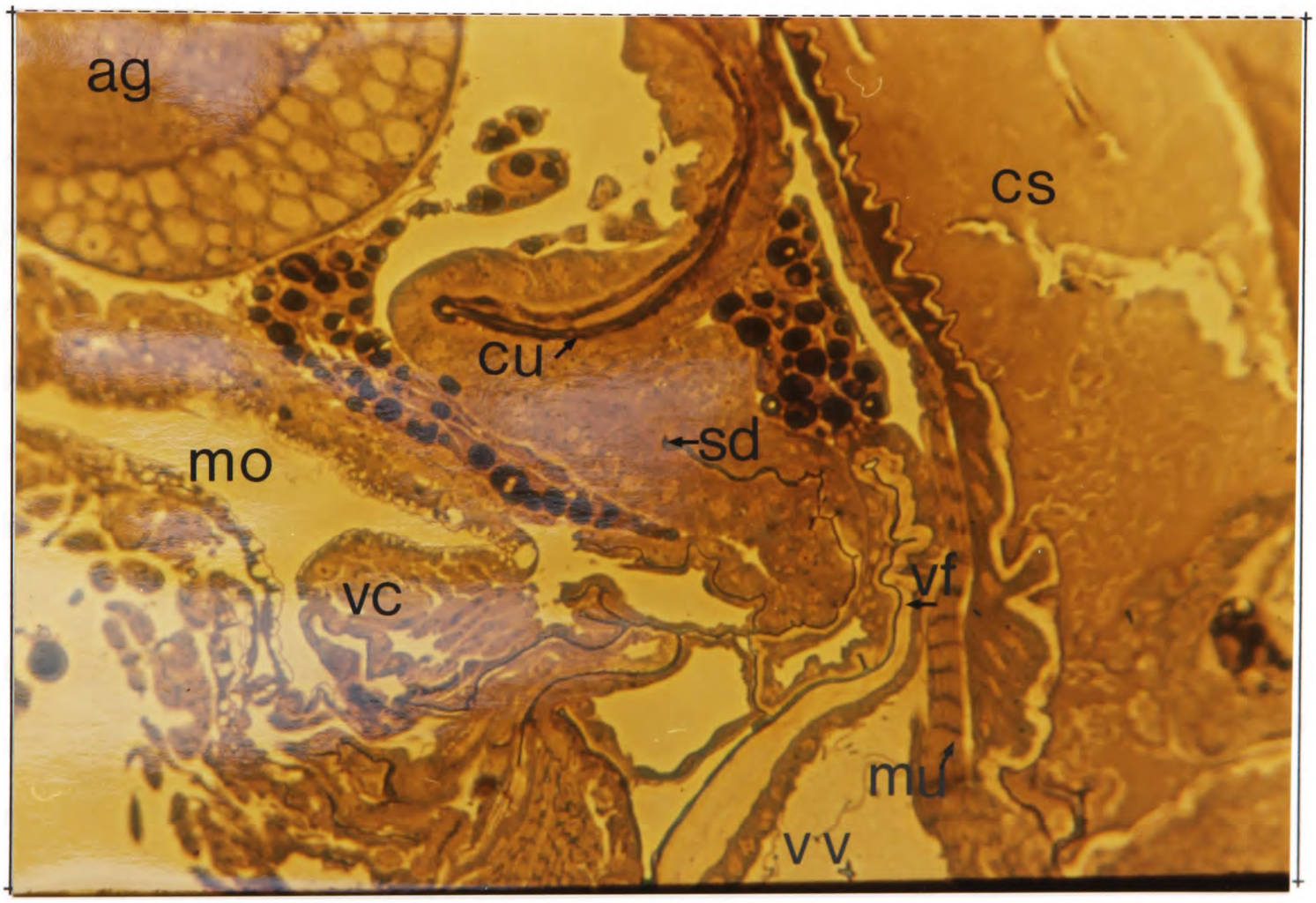


Plate 5.20. Section through the spermatheca of Pontania proxima. Thickness five microns. Fixation using Protocol two. Stain p-phenylenediamine; a. abdominal ganglion; cs. colleterial sac; cu. cuticle; mo. median oviduct; mu. muscle; sd. spermathecal duct; vc. vaginal clamp; vf. vaginal fold; vv. vaginal valve (Mag. x560).

The main regions of the vaginal pouch have been named using terms that aim to indicate a possible function. These functions will be described in the discussion to this Chapter. Plate 5.21 and Figure 5.3 (p. 132) illustrate the major structures in this area. Positioned ventrally to the spermatheca was the median oviduct, its proximal end was highly constricted and it was often difficult to trace a pathway to reach the vaginal pouch. Part of this constriction was caused by a muscular body, the vaginal clamp. At this position, on the ventral surface of the oviduct were thick bands of striated muscle.

Posterior to the vaginal clamp, the spermathecal duct and the median oviduct joined and led into the vaginal pouch. Most of this cavity was filled with a muscular organ, the vaginal valve, which was covered with a thick layer of cuticle. The muscles of this organ originated from a sheet of muscle on the dorsal surface of the spermatheca. From here they ran down past an infolding of the cuticle-lined epithelium, the vaginal fold and, in longitudinal sections, passed down the anterior and posterior internal surfaces of the vaginal valve. The muscle block in the posterior portion of the vaginal valve was much thicker than that in the anterior portion. Opposite to the vaginal fold was a small hook-like structure, the vaginal lip. This was situated adjacent to an infolding of the wall of the vaginal pouch which, at this point received the curved cuticle-lined duct leading from the colleterial sac. In Plate 5.21 the vaginal valve is positioned in the central region of the vaginal pouch. However, in sections from different specimens of Pontania proxima, contraction of either the anterior or posterior bands of muscles resulted in a distortion of its shape. Plate 5.22 shows the upper part of the posterior muscles contracted, pulling the base of the vaginal

valve upwards into a position where the lip of the valve connects with the infolding of the wall of the vaginal pouch. When in this position the vaginal valve seemed to close the duct leading from the colleterial sac. In other sections the vaginal valve was positioned so that the colleterial duct was more open; in this position the vaginal valve was pushed hard against the openings of the spermatheca and the oviduct.

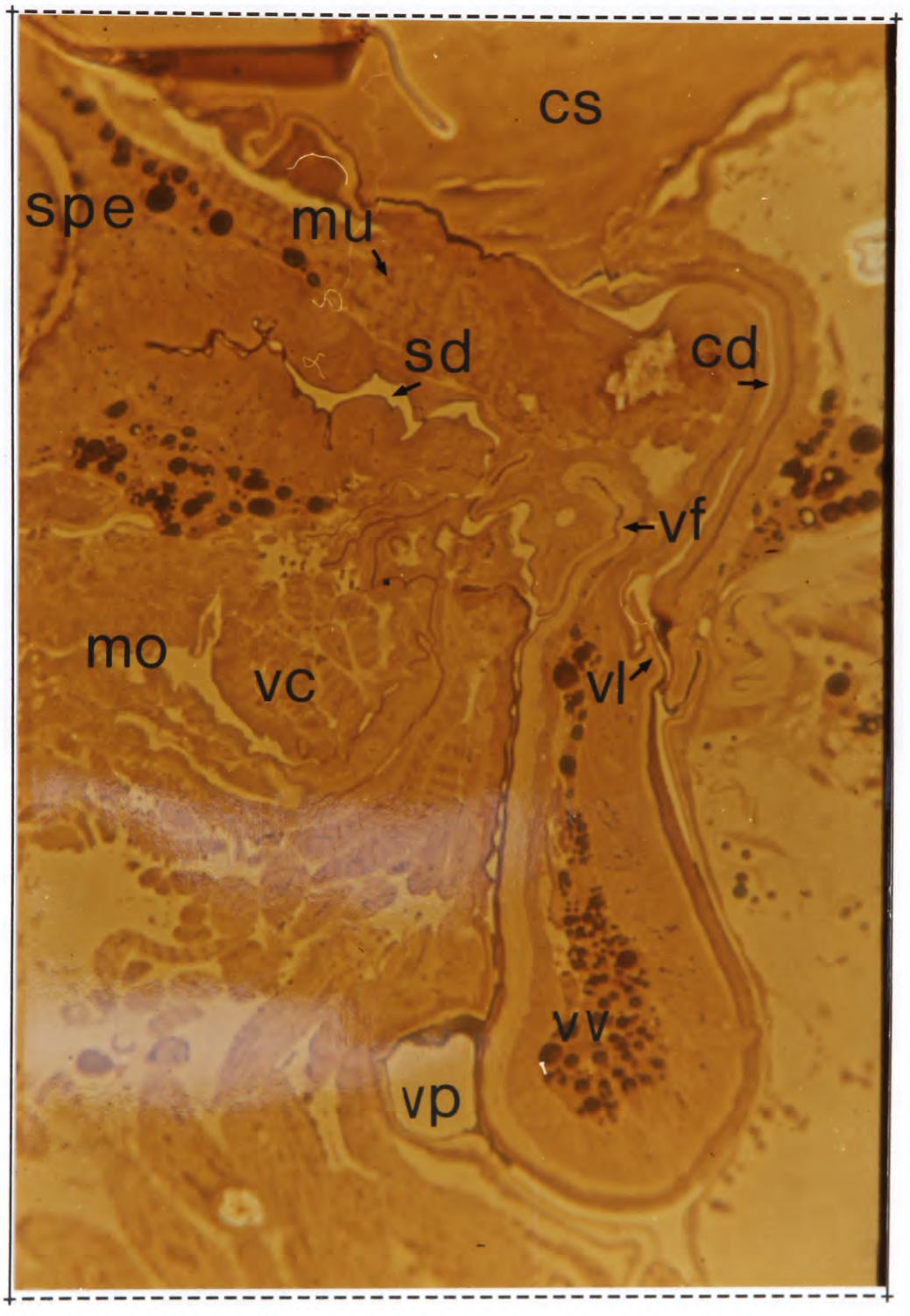


Plate 5.21. Section through the vaginal pouch region of Pontania proxima. Thickness two microns. Fixation using Protocol two. Stain p-phenylenediamine; cd. colleterial duct; cs. colleterial sac; mo. median oviduct; mu. muscle; sd. spermathecal duct; spe. spermatheca; vc. vaginal clamp; vf. vaginal fold; vl. vaginal lip; vp. vaginal pouch; vv. vaginal valve (Mag. x515).

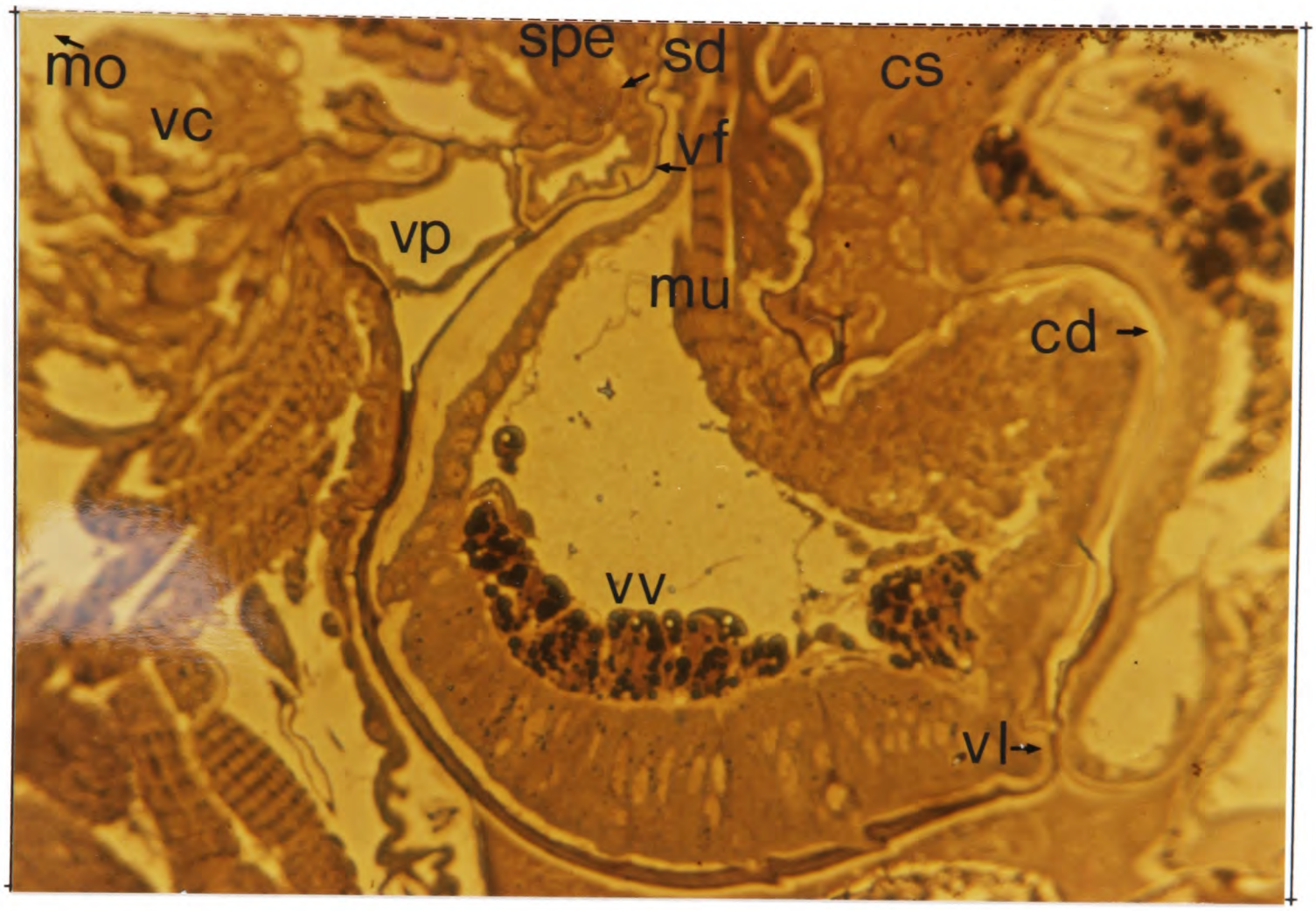


Plate 5.22. Section through the vaginal pouch region of Pontania proxima showing the change in shape of the vaginal valve, so possibly closing the colleterial duct. Label vp corresponds to the same area in Plate 5.21. Thickness five microns. Fixation using Protocol two. Stain p-phenylenediamine; cd. colleterial duct; cs. colleterial sac; mo. median oviduct; mu. muscle; sd. spermathecal duct; spe. spermatheca; vc. vaginal clamp; vf. vaginal fold; vl. vaginal lip; vp. vaginal pouch; vv. vaginal valve (Mag. x560).

5.3.2 The gall.

a) The young gall.

At the time of oviposition, the structure of the leaf of Salix fragilis was slightly less developed than that shown in Plate 5.23. Usually there were seven to eight layers of cells, each layer undergoing both periclinal and anticlinal divisions. The main features of the young leaf were a layer of provascular tissue above a single layer of mesophyll (lower parenchymal layer or subepidermal layer) that developed into the spongy parenchyma tissue. For a normal leaf most divisions were anticlinal, growth occurring mainly along the axis of the leaf. Periclinal divisions did occur, but a fully expanded leaf was only ten to 11 cells thick, with a single layer of cells forming the upper epidermis, two to three layers forming the palisade parenchyma, approximately five layers forming the spongy parenchyma and a single layer forming the lower epidermis. The outer leaves of a bud took, on average, 15 days to reach maturity (see Appendix Seven).

Plate 5.24 is of the same leaf as in Plate 5.23, but shows the first stages of gall development, approximately 24-36 hours after oviposition by Pontania proxima (a gall of similar age is shown in Plate 5.33). During this time all the cell layers had undergone periclinal divisions, locally increasing the leaf's thickness to approximately 37 cells. Hypertrophy of cells away from the cavity containing the egg had occurred and was especially prevalent at the site of vascular tissue. The egg had been inserted from the abaxial side the leaf, into the provascular tissue. It was horizontal to the surfaces of the leaf and lay nearer the adaxial surface.

Plate 5.23. Leaf tissue 24 to 36 hours after oviposition by Pontania proxima, but away from its site. Thickness 0.5 microns. Fixation using Protocol one. Stain toluidine blue; le. lower epidermis; lp. lower parenchymal layer; pp. palisade parenchyma; pr. provascular tissue; ue. upper epidermis (Mag. x400).

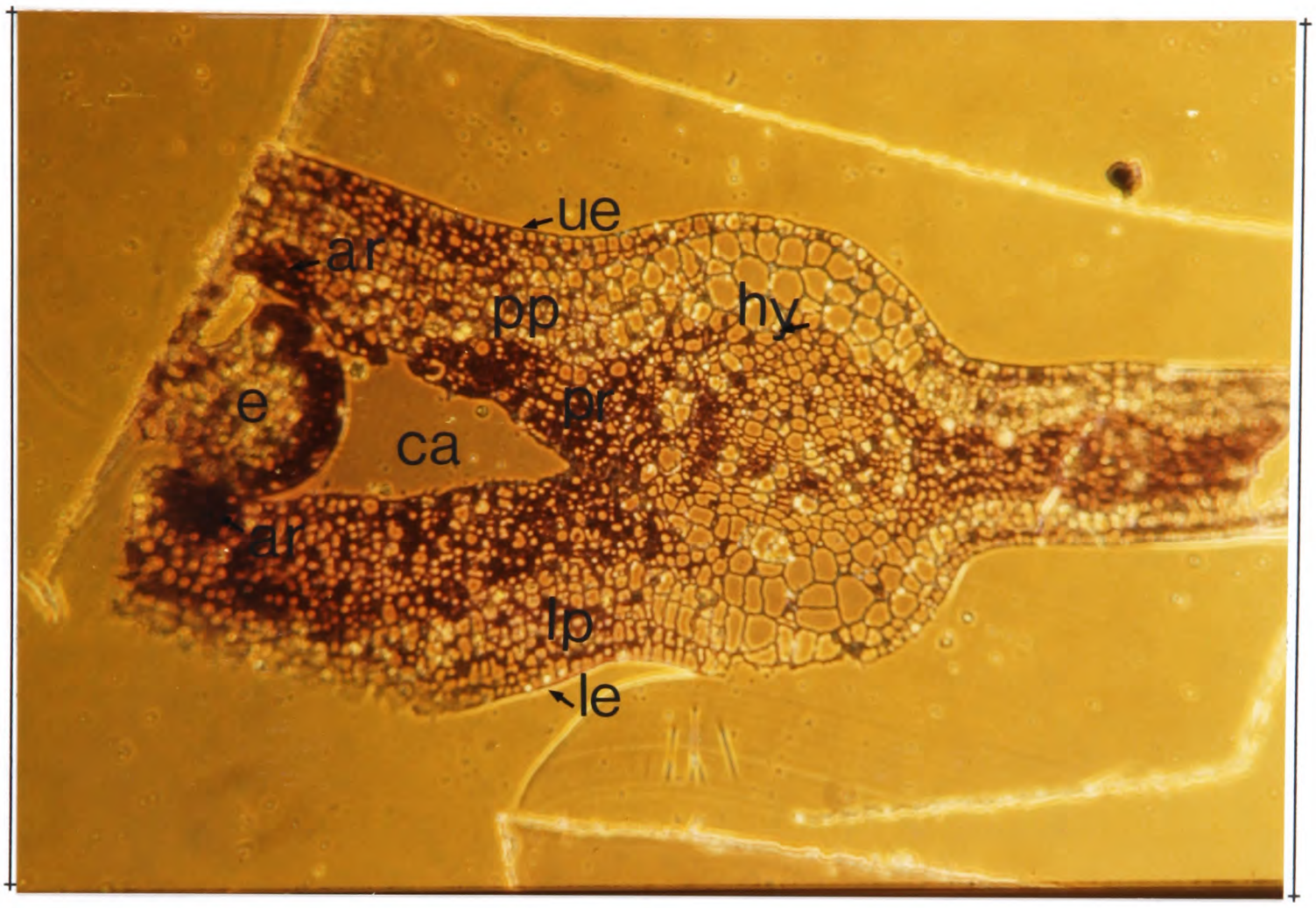


Plate 5.24. Leaf tissue 24 to 36 hours after and at the site of oviposition by Pontania proxima. Thickness 0.5 microns. Fixation using Protocol one. Stain toluidine blue; ar. area of rapid cell division; ca. cavity; e. egg; hy. hypertrophy of vascular tissue; le. lower epidermis; lp. lower parenchymal layer; pp. palisade parenchyma; pr. provascular tissue; ue. upper epidermis (Mag. x140).

Within the leaf, the most active area of cell division was in the provascular tissue where areas of greater meristematic activity were noted adjacent to the ends of the egg, where it was in contact with the leaf tissue (see Plate 5.25 and 5.26). Divisions were mainly periclinal and there was no merging of the different tissues within the leaf. Therefore, files of cells were produced that ran perpendicular to the cavity and did not radiate from the egg.

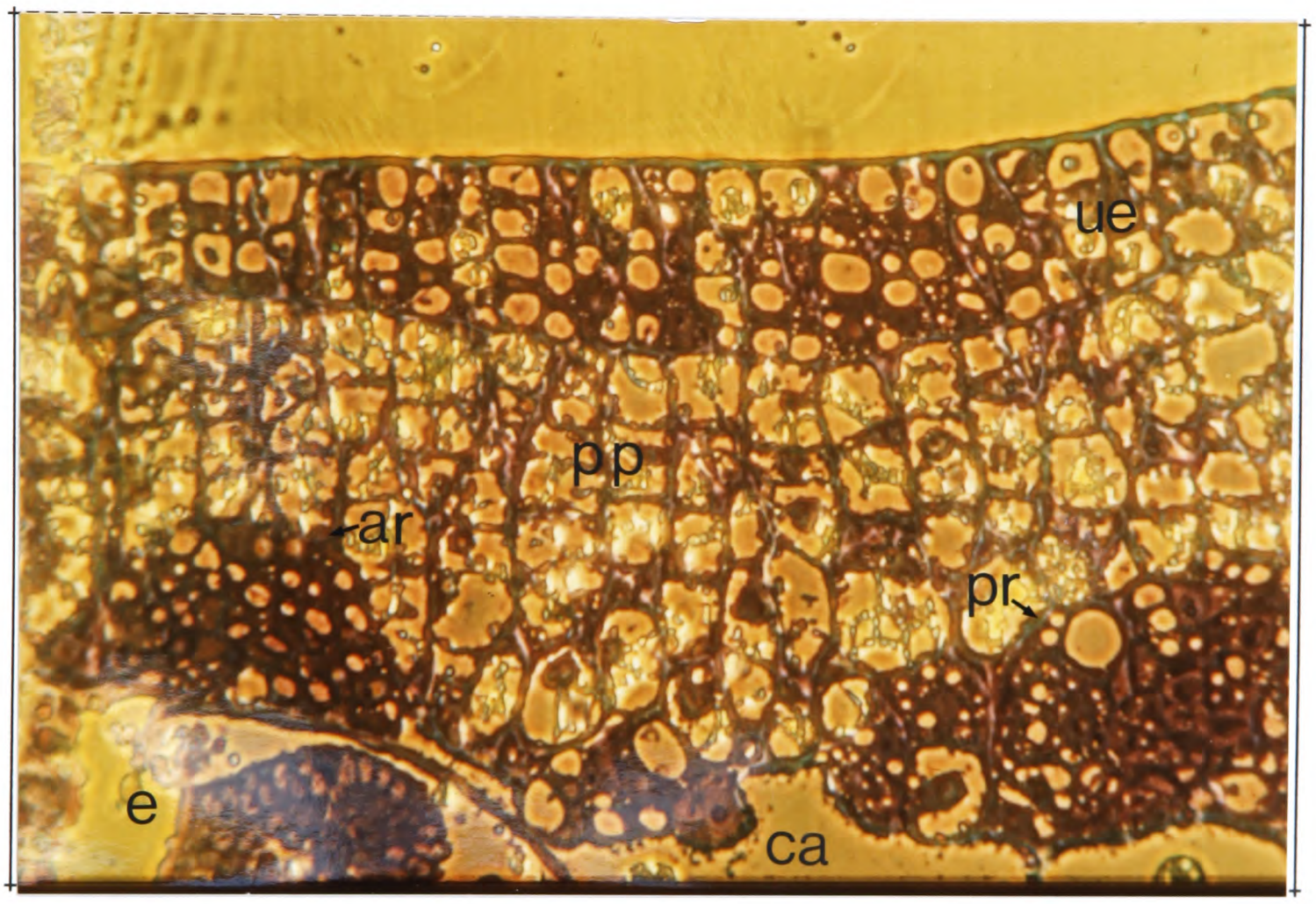


Plate 5.25. Leaf tissue 24 to 36 hours after and at the site of oviposition by Pontania proxima. Thickness 0.5 microns. Fixation using Protocol one. Stain toluidine blue; ar. area of rapid cell division; ca. cavity; e. egg; pp. palisade parenchyma; pr. provascular tissue; ue. upper epidermis (Mag. x400).

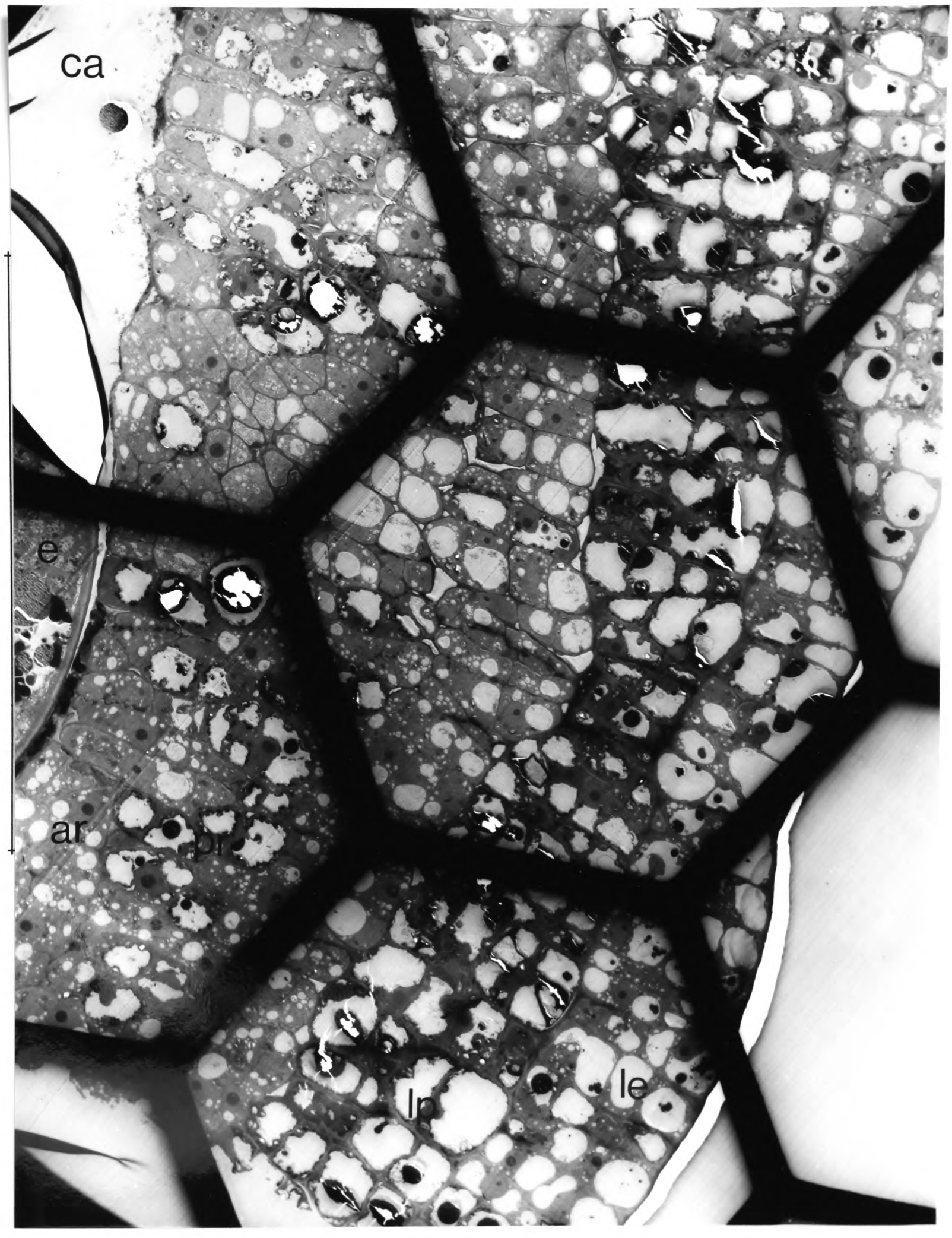


Plate 5.26. Leaf tissue 24 to 36 hours after and at the site of oviposition by Pontania proxima; ar. area of rapid cell division; ca. cavity; e. egg; le. lower epidermis; lp. lower parenchymal layer; pr. provascular tissue (Mag. x750).

A survey of the cavity and surrounding tissues was made for microorganisms, but none was found, neither was there evidence of colleterial fluid within the cavity. However, laid against the sides of the cavity and at its deepest part was electron-dense material, different in structure from that observed in the intercellular spaces of normal leaf tissue. Other fibrillar material was seen between the cells lining the cavity. Some cells adjacent to the egg seemed compressed, with their cell walls distorted (see Plate 5.27), to a greater extent than in normal leaf tissue of the same age (see Plate 5.29b).

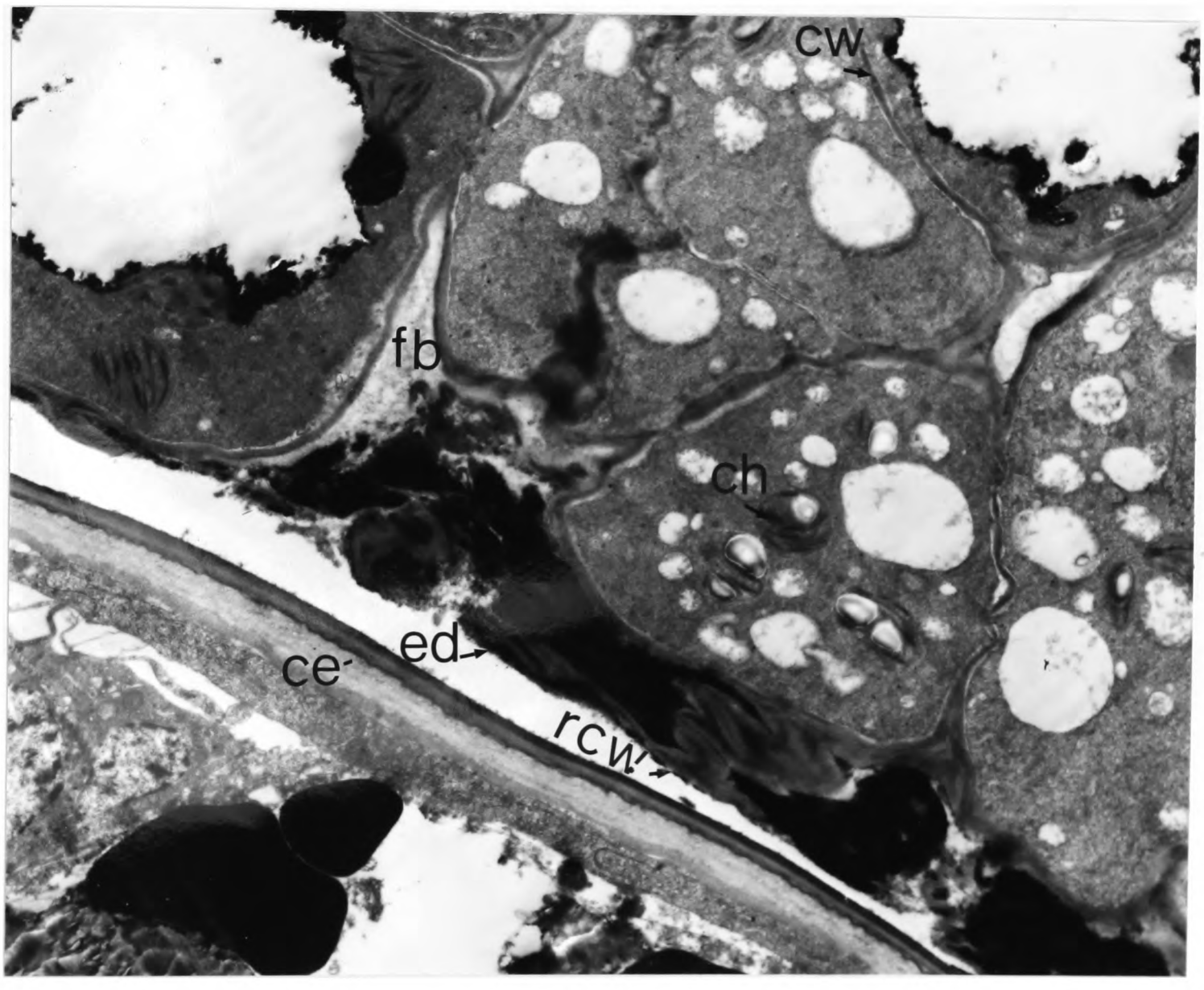


Plate 5.27. Provascular cells lining the cavity adjacent to the egg; ce. chorion of egg; ch. chloroplasts; cw. cell wall; ed. electron-dense material; fb. fibrillar material; rcw. remains of cell wall (Mag. x5000).

Sections through eggless procecidia confirmed that the presence of an egg was not necessary for the basic pattern of gall formation to occur (see Plate 5.28). The tissues involved demonstrated a similar degree of division, with the different layers maintaining their respective orientations and position. However, the patterns of division were less organized. The files of cells were not as distinct and the regions of greater meristematic activity were more randomly orientated.

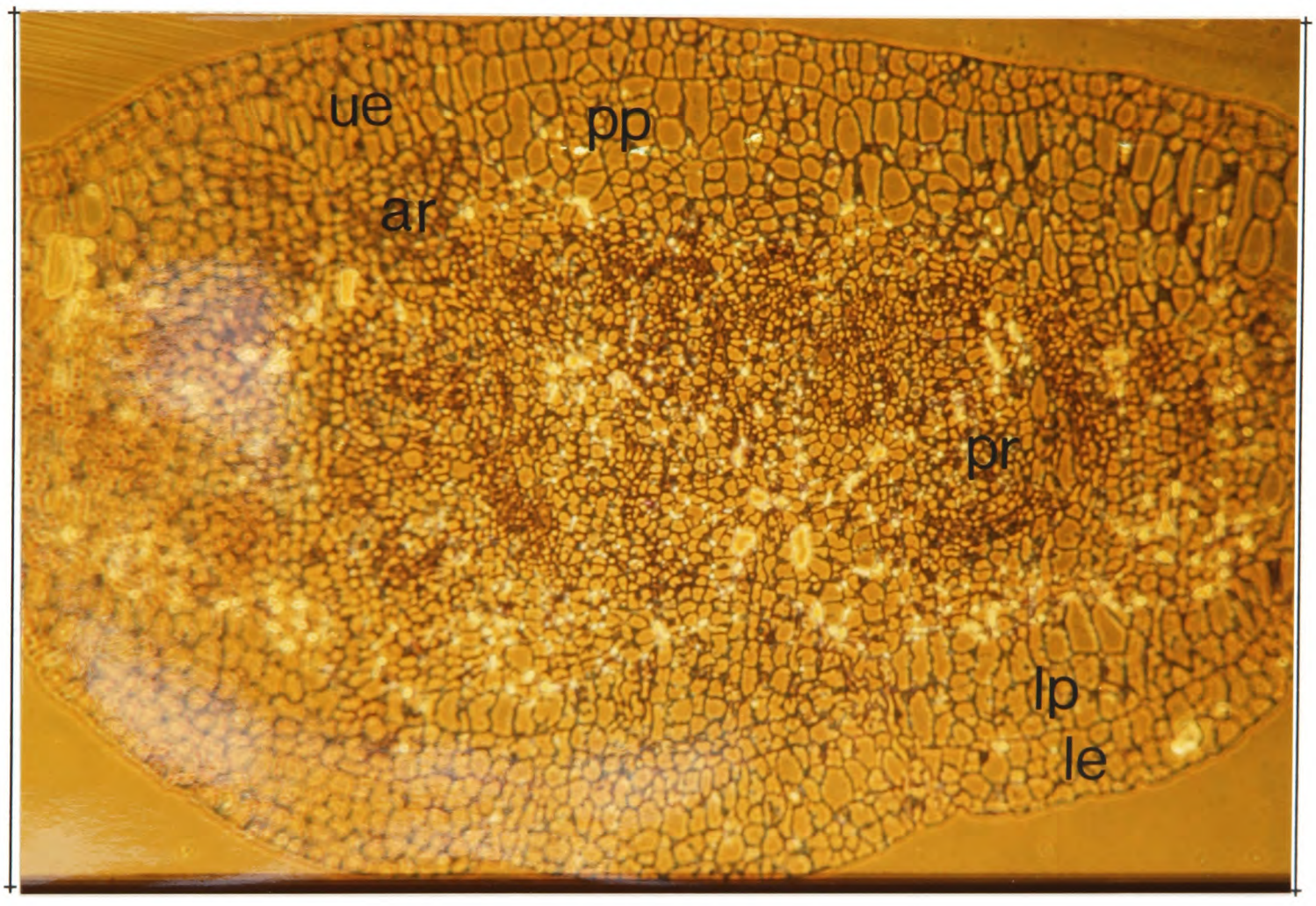


Plate 5.28. Eggless procecidium after an abortive attempt at oviposition by Pontania proxima; ar. area of rapid cell division; le. lower epidermis; lp. lower parenchymal layer; pp. palisade parenchyma; pr. provascular tissue; ue. upper epidermis (Mag. x140).

b) The chloroplasts.

Chloroplast development was compared with that in normal leaf tissue. The young chloroplasts (see Plate 5.27 and Plate 5.29) demonstrated

poor delineation of the plastid envelope and reversed staining of the thylakoids, fairly common features which may be due to the physiological state of the plastid membranes. These features have been observed in other species e.g. Theobroma cacao L. (Sterculiaceae).

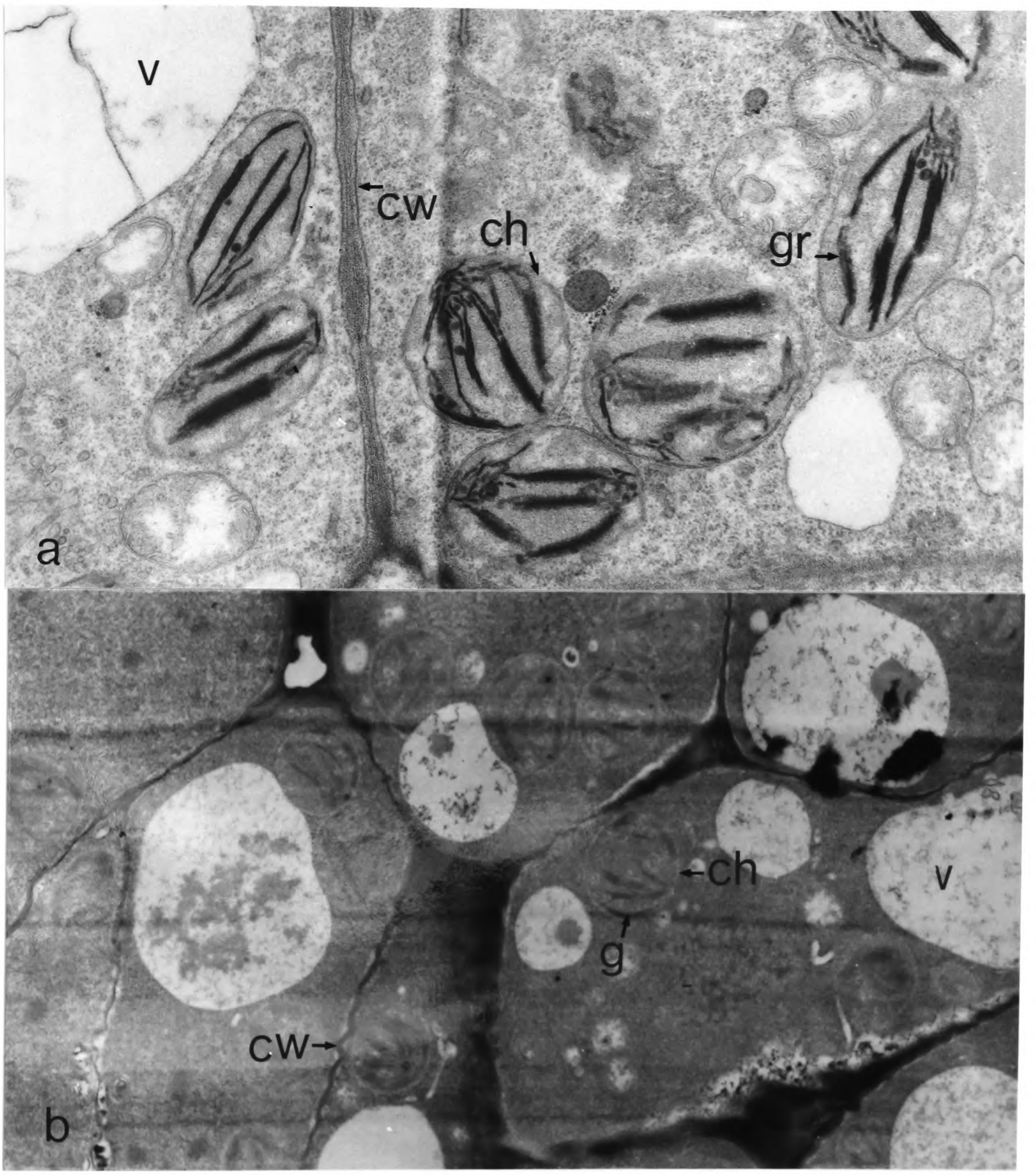


Plate 5.29. (a) Young chloroplasts developing in leaf tissue of Theobroma cacao (Mag. x22000). Reproduced by kind permission of Dr. J. Whatley, University of Oxford. (b) Young chloroplasts developing in normal leaf tissue of Salix fragilis (Mag. x7500); ch. chloroplasts; cw. cell wall; gr. granum; v. vacuole.

From about 60 hours after oviposition, it was observed that the chloroplasts of the central region of the gall contained electron transparent globules and invaginations of the inner envelope (see Plate 5.30). These chloroplasts had a structure similar to those observed by Rey (1972, 1973 and 1974), who suggested that these artifacts were a feature of gall development. However, chloroplasts like this can be found in other species (Whatley, personal communication, see Plate 5.31). Leaf chloroplasts in Photinia x fraseri, Dress `Red Robin' (Rosaceae) show the same features including poor clarity of plastid membranes. These features are probably fixation artifacts and are common in plastids from leaves with a high content of phenolics.

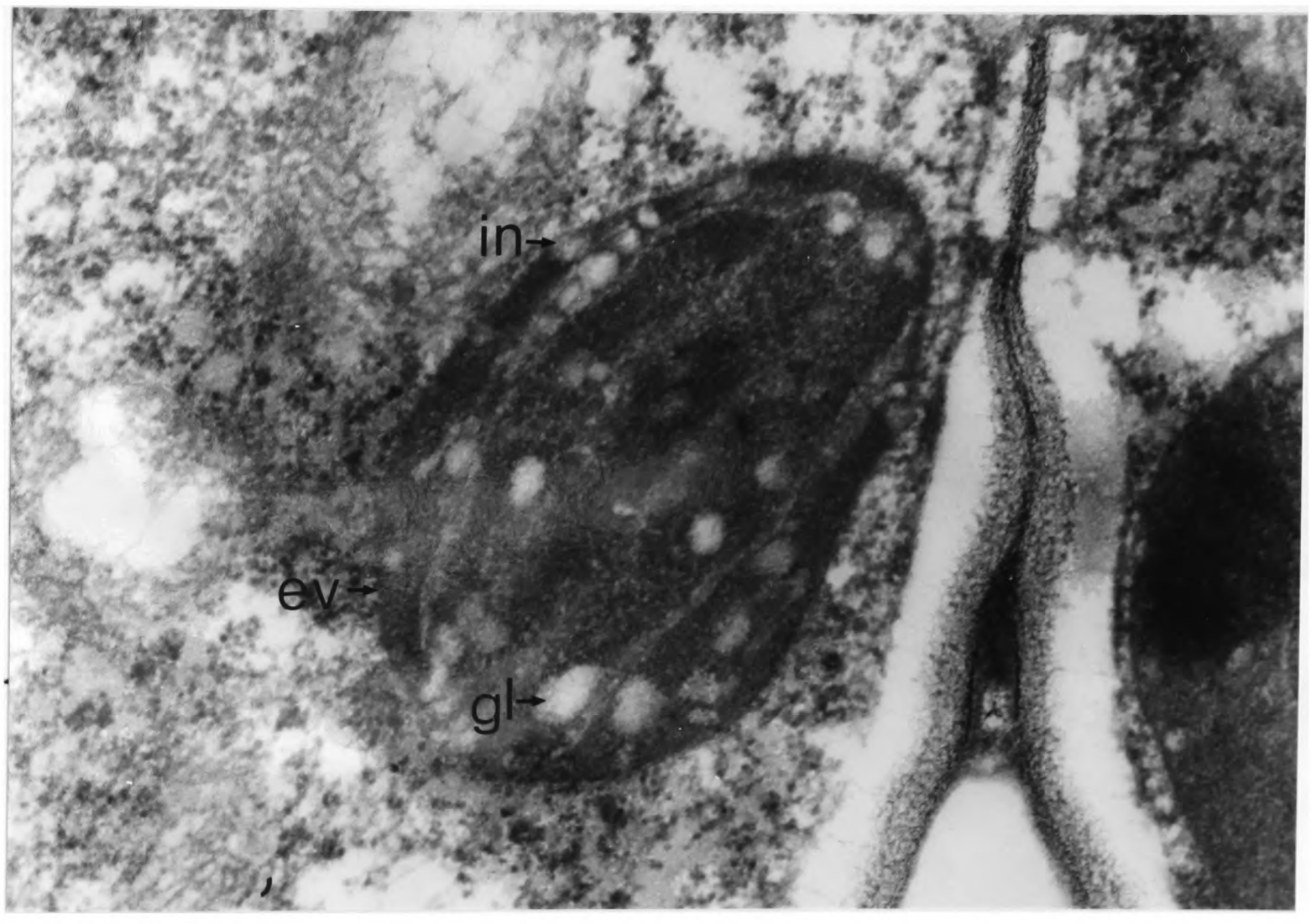


Plate 5.30. Chloroplast of a cell from the central region of the gall after approximately 60 hours of development; ev. plastid envelope; gl. globule; in. invagination (Mag. x50000).

Plate 5.31. Chloroplast of Photinia x fraseri 'Red Robin', showing the same characteristic as those of a young chloroplast in the gall of Pontania proxima; cw. cell wall; ev. plastid envelope; gl. globule; in. invagination (Mag. x50000). Reproduced by kind permission of Dr. J. Whatley, University of Oxford.

Fully-developed gall cells contained chloroplasts with a well preserved structure. They were unusual for mature chloroplasts, in that they were pleomorphic in shape, rather than discoid, and contained cytoplasmic intrusions, but no starch granules. The cells themselves were diametric in shape and surrounded by large air spaces. It was these gall cells that formed the inner nutritional layer of the gall.

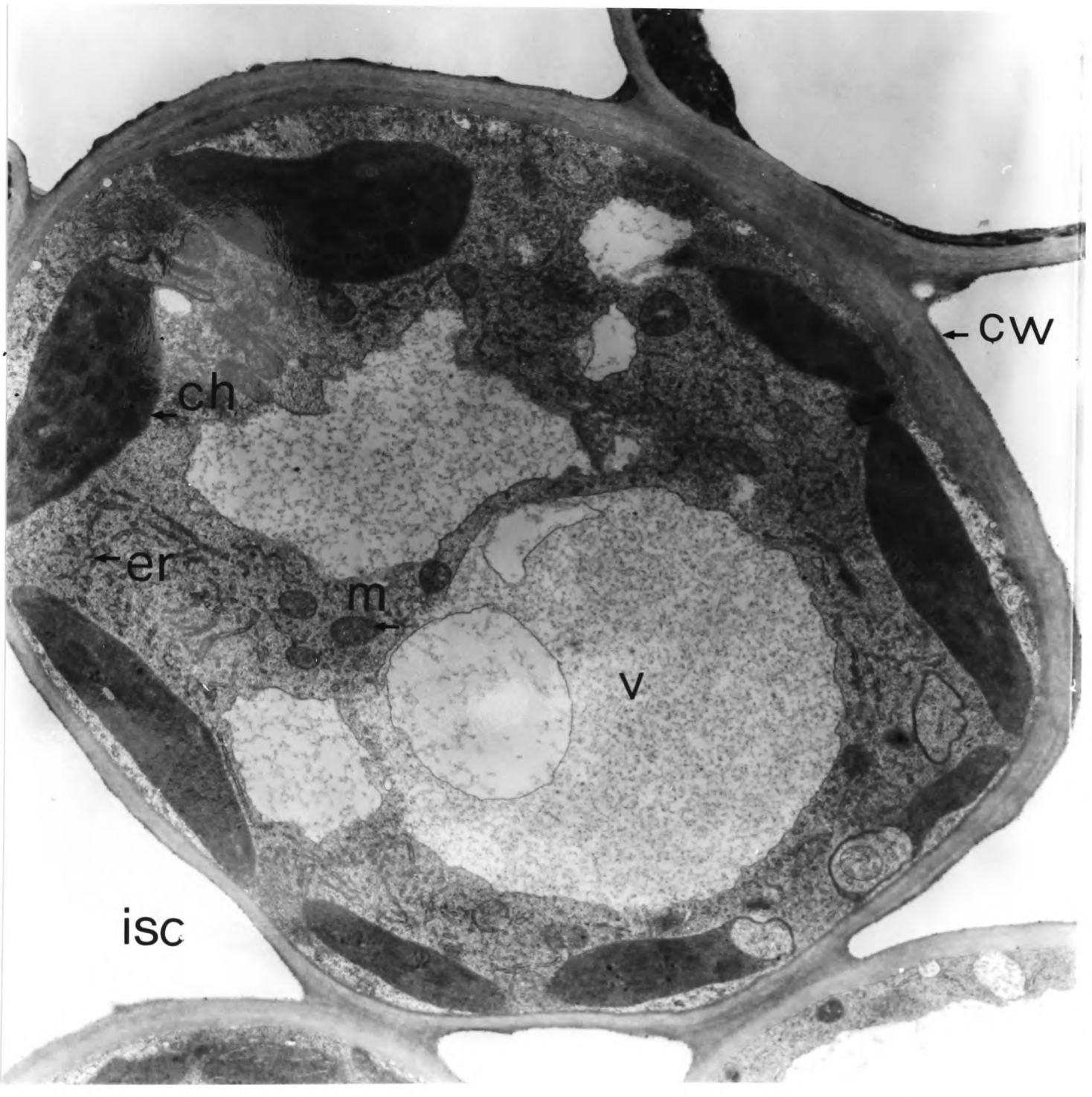


Plate 5.32. Chloroplasts from the central region of the gall; ch. chloroplast; cw. cell wall; er. endoplasmic reticulum; isc. intercellular space; m. mitochondrion; v. vacuole (Mag. x8000).

5.4 **Discussion.**

From a visual survey of the fluids within the reproductive organs and associated gland of Pontania proxima, together with a similar survey of the young gall, there does not seem to be any evidence to suggest that, at least for this species pair, there is any association between the sawfly and a species of microorganism. If there were, this would not have established a mutualistic relationship as responsible for gall formation, but the suggestions of Magnus (1914: 63) and Cornell (1983) (see Section 3.2.4), would have been worth pursuing. Therefore, unless the presence of an organism has been missed, or it is not susceptible to the methods of fixation and staining adopted in this study, then the observations made here would suggest that the cecidogen has to be searched for at the biochemical level. This search will form the basis of the next Chapter in this thesis.

However, the observations made in this Chapter have yielded much information concerning the structure of the reproductive system of Pontania proxima and they represent the first detailed account for any member of Tenthredinidae. The anatomical separation of the accessory gland from the oviducts, demonstrated in this study, is a point noted by Severin and Severin (1908) for Cimbex americana and by Tait (1962) for Perga affinis affinis, but was missed by other authors, in their work on species of Pontania. For example, Beyerinck (1888) produced a diagram of the reproductive system of Pontania triandrae (see Plate 2.1), which showed no spermatheca and the colleterial sac entering the median oviduct. McCalla et al. (1962) working on Pontania pacifica also produced a diagram of the reproductive organs that suggested a similar convergence (McCalla et al. Figure 1). In a criticism of Dahlgren's

classification of angiosperms, Corner (1981) used the phrase "on repetition they are apt to seem proven", this phrase would also seem the be applicable in this discussion; as well as it was for the belief that, for continued development, the galls of Pontania proxima required the presence of a feeding larva. Indeed, this type of error is probably due to the wide range of disciplines needed by those who study cecidology and provides further justification for an holistic approach. The intricacies of the structure of the reproductive system of this sawfly may seem to be of little consequence, but when linked to other factors, they assume far greater importance. For example, previous authors have observed that the presence of an egg in the leaf was not necessary for the initial stages of gall formation (Beyerinck, 1887; Magnus, 1914: 76-77; Rey, 1967). For the first time, this study has demonstrated that, at a cellular level, the eggless procecidium had many of the characteristics of a normal gall (see Plate. 5.28).

These two observations deserve further consideration because they raise the question of what actually happens when the sawfly inserts her ovipositor into a leaf. The stimulus to gall formation could either come from the oviducts or from the accessory gland; the assumption, in the past, was that it came from the colleterial sac (Beyerinck, 1887 and hence to McCalla, 1962), but neither had proved this. Beyerinck assumed it and McCalla used the fluid on already growing galls. One of the primary aims of the next Chapter will be to try to establish in which region of the reproductive system the cecidogen is situated.

Unless the variations in shape of the vaginal valve from different specimens were caused by the processes of fixation and embedding, this organ would seem to act in a way that could block off either the oviduct or the duct leading from the colleterial sac. A similar organ

does not appear to exist for Cimbex americana (Severin and Severin, 1908), which was the only microscopical study of any other member of the Tenthredinoidea that could be found; they describe a similar structure termed the ventral fold, but it is smaller and they describe its function as opening and closing the colleterial duct and would appear to be the area to the left of label cd in Plate 5.21.

Although transverse sections of this region would add further information, the series of events that may occur during oviposition is described as follows: the well-developed longitudinal muscles of the oviducts contract, pushing the egg downwards, its return being prevented by the downward-pointing cuticular spines. Copland and King (1971) suggested that the spines in the oviducts of some Eulophidae and Tetracampidae may have a sensory function; it is unclear whether this is the case for the spines in the oviducts of Pontania proxima, since there was no evidence of innervation (Rothschild et al., 1986: 125) or the characteristic membranous articulatory socket associated with setae. The passage of the egg past the vaginal clamp would be aided by the contraction of the broad band of striated muscle lying along the ventral surface of the oviduct (see Plate 5.21, label mu). Also, contraction of the muscles of vaginal valve (as in Plate 5.22) would seal the colleterial duct and facilitate the movement of the egg into the vaginal pouch and hence to the saw.

Togashi (1970) described the spermatheca of the Tenthredinidae as hemispherical, spherical, globose, ovoid, obovoid, club-shaped ellipsoidal, obpyriform or hook-like in shape, and derived from a bulging out of the common oviduct or genital chamber. In Pontania proxima it would seem to be more bell-like in shape than that described for Cimbex americana by Severin and Severin (1908) and, due to its

small size (approximately 0.1 mm in diameter), it has often been overlooked. Magnus (1914: 64) observed a second non-branching gland associated with the reproductive organs of Pontania triandrae: this could have been the spermatheca, because no other such gland was observed in this study. Because Pontania proxima reproduces by parthenogenesis (see Section 2.7), it is not known whether the spermatheca has a function in this insect, although there did appear to be a glandular secretion in the lumen of the spermatheca. The fact that no spermathecal gland could be found agrees with the observations of Baird (1921) on the reproductive anatomy of Tenthredinoidea.

The secretory rôle of the tissues of the oviducts would seem to be the first observation of this type for any member of the Tenthredinoidea. Severin and Severin (1908) noted that for Cimbex americana the entire inner surface of the lateral oviducts was covered with cuticle. There are few papers that deal with the these aspects of the reproductive system of Hymenoptera. Of the few that do, King and Ratcliffe (1969) noted a structure similar to the median oviduct of Nasonia vitripennis (Walker) (Pteromalidae), with a finely folded cuticular lining, above which were epidermal cells with microvilli borders, but they noted that there was no suggestion of secretory globules. However, they did hypothesize that these cells provided a lubricant for the egg as it passes through the oviduct. Copland and King (1971) observed a similar structure to the median oviduct of some parasitic Eulophidae and Tetracampidae and here they identified large secretory droplets near the apex of the epidermal cells of the oviduct. Again, the suggestion of lubrication was made, together with the possibility of the secretion aiding the prevention of a host reaction to the egg. Specialized secretory cells were observed in the distal

portion of the median oviduct of the parasitoid Telenomus heliothidis Ashmead (Scelionidae) by Strand et al. (1986), who noted that they knew of no other example of an oviduct serving as an exocrine gland.

In Pontania proxima there appears to be a gradation of development within the oviducts. At the distal portion of the lateral oviducts the cuticular lining is absent; whether these cells are derived from endoderm or whether they are modified ectodermal cells is not clear. The cells that line the oviduct, in this area, appear to have a secretory function. Their folded basal membranes may facilitate the uptake of substances from the haemolymph, since they seem to be only loosely attached to the overlying muscle layers. Internally these cells have abundant smooth-surfaced endoplasmic reticulum, golgi complexes and vesicles: this suggests a steroid or lipid secretory function. When a cuticle lining did appear, approximately half way along the lateral oviduct, the vesicles within the cells were still observed, but the number of mitochondria adjacent to the apical surface of the epidermal cells increased, and rough-surfaced endoplasmic reticulum was more evident. These features are, perhaps, related to the formation of the cuticle lining. At first the cuticle was very thin; as it thickened, spines were formed; these are present first in the lateral oviducts and increased in frequency in the median oviduct. In the median oviduct the lining of the lumen of the duct was folded, presumably to allow for expansion in width as the egg passes through. The cell lining of the lumen still possessed a microvilli brush border and had the structural features characteristic of a secretory function.

There is much scope for studies of comparative anatomy of other closely allied species, both gall-formers and non-galling insects. Togashi (1970) considered that the duplex type of colleterial sac was

the most primitive form, with the simplex type, as in Pontania proxima, having developed from this. Similarly with the proximal parts of the accessory glands; the primitive form being two separate glands that evolved into the combined condition.

The end-apparatus represents a common type of secretory cell within the accessory glands of insects and other glands of Hymenoptera, e.g. the acid gland of Nasonia vitripennis (King and Ratcliffe, 1969). The most detailed study of these cells was by Mercer and Brunet (1959) on the left colleterial gland of Periplaneta americana (L.) (Blattinae). In Pontania proxima the predominant type of cell was one similar to Brunet's type three cell, with a long duct from the secretory cell to the lumen of the accessory gland tubule. As in the cells described from Periplaneta americana, the secretory cells in the accessory gland of the sawfly contained much rough-surfaced endoplasmic reticulum and numerous mitochondria in the region of the end-apparatus. Also, electron-dense material was observed in the tips of some microvilli, but the felted appearence of the lumen of the end-apparatus was not noticed. Further studies are needed to establish whether the observed differences of morphology of the secretory cells denote different cell types, or whether they were due to the plane of the sections surveyed.

The observations of cells that were, perhaps, undergoing lysis, was not a common condition, but it was a persistent feature of all parts of the accessory gland and was observed in different specimens. The cells that were affected were in the same region as the secretory cells, but they were too disrupted to be identified as secretory cells. No observations were made of invagination cuticle lined ducts leading into these cells. If the assumption is made that they were derived from secretory cells then the reason for their lysis could either be due to

a pathogenic infection; however, no pathogen was observed. Alternatively, judging by the volume of secretion produced by these cells, they must have a high metabolic rate, perhaps leading to a high rate of cell death.

Finally, with reference to the insect, debris was observed in the lumen of the gut in sectioned material. Restrictions on time prevented efforts to try to identify this material e.g. whether it contained pollen grains. This would, however, be an interesting exercise, because there are few data on the food sources for adult Tenthredinidae; pollen has been mentioned as one source of food (see Section 2.5), but the second flight of adults of Pontania proxima would not have access to this, because they emerge after the catkins have dropped. The sawflies used in this study were raised in the laboratory (see Section 4.3.2) and it is unclear as to what they could have found to eat.

In a study of this type, limitations of time prevented further investigations into these structural aspects of the reproductive system of Pontania proxima. However, those parts that were completed have produced hypotheses concerning the functions of the structures observed. These can only be answered by a fuller and more extensive study.

The observations made on the early stages of development of the gall, agree with those of the authors mentioned in Section 5.1.3. Less importance should be paid to the observation made by Rey (1972, 1973 and 1974), as it has been shown that similar globules and invaginations are to be found in non-galled tissue from a different species of plant.

A few of the observations made by other authors and confirmed by this study were important for its future direction. They formed the

basis upon which the work detailed in the next Chapter was developed. These points were:-

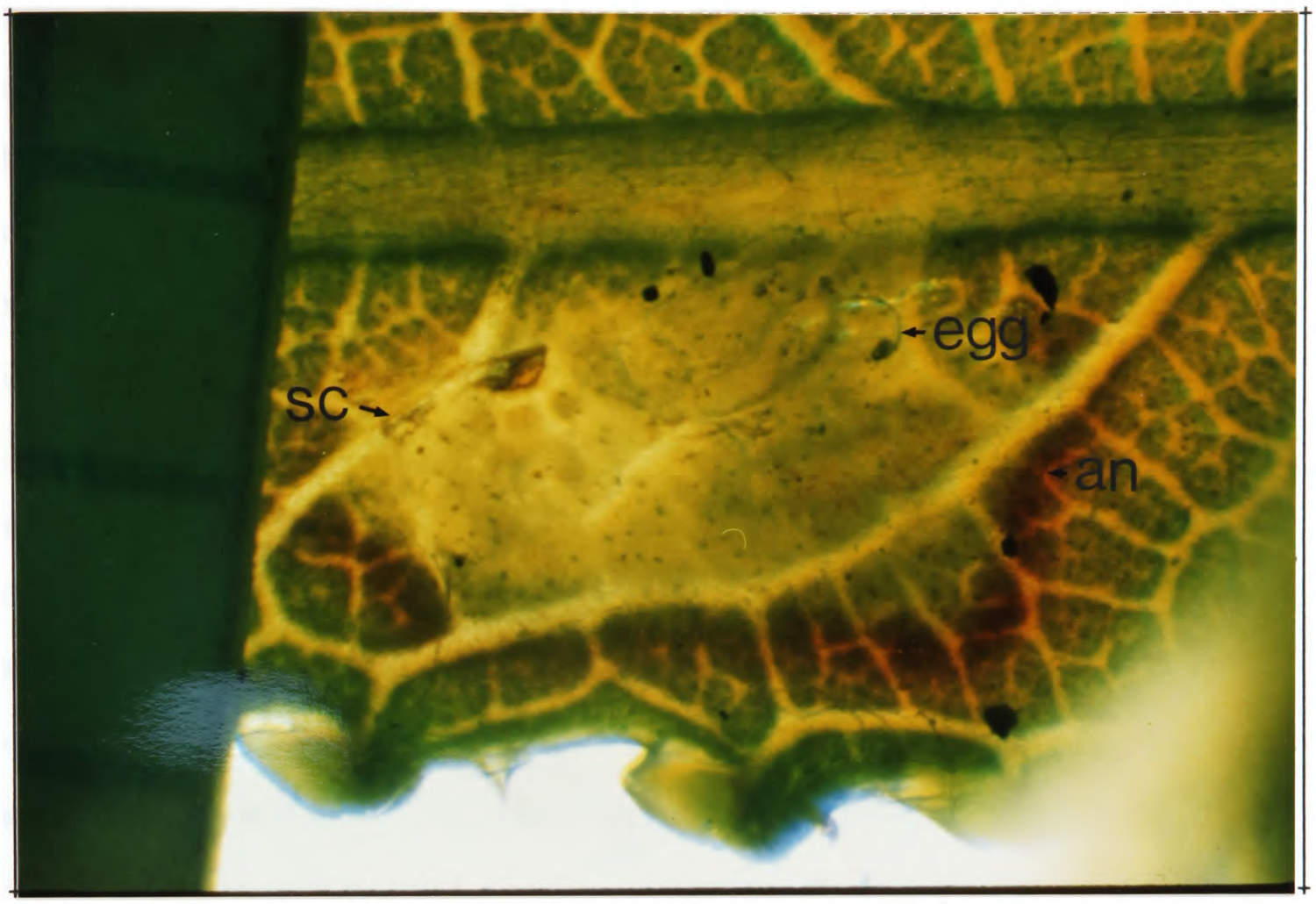


Plate 5.33. Procecidium 24 to 36 hours after oviposition. Abaxial surface of leaf. an. anthocyanin ring; e. egg. sc. scar of oviposition. (scale divisions mm).

1) The gall effect was limited: it did not spread, during the development of the gall, further than the original wound and a small surrounding area. This area was delineated by a ring of anthocyanin that formed round the site of the wound within 24 hours of its occurrence (see Plate 5.33). The rate of anticlinal divisions within the unaffected leaf and the gall tissue must have remained similar, with the effect of the cecidogen dramatically increasing periclinal

divisions. If these observations are correct, they would explain why different sized leaves produce similar-sized galls.

2) At the start of gall development, the leaf is no more than seven to eight cells in thickness. Material, from the gall cavity, was seen between the surrounding cells, but no colleterial fluid was observed in the cavity. Assuming that the cecidogen is contained in this fluid, as would be suggested by the eggless procecidia observed, then during oviposition or soon after the colleterial fluid moves into the surrounding tissues and the cecidogen exerts its effect.

3) The effect of the cecidogen is only on the rate and direction of cell division. In the gall of Pontania proxima there is no complex differentiation of cell layers, as in Cynipinae, to be considered. Neither does it appear that the cecidogen is the result of a mutualistic relationship. The stimulus for gall formation in this species pair must be searched for in the fluids of the adult insect itself.

CHAPTER SIX

THE CECIDOGEN

> "No entirely satisfactory explanation accounts for gall production, and nothing will be proven until one has succeeded in creating a completely artificial gall on a plant".
>
> (Jolivet, 1986: 107).

6.1 Introduction.

The quotation above summarizes the major objective of any mechanistic investigation into gall formation. At the present time, the limited level of knowledge concerning the induction of any insect gall suggests that this objective will not be reached in the near future.

The first step towards a "satisfactory explanation", therefore, lies not in the creation of a completely artificial gall, but in the creation of a gall using fluids extracted from the insect. Once this has been achieved, then analysis of the fluids involved could lead to the final stage i.e. the induction of a completely artificial gall.

The necessity of initiating a gall using fluids from the insect is important and can be summarized by the aims of the work in this Chapter:-

a) Although much of the literature (Beyerinck, 1887; Carleton, 1939; Hovanitz, 1959 and McCalla et al., 1962) supplied strong evidence for

the rôle of the colleterial fluid in gall formation, it has never been proved. All these workers failed to induce galls; indeed Magnus (1914: 88) concluded that the colleterial fluid played no part in cecidogenesis (see Section 2.2). Until a gall has been induced using an insect extract, the question of where the cecidogen lies remains open. It cannot be accepted that it is the colleterial fluid. The work in Chapter Five, demonstrated the division between the ovaries and the accessory gland. Fluids from the oviduct may carry the cecidogen. It was the major aim of the work in this Chapter to identify that part of the insect which contained the cecidogen.

Whatever the source of the cecidogen, further questions remain. Perhaps, passage through the reproductive system is essential for its activation; perhaps it is of a temporal nature; perhaps a mixture of fluids is needed; perhaps there are other glands not yet identified. What has been shown is that it is probably not cellular in nature. These questions are complicated further by the nature of the leaf. For example, is the developmental stage of the leaf, at oviposition, an important consideration for gall production; what are the problems of physical damage to the leaf and is the associated wound response important? It has been shown, by this study, that gall initiation occurred in young leaves containing actively dividing procambial tissue, and that oviposition was always through a secondary vein. As long as these conditions were met, then the position of the gall on the leaf lamina was not important.

b) To identify the cecidogen it was necessary to develop a bioassay to test for its presence. For this, techniques were devised for the inoculation of Salix leaves using micropipettes. These techniques were

designed to limit physical damage to the leaf and to copy oviposition as closely as possible.

c) Once the aims listed above had been achieved it was then possible to begin an analysis of the fluid containing the cecidogen. The criteria used in choosing techniques for this analysis were dependent upon the maintenance of biological activity in the extracted material. The implementation and testing of the effectiveness of these techniques took much time. However, this aim was achieved and working techniques were established. This work made it possible to begin an analysis of the fluid involved. The preliminary results obtained were sufficient to provide useful data for future investigators.

The final identification of the causal agent/s must be part of that future. When this has been achieved, the synthesis of an artificial gall will be possible.

6.2 **Methods of injection and the development of a bioassay.**

6.2.1. Introduction.

Experiments to induce gall formation in the leaves of Salix fragilis have been hampered by the difficulty of injecting material into the young, delicate leaves, of the type preferred by Pontania proxima for oviposition (see Plate 4.9). Beyerinck (1887) found it impossible to duplicate the precision of oviposition as carried out by the sawfly. Magnus (1914: 81) also found the technical difficulties too great and resorted to the use of young root tissue as an alternative, but he was unsuccessful in his attempts at cecidogenesis. Carleton (1939) tried to inoculate the leaf and was partially successful. Using a glass needle, she was able to make a small incision into the lower epidermis of the

leaf. Into this she inserted an ovipositor, which had been dissected from a sawfly and glued to a stiff bristle. Unfortunately, she was unable to inject any glandular fluid without causing excessive damage to the leaf tissue; she commented, "In every case the damaged cells died and the full grown leaf had a large torn area surrounded by dead tissue".

The use of insect extracts to induce gall formation has been attempted by numerous investigators. Boysen Jensen (1948) investigated whether the larvae of Mikiola fagi (Hartig) (Cecidomyiidae) secreted substances that may cause gall formation on the leaves of Fagus sylvatica L. (Fagaceae) and in his experiments he observed some physiological response. Leatherdale (1955), using an ultra-fine hypodermic needle succeeded in producing a few cataplastic hyperplasia in Urtica dioica L. (Urticaceae) stems from whole body and head extracts of the dipteran Dasyneura urticae (Perris) (= Dasineura urticae; Cecidomyiidae). However, like Boysen Jensen (1948), Leatherdale (1955) acknowledged that his results did not resemble a real gall. Other investigators have also achieved varying degrees of success (Birch, 1974: 4), but no one has produced, unequivocally, a gall. Indeed, McCalla (1962) considered that the artificial production of normal galls would depend on precise amounts being placed in a precise place. This was something he considered to be difficult, if not impossible.

The use of micropipettes, however, has been tried, and with some success. Schäller (1969) recorded the production of artificial gall-like tumors in the petioles of species of Vitis L. (Vitaceae) after using glass cannulae to insert various solutions into the petioles of the vine.

The main aims of work in this Section were: firstly to assess the viability of using micropipettes as a method of delivery for material extracted from the insect into the leaf lamina of Salix fragilis, then, secondly to identify the area of the insect that contained the cecidogen.

6.2.2 Methods.

Throughout this work, rooted cuttings of known origin were used (see Section 4.2.2). The trees from which these cuttings had been collected had all shown a susceptibility to gall formation. Further, where insect-derived materials were injected into the leaves of these cuttings, it was ensured that both the insect and the cutting had been raised from the same "parent" tree.

The specimens of Pontania proxima used in these experiments were either recently emerged (within 48 hours) or had been stored, in a chest freezer, at a temperature not exceeding -75° C (model LTF-6.5-85, Harris, No. Billerica, Massachusetts, USA).

a) Development of micro-injection techniques.

It was found to be impossible to inject directly into the leaf, as the bore of the micropipette became blocked with cellular debris. To overcome this problem, an incision was made into the leaf, to facilitate the entry of the pipette. It was decided that this wound should approximate the wound made by the ovipositor of the sawfly during oviposition.

Initial trials were conducted using dissected ovipositors, glued to extruded glass capillary tubing, but ovipositors were too flexible to break through the epidermis of the leaf. Therefore, a variety of instruments was constructed to cut into the leaf tissue. The choice of

materials used was governed by the requirement for strength coupled with an ability to be of a size similar to that of the ovipositor of the insect.

The dimensions of the insect's saw, when measured one millimetre from the tip were, width 0.17 mm and thickness 0.022 mm (n = 20). The length of the wound caused during oviposition was approximately 1.25 mm (Magnus, 1914: 60; personal observations). To reproduce these dimensions, as closely as possible, an A3 sized entomological pin (Bio-Science Supplies, Wednesfield, West Midlands. WV11 1JD) was ground down, so that its dimension, one millimetre from the tip, were width 0.209 mm and thickness 0.01 mm. The thickness of this micro-scalpel was judged to be important, since the size of the cells in the provascular layer were between 0.09 mm and 0.11 mm in diameter and observations, detailed in Chapter Five, suggested that during oviposition the sawfly usually disrupted one, sometimes, two layers of cells. The use of this instrument was partially successful, but it was so thin that it soon became distorted and unusable. Finally, in order to gain greater strength a compromise had to be reached between size and the material used.

The most successful type of micro-scalpel constructed was made from tungsten. Used filaments, approximately one mm in diameter, from an electron microscope were etched thinner using heated and fused sodium nitrate. This was achieved by heating sodium nitrate (Sigma) crystals, in a crucible, until they were molten. Then by trial and error, the temperature of the fused sodium nitrate was controlled so that, when a tungsten filament was dipped into it, the surface of the filament was gradually etched to a needle sharp point, with a diameter, one millimetre away from the tip of approximately 0.1 mm. This was found to

possess the strength and durability required. With this type of micro-scalpel it was possible to cut into the leaf, causing as little disruption to the tissues as possible.

To facilitate micro-injection a micro-scalpel was used to make a pocket, approximately one millimetre in length, in the leaf. A young leaf was chosen, usually one that had not yet fully separated from the bud (see Plate 4.9). This leaf was carefully separated and laid along a narrow metal tube, into which the remaining terminal leaves were placed. The metal tube was covered with an outer coat of plastic tubing to cushion the young leaf. A wound was then made in the leaf, using the micro-scalpel, the micropipette inserted and its contents expelled into the leaf tissue; the micropipette was then removed and the wound sealed. The wound was always initiated through a secondary vein on the abaxial surface of the leaf and ran parallel to the midrib, in the direction of the petiole. This type of wound was similar to that caused by Pontania proxima during oviposition (see Section 4.4; Magnus, 1914: 55-88). Because it was often difficult to ensure that sufficient fluid had entered the leaf, it was found that the insertion of acid-washed beads (75-150 micron diameter, Sigma) would approximate the size of an egg (270-640 microns, Carleton, 1939; personal observation). The glass beads functioned to hold the mesophyll layers apart, allowing the fluid to enter. When it was judged that this had been achieved, a ring of lanolin was placed round the wound and covered with a fragment of a glass coverslip, this sealed the operation and minimized desiccation of the tissues (see Plate 6.1).

The micropipettes were made from eight inch lengths of Pyrex glass tubing (outside diameter one millimetre, internal diameter 0.85 mm; Horwell Ltd., West Hampstead, London. NW6 2BF), using a micropipette

puller at 390 microamps. The tips were then broken and smoothed using a microforge (ref 4080. Beaudouin, 92120, Montrouge, France). Using a Hamilton syringe (Howe and Co., St. Ann's Crescent, London. SW18 2LS), the micropipette were then calibrated by filling with a known volume of water at 21° C. Various tip diameters were evaluated and a compromise of approximately 0.125 mm was chosen. This minimized damage to the leaf, while being large enough to draw up the rather viscid colleterial fluid extracted from the insect.

Before use, the micropipettes were placed in a Narishe H1-4A micropipette holder (Micro Instruments, Little Clarendon Street, Oxford. OX1 2HP), held in a Prior micromanipulator. The micropipette holder was connected to a three-way tap using 1.07 mm (inside diameter) Teflon tubing (Caldy Science Associates, Heswell, Merseyside. L60 9LL). The remaining ends of the tap were, in turn, connected to a 10 μl Hamilton syringe and a one mm^3 glass tuberculin syringe. Light mineral oil was used to fill the apparatus, with the tuberculin syringe acting as a reservoir. The three-way tap was used to isolate the tuberculin syringe. With the tap in this position, the Hamilton syringe was used to draw up material into the micropipette or expel it. Air was not used in the system, as it was too compressible, and may have caused the oxidation of collected biological material. However, it was recognized that problems could occur if the cecidogen was found to be soluble in lipid.

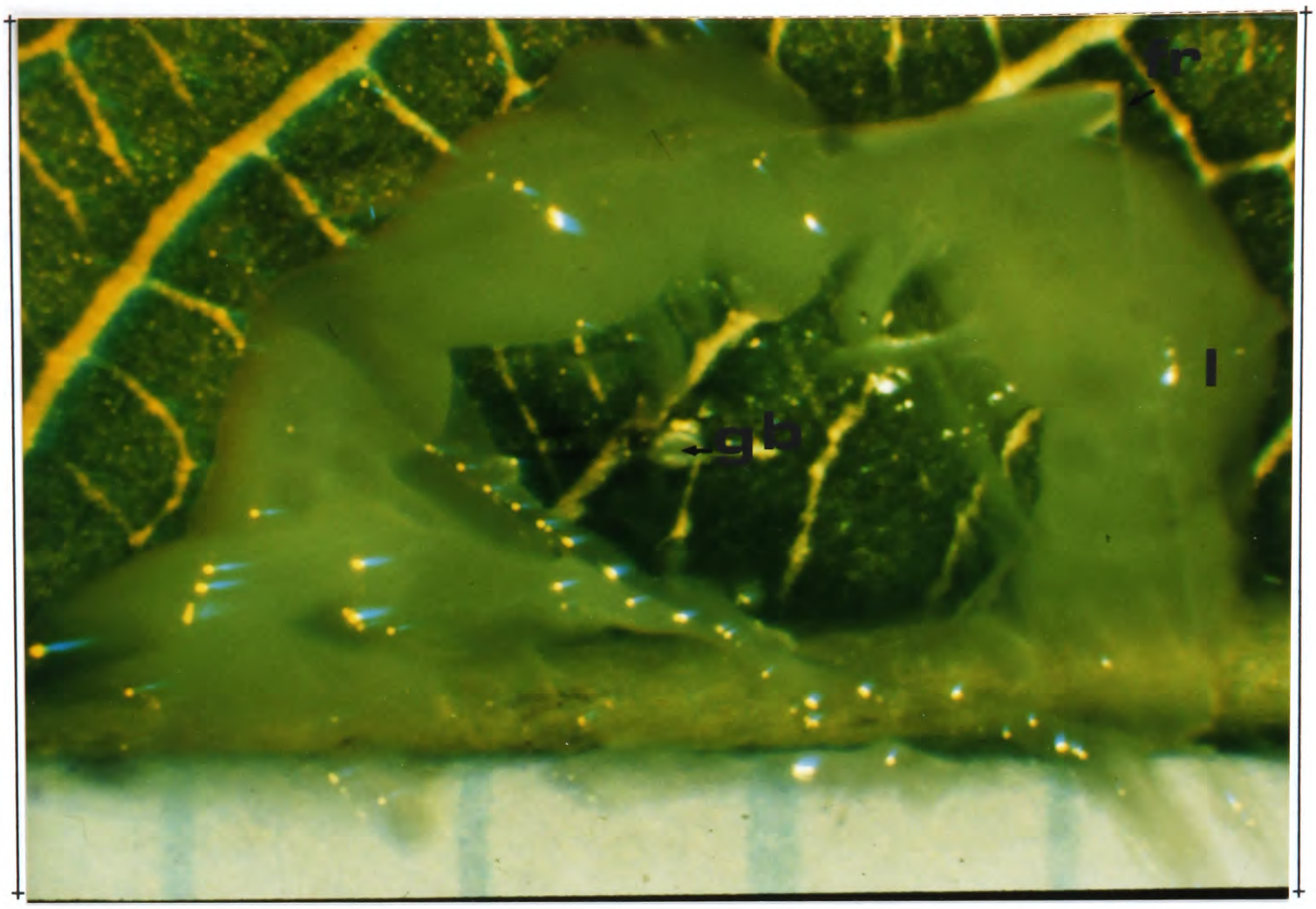


Plate 6.1. Completed injection into the leaf tissue of Salix fragilis. A glass bead has been placed in the wound to keep the mesophyll layer apart. The wound has then been sealed with lanolin and a fragment of glass coverslip; fr. fragment of glass; gb. glass bead; la. lanolin (scale divisions mm).

The insects were dissected, under either distilled water or Ringer's solution (see Appendix Six). Watchmaker's forceps were used to hold the upper thorax and the ovipositor. These were then pulled apart, exposing the colleterial sac, accessory gland, ovaries and oviduct. Trials were conducted of this delivery system, using 1% methylene blue as a marker. A small drop of light mineral oil was first drawn into the pipette; this was then followed by methylene blue, which had previously been filtered through a 0.2 micron pore size disposable filter (Sigma). The pipette was inserted into a wound, made as detailed above, and

sealed in position with lanolin paste. The small volume of mineral oil prevented evaporation of the liquid from the open end of the pipette. The pipettes were left in the leaf for four hours, after which they were removed and the area of the stain calculated. The leaves were then removed and the surface area of the abaxial side measured together with the area of the main body of absorbed stain.

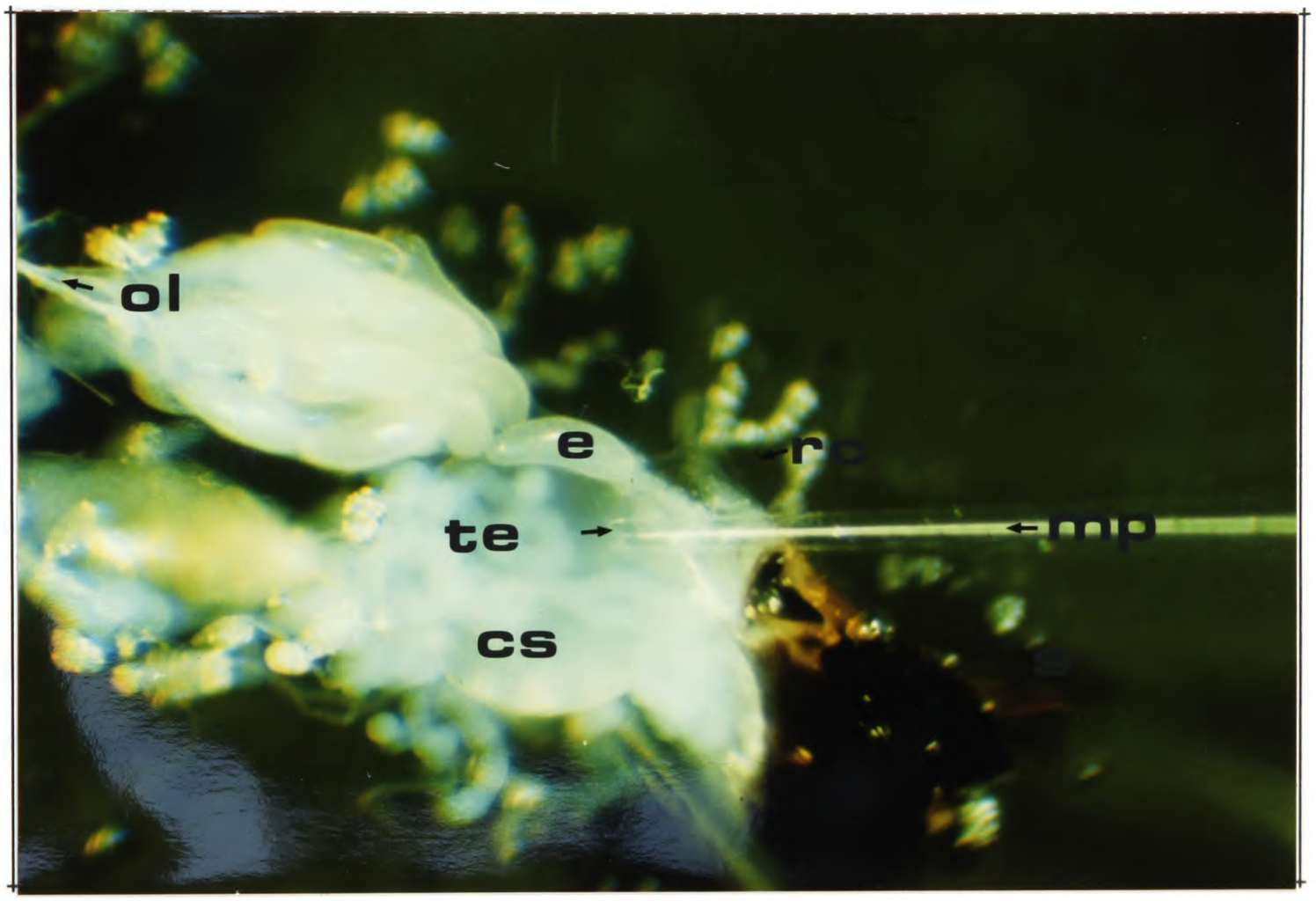


Plate 6.2. Use of a micropipette to remove colleterial fluid from the colleterial sac of Pontania proxima; cs. colleterial sac; e. egg; mp. micropipette; ol. ovary ligaments; rc. released colleterial fluid; s. saw; te. tear made with pin (Mag. x40).

b) Assessment of the physical damage caused to the leaf by wounding and the insertion of the micropipette.

The previous experiment was repeated using new plants, but distilled water was used instead of methylene blue. The pipettes were left in the leaf for two to four hours then removed and the plants returned to the greenhouse. They were then inspected for damage after four days, by which time the leaves had expanded sufficiently for this to be assessed.

c) The site of the cecidogen.

To establish the area of the insect that contained the cecidogen, either colleterial sacs or oviducts and ovaries were broken on the surface of the leaf. A series of dissections was performed and either the ovary, eggs or ducts of the reproductive system were implanted as effectively as possible in a wound produced as detailed above. Any affect on the leaf tissue was noted over a period of two weeks.

Similarly, colleterial sacs were dissected out on to the leaf and, using a micro-scalpel, their contents were encouraged to enter the wound. Control experiments were carried out using distilled water.

6.2.3 Results.

a) Development of micro-injection techniques.

1. Calibration of micropipette at 21° C. At its widest part, one millimetre of the pipette held 0.45 μl of distilled water.

2. Uptake of methylene blue into the leaf.

On average, approximately 0.77 μl of methylene blue was taken up into the leaf. This resulted in approximately 1.55% of the surface of the leaf showing evidence of staining.

leaf	diameter of micropipette tip (mm)	volume of liquid absorbed (μl/hr)	area of leaf (mm^2)	area of stain (mm^2)
1	0.125	0.78	253	4.0
2	0.125	0.78	623	10.0
3	0.075	0.90	246	4.0
4	0.100	0.55	437	5.0
5	0.075	0.78	222	4.0

Table 6.1. Results of the uptake of methylene blue from micropipettes into the tissues of the leaf.

b) Assessment of the physical damage caused to the leaf by wounding and the insertion of the micropipette.

LEAF	AREA OF LEAF (mm^2)	AREA OF DAMAGE (mm^2)	$\frac{\text{AREA OF DAMAGE}}{\text{AREA OF LEAF}}$ (%)
1	825.0	4.0	0.48
2	950.0	3.0	0.32
3	675.0	3.0	0.44
4	675.0	3.0	0.44
5	1100.0	4.0	0.36
6	925.0	4.0	0.43

Table 6.2. Results of the amount of physical damage caused to the leaf tissue by the insertion of micropipettes.

At each position where the micropipette had been there was evidence of reduced amounts of mesophyll, making that area of the leaf lighter in colour than the rest. In only one instance had a hole been formed. In each of the other leaves, their adaxial epidermises were whole and the disturbance to their abaxial epidermis showed no necrosis of tissue

except for a few small spots only visible using a x40 magnification on a dissecting microscope. However, in three of the leaves there was evidence that the normal expansion of the leaf had been arrested or slowed at the site of insertion. The leaf lamina on either side of the damage, caused by the micropipette, had continued to expand, but the area of damage had not. This resulted in an undulation on the margin of the leaf nearest the insertion and the whole leaf becoming falcate due to the normal expansion of the lamina on the undamaged side.

Removal of the fluid from the colleterial gland was complicated by its viscid nature. A pipette narrow enough to pierce the glandular sac is too narrow to draw up the exposed fluid. However, the sac could be punctured using a fine needle and the pipette inserted through this hole. During this process dilution took place as the fluid, in which the insect was being dissected, entered the pipette. It was possible to remove larger amounts of colleterial fluid from the colleterial sac, using a wider bore pipette, but it was found that a wider pipette did greater damage to the leaf during the injection phase of this process. With practice, it was found that it was possible to carry out the complete process of extraction and injection in approximately four minutes from the removal of the wasps from the freezer.

The colleterial fluid had the appearance of granular egg white. If exposed to air it hardened quickly, into what appeared to be a yellowish, crystalline mass similar to dried egg albumen. If it dried on the surface of a leaf it formed transparent crust, capable of imprinting the outlines of the epidermal cells it covered. Colleterial fluid collected from specimens of Pontania proxima, that had been frozen, had different properties from that collected from living specimens. The colleterial fluid from fresh sawflies was transparent:

it was possible to look down through the gland and obverse the tissues lying under it. When it hardened, which it did so in approximately two minutes (depending on the volume of the liquid and its temperature), it lacked the yellow hue of previously frozen material.

c) The site of the cecidogen.

Over 100 dissections were carried out to establish the area of the insect that contained the cecidogen. In all cases a response, by the tissues of the leaf, over and above that normally occurring at the site of a normal wound was looked for. Recording these observations, in a meaningful manner, proved impossible. However, three criteria were chosen to record a positive response i.e. a response over and above that expected from a normal wound. These were: firstly callus formation being estimated as above that for a normal wound; secondly that the callus should remain for the full life of the leaf - in control experiments it was found that wound callus soon underwent necrosis; thirdly that there should be visual evidence of a red pigment in the callus.

In no cases did a "normal" gall form but, by using the criteria listed above, differences in the response of the leaf tissue to the types of implanted material were observed. In only a few cases were all three criteria met, and in all of these, the material that had been implanted was colleterial fluid. Also, the leaves injected with colleterial fluid suffered much less necrosis than that for any other type of implant.

A typical result of one of the successful experiments was obtained from using the colleterial fluid from a living specimen which had emerged from a gall collected at Little Wittenham on 14.9.88 (see Appendix Two) and is shown in Plates 6.3 and 6.4. This growth remained

on the leaf for the life of that leaf, but did not increase in size. It was considered to be comparable to the eggless procecidium sometimes formed during abortive attempts at oviposition (see Section 5.3.2 and Plate 6.5).

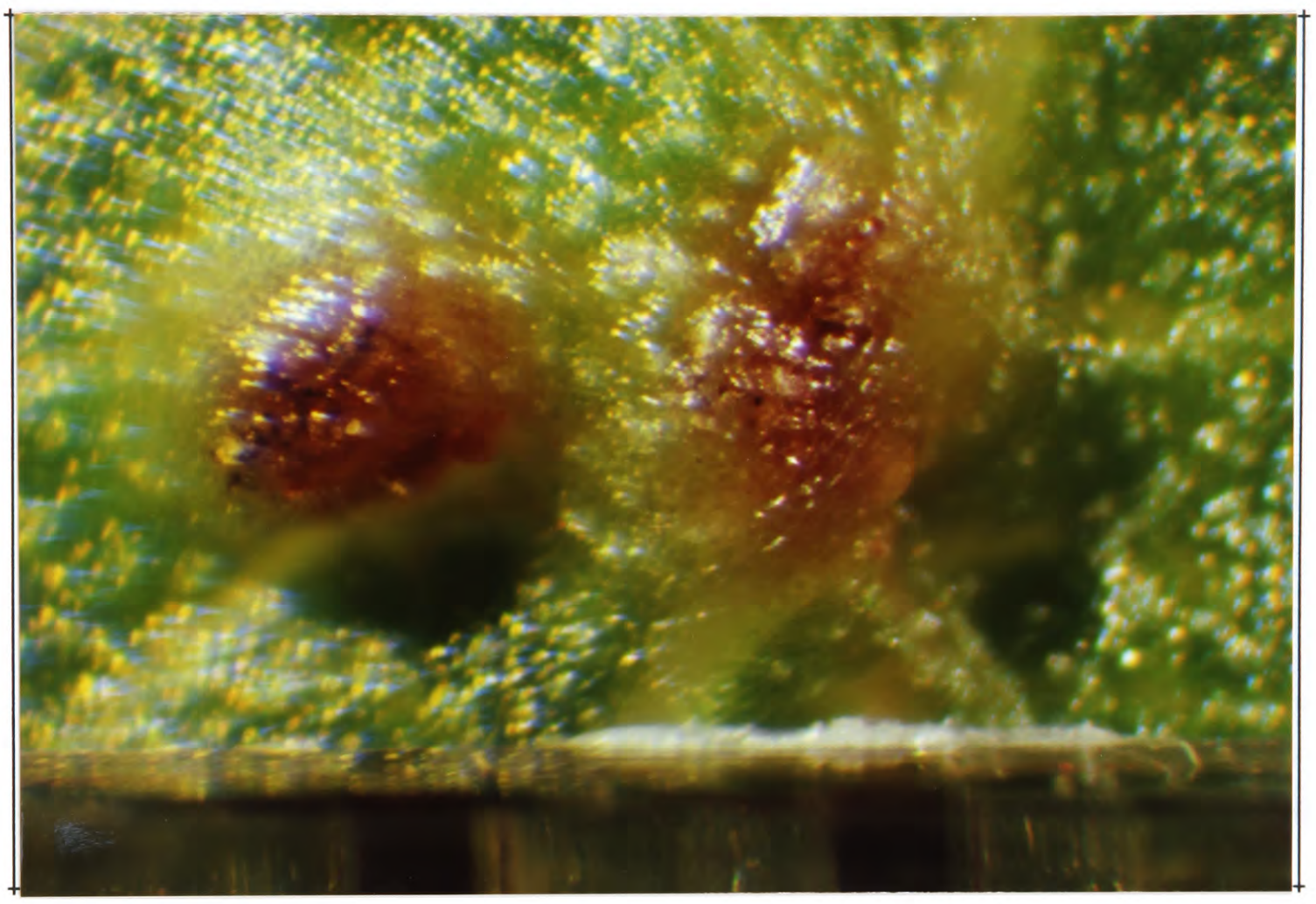


Plate 6.3. Callus tissue induced by the implantation of colleterial fluid. View of adaxial surface of leaf after eight weeks (scale divisions mm).

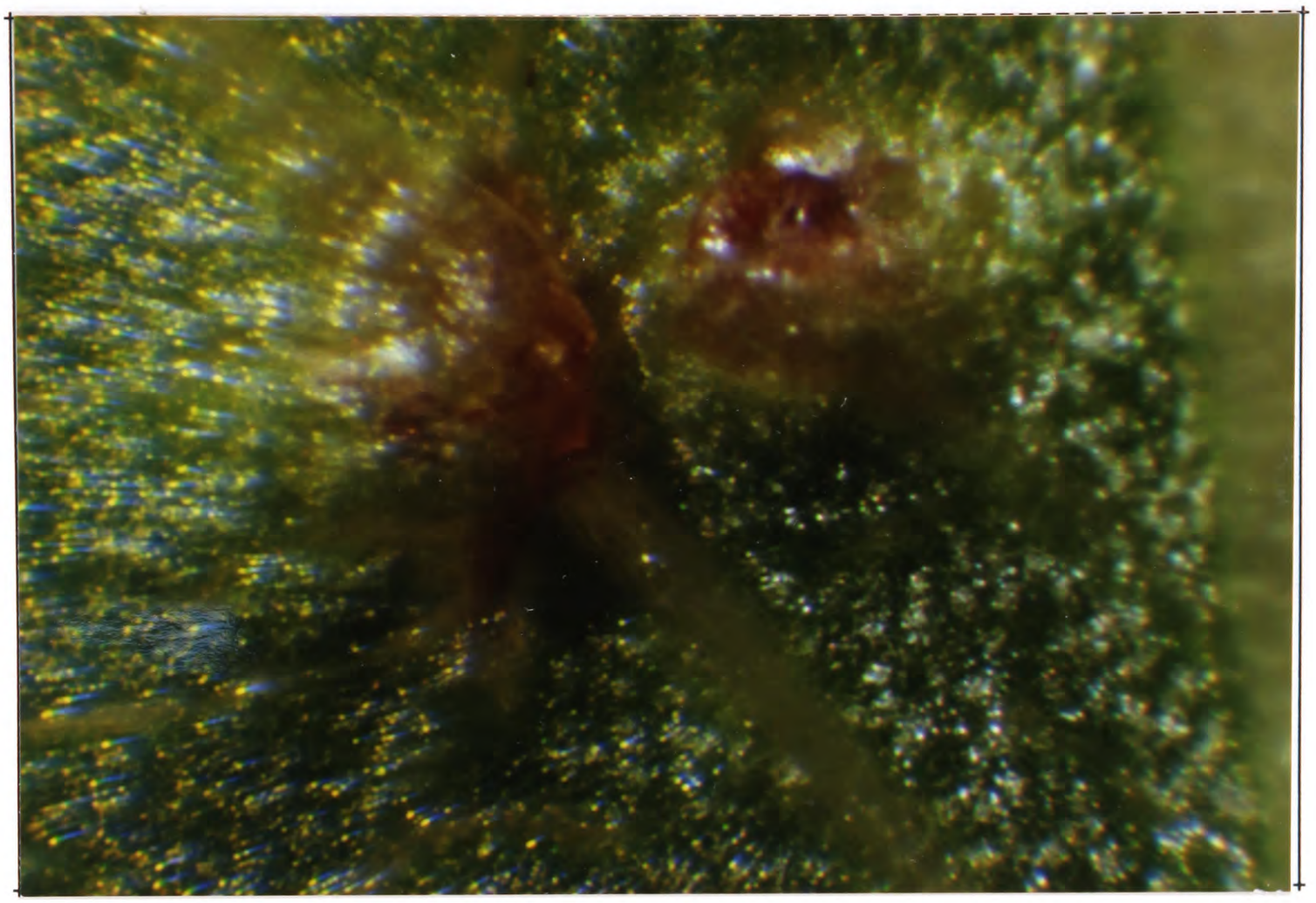


Plate 6.4. Callus tissue induced by the implantation of colleterial fluid. View of abaxial surface of leaf after eight weeks (scale divisions mm).

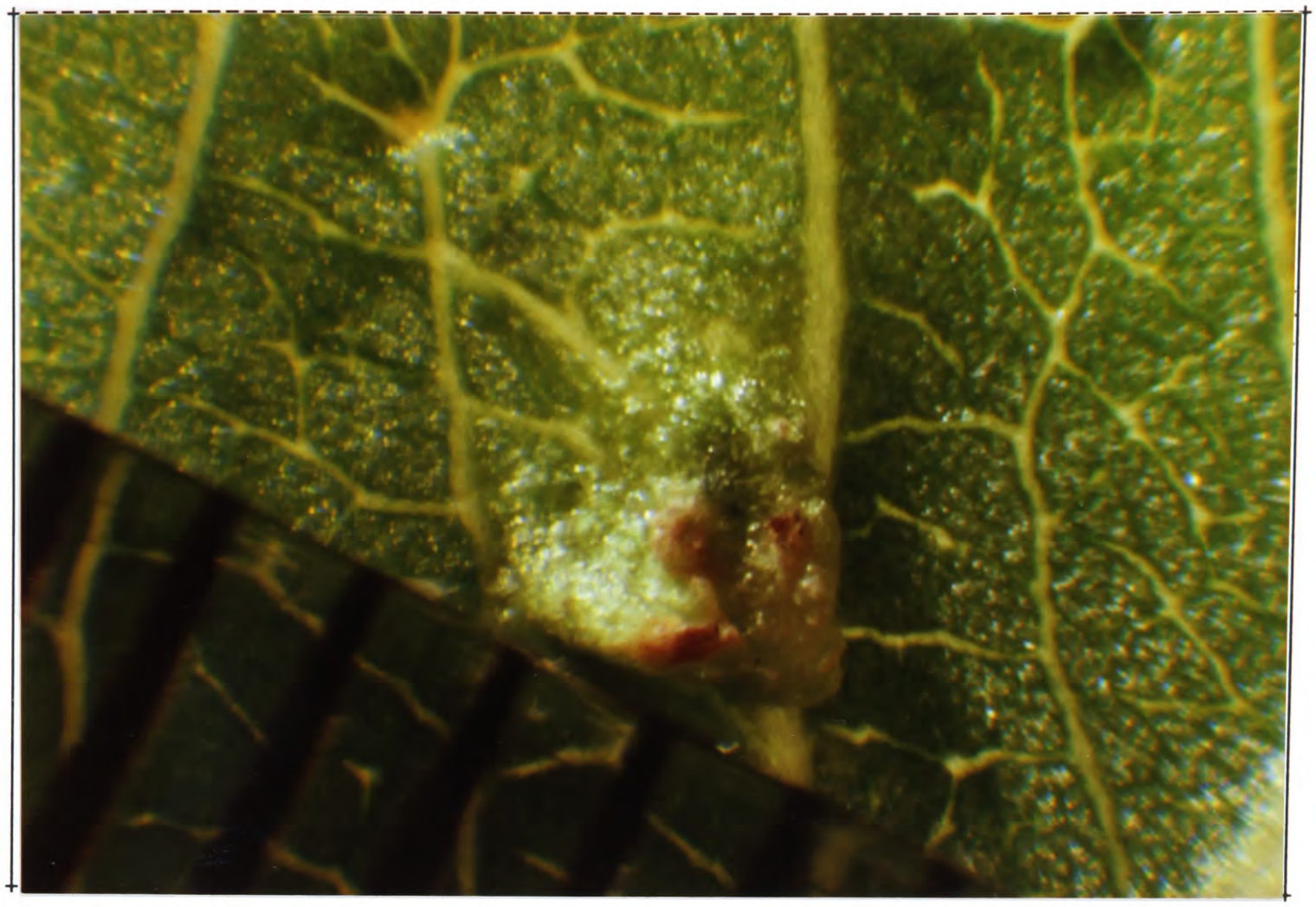


Plate 6.5. Callus produced on the abaxial surface of a leaf, by an abortive attempt of a sawfly to oviposit. Growth after eight weeks (scale divisions mm).

6.2.4 Discussion.

The methods for the micro-injection of material into the leaf tissues of Salix fragilis, developed in this section, together with the response of those tissues, formed the basis of a bioassay that was used throughout the experiments described in the rest of this Chapter.

Beyerinck (1887) described Pontania as producing, during oviposition, a very precise wound with a volume of approximately 0.06 mm^3. The insect laid its egg in the cavity and surrounded it with colleterial fluid. It has proved impossible to duplicate this process exactly, but the trials conducted suggested that the methods developed may have provided a reasonable approximation to the process of

oviposition. Micropipettes have been shown to be effective in the delivery of material into the tissue of the leaf and that, at least for methylene blue, once material has been placed in the leaf tissues, it then spread into the surrounding areas, but was restricted in the degree of this spread. The amount of damage caused to the leaf tissue the trials of these methods, was much less than that observed by others working with similar species (see Section 6.2.1).

Unless great care was taken, the outer leaves of the Salix leaf-bud were easily bruised. For this reason, the first leaf after the bud was also used in these experiments. It was found that this leaf was easier to handle and injections into the lamina produced the same responses. However, from the observations made in the previous Chapter, it was recognized that young meristematic tissues were essential, so the leaves were as young as possible.

Using the methods detailed in this Chapter, it was not found possible to induce a growth, in the tissues of the leaf, that resembled a natural gall. This was not unexpected but, the results obtained suggested that insect-derived material did possess growth-promoting properties. It was also a consistent observation that these growth-promoting properties were contained in the colleterial fluid. It was found that the physical manipulation of the colleterial fluid was hampered by the rapidity with which it solidified, a process which may be caused by oxidation of -SH bonds on exposure to air, and prevented by the addition of dithiothreitol (Sigma). The addition of DTT was tried, but in the concentrations used, was found to be ineffective. It would have been useful if there had been enough time to continue this aspect of the work. Many authors (Magnus, 1914: 87; Beyerinck, 1888 and Caltagirone, 1964), have pointed out that the hardening of the

colleterial fluid would result in the sealing of the wound caused by the ovipositor. It is interesting to note that, from the study made in Chapter Five, no colleterial fluid was observed, in the cavity of the procecidium, 24-36 hours after oviposition and that there were visual suggestions, that this fluid may have dispersed into the surrounding tissues of the leaf.

The criteria used to judge the initiation of growth, over and above that expected for a wound response, were rather subjective e.g. Jacobs and Rubery (1988) explained that the accumulation of anthocyanins was part of a normal wound response. Despite this criticism, the abundant nature of the callus material, initiated by the colleterial fluid, which contained cells that had undergone hyperplasy and hypertrophy, has convinced this author that it is the colleterial fluid alone that contains the cecidogen. Further to this, it is also believed that the methods developed and the criteria chosen are valid tools to use in a bioassay for the cecidogen.

The smaller amount of callus material that was produced by wound response mechanisms alone, may play some part in the initial stages of gall-formation. Taft and Bissing (1988) studied the development of the horned oak gall. This gall developed under the influence of the larva of Callirhytis cornigera (Osten Sacken) (Cynipidae) in twigs of Quercus palustris Muenchhausen (Fagaceae). It was observed that oviposition caused the development of wound response phellogen. Taft and Bissing (1988) concluded that this arrangement of tissues formed the framework, from which the gall would develop after the hatching of the larva. They observed that the resulting gall was a complex structure, with a definite morphology, that went far beyond a simple wound response. The gall formed by Pontania proxima is much less complex, in structure,

than any gall formed by members of Cynipinae. This fact does not preclude a wound response mechanism as having a rôle to play in the formation of the Pontania gall.

6.3 Initial studies of the colleterial fluid.

6.3.1 Introduction.

From a search of the literature, it appeared that there were few papers that provided any details concerning the composition of the colleterial fluid of any species of Pontania. Hovanitz (1959) described the fluid as rich in nucleic acids and proteins, and containing carbohydrates and the precursors of silk. The silks of Hymenoptera show a variety of structural forms: Atkins et al. (1966) described the silk of the closely allied species Nematus ribesii (Scopoli) (Tenthredinidae) as having a structure similar to that of collagen, while that of Cimbex femoratus (L.) (Cimbicidae) was of the parallel beta-sheet conformation.

The only other reference that could be found, relevant to the work in this Section, was that of McCalla et al. (1962), who carried out a series of bioassays on material extracted from the colleterial sacs of Pontania pacifica. McCalla et al. (1962) prepared a "crude extract" by removing the accessory gland and the colleterial sac from the insect. These were then frozen, in batches of 50 to 100, and stored at -20° C. When required, small amounts of 80% ethanol were added to the frozen sacs, the mixture macerated in ethanol and the grindate centrifuged at 2500 rpm. The supernatant was then decanted and the residue washed and recentrifuged. The two supernatants were combined and allowed to

evaporate; water was added to the dried supernatant and the mixture stirred, centrifuged and the pellet discarded.

McCalla et al. (1962) found that the ultraviolet absorption spectrum of the "crude extract" demonstrated an increase in absorption from 300 nm downwards, with a peak occurring at 260 nm. From this fraction they isolated six compounds and identified uric acid, two unidentified adenine derivatives, glutamic acid and possibly uridine. They believed that these compounds were in sufficiently large quantities to have significant growth-promoting activity. Their view was not shared by others (see Section 3.2.1), but the work of McCalla et al. (1962), represented the furthest stage anyone had reached in the analysis of the colleterial fluid of any species of Pontania.

The aim of this Section was to complete an initial characterization of the colleterial fluid of Pontania proxima. This included basic measurements of pH and U/V spectra and, from the latter, an estimation of protein concentration. The spectrum obtained from the colleterial fluid was also compared with those obtained from the frass of the insect (see Section 3.2.1) and the inner tissues of the gall. Proteins from the colleterial fluid were separated using gel electrophoresis and measurement of molecular weight was made for each of the bands obtained.

With the exception of the work using gel electrophoresis, it was impossible to collect enough specimens of Pontania proxima, at any one time, to enable fresh material to be used. Emergence of the adults was limited to a few months of the year and during these months the main task was the collection of galls. Therefore, on emergence the adult sawflies were frozen and stored at a temperature of -75° C (see Section 6.2.2). The temperature (-20° C) used by McCalla (1962) to store the

colleterial sacs was judged to be too high; Scopes (1978: 13-17) made the point that at -20° C cell proteases could still function. From personal observations, the body contents of insects kept in a domestic freezer soon decomposed.

Gordon (1971: 38) described the use of polyacrylamide gel electrophoresis (PAGE) as a method whereby an estimation of the molecular weight of a small amount of protein could be determined. Initially, in this work, the methods described by Fehrnström and Moberg (1977) were used, but the most successful results were obtained using the semi-automated PhastSystem developed by Pharmacia LKB Biotechnology (S-751 82 Uppsala, Sweden). It is the results from the use of this system that are described in this Section. Three PAGE techniques were applied; two used gradient gels, which allowed proteins with a wide range of molecular weights to be separated on a single gel. These techniques were: firstly, separation using native-PAGE, which separated intact proteins by size, charge and conformation and had a molecular weight range of 50 to 500 kDa; secondly, SDS-PAGE with a discontinuous buffer system with a separation range of between 6 and 300 kDa - for this proteins were first separated into polypeptide subunits by sodium dodecylsulphate (SDS) and beta-mercaptoethanol, then separated further on the gel by size only (since the addition of SDS resulted in all the polypeptide subunits having the same net negative charge density); finally, separation by isoelectric focusing (IEF) was achieved, this technique allowed for the high resolution and concentration of proteins at their isoelectric points.

6.3.2 Methods.

a) Preparation and initial characterization.

Batches of approximately 50 frozen sawflies were dissected in cold DDH_2O and their colleterial sacs and accessory glands placed in 0.5 ml of cold DDH_2O. The samples were then macerated, using a glass rod, and sonicated using a Dawe soniprobe (Model 7530A, Dawe Instruments, Concord Road, London. W3) on setting eight for one minute. Both these procedures were designed to disrupt and release the contents of the glands and sacs; during disruption the insect material was surrounded with ice. The macerate was then centrifuged at 6000 rpm for 15 minutes and the supernatant decanted. This process was repeated and the two supernatants combined. The absorption spectrum was obtained for both the combined supernatants and the pellet, after appropriate dilution. The samples were placed in a one millilitre quartz cuvet and the U/V absorption spectrum obtained between 190 nm and 400 nm (bandwidth 2.0 nm, scan speed 250 nm/min, with no smoothing), using a Philips PU8700 Series UV/Vis. Spectrophotometer (Pye Unicam). The pH of the insect extract was measured using a Beckman combined pH electrode (No. 9505, Beckman Ltd., Glenrothes, Scotland) and a newly calibrated Pye pH meter (Model 292, Pye Unicam).

To assess the stability of the extracted material, the supernatant was frozen in a dry ice/actetone mixture, covered with perforated parafilm and freeze-dried in an Mudalyo Freeze Dryer (Edwards High Vacuum, Crawley, West Sussex. RH10 2LW).

Protein concentration was estimated using the method outlined by Dawson et al. (1986: 541-542). A one centimetre path-length quartz cuvet was used and the absorbance of an appropriately diluted extract

was measured at both 260 nm and 280 nm. Protein concentration was estimated by the formula:-

Protein concentration (mg/ml) =

(1.55 x Absorbance at 280 nm) - (0.76 x Absorbance at 260 nm)

Protein concentration was calculated, on another sample, using the methods of Warburg and Christian detailed in Dawson et al. (1986: 541-542).

b) Comparison of colleterial fluid extract with frass and gall tissue.

Similar procedures were used in the preparation of frass and gall tissue, prior to U/V spectroscopy. In this way a comparison of the spectra of frass (collected from sectioned galls), gall tissue and colleterial fluid was achieved.

c) Polyacrylamide gel electophoresis.

The gels and buffer strips used in this work were prepared by Pharmacia LKB Biotechnology for use with their PhastSystem.

1. Native-PAGE.

PhastGel Gradient 8-25 gels were used; these had a 13 mm stacking gel zone (6% T, 3% C), where T = the total concentration of monomer (acrylamide monomer + N,N'-methylene-bis-acrylamide) w/v (expressed as a percentage), and C = percentage by weight of total monomer T which is due to crosslinker. The stacking zone was followed by a 32 mm continuous 8% to 25% gradient gel zone with 2% crosslinker. The gels were approximately 0.45 mm thick. The buffer system in the gels was 0.112 M acetate and 0.112 M Tris, pH 6.4; that of the PhastGel Native Buffer Strips was 0.88 M L-alanine and 0.25 M Tris, pH 8.8 (Pharmacia LKB Biotechnology, Separation Technique File No. 120).

Colleterial sacs were dissected out from live sawflies in Tris-HCl buffer, pH 8.8. Ten sacs were then transferred to 50 μl of buffer

containing 50 mM Tris, 20 mM DTT, one mM EDTA (to remove heavy metals) and two μg/ml leupeptin (acetyl-Leu-Leu-Arg-al) to inhibited protease activity. A sample applicator was loaded with various dilutions (see Plate 6.5), at a volume of 0.5 μl for each lane. Molecular weight markers for nondenatured polyacrylamide gel electrophoresis were prepared (Sigma, Technical Bulletin No. MKR-137) and added at the same time. The run conditions were as shown in Table 6.3.

2. SDS-PAGE with discontinuous buffer system.

PhastGel Gradient 8-25 gels were used as described above; the PhastGel SDS Buffer Strips contained 0.20 M tricine, 0.20 M Tris and 0.55% SDS, pH 8.1 (Pharmacia LKB Biotechnology, Separation Technique File No. 110). The colleterial sacs from live sawflies were dissected out into a small volume of buffer (10 mM Tris/HCl, pH 8.0, one mM EDTA, SDS to 2.5% and beta-mercaptoethanol to 5.0%), then evaporated to dryness under a bench lamp: this process was designed to dissociate the proteins into their polypeptide subunits. These were resuspended in buffer at a concentration of one gland per three μl of buffer. A small volume of bromophenol blue was added as a tracking dye and 0.5 μl loaded onto the gel as before. Molecular weight markers were also added (Sigma and Pharmacia; see Figure 6.3); these were prepared as detailed by the suppliers (see Sigma Technical Bulletin No. MWS-887L, Pharmacia LMW Calibration Kit). Details of the run conditions are given in Table 6.3. The end step reduced the risk of the proteins migrating off the gel.

3. IEF.

PhastGel IEF 3-9 (5% T, 3% C) gels were used: these covered the pH range of 3 to 9 and contained carrier ampholytes which generated a stable, linear pH gradient within the gel during the run (see Pharmacia

LKB Biotechnology, Separation Technique File No. 100). The colleterial sacs were dissected out in a 100mM solution of DDT at a concentration of eight sacs per 50 μl. Isoelectric point markers were prepared as detailed by the supplier (Sigma Technical Bulletin No. IEF-100); these together with various dilutions of the colleterial fluid were loaded onto the sample applicator and run from the anode; details of the run conditions are given in Table 6.3.

	Volts (V)	Amps (A)	Watts (W)	Temperature (° C)	Volt hours (Vh)
native-PAGE					
prerun	400	10.0	2.5	15	10
loading	400	1.0	2.5	15	2
run	400	10.0	2.5	15	108
SDS-PAGE					
loading	100	3.0	3.0	15	15
run	250	10	3.0	15	75
end	50	0.1	0.5	15	0
IEF					
prefocusing	1910	1.8	3.4	16	77
loading	200	0.1	0.0	16	80
focusing	1990	1.8	3.5	15	500

Table 6.3. Run conditions (maximum limits) for native-PAGE, SDS-PAGE and isoelectric focusing, using the PhastSystem developed by Pharmacia LKB Biotechnology.

4. Silver staining.

The protocols detailed in PhastSystem Development Technique File No. 210 (Pharmacia LKB Biotechnology) were estimated by the manufacturer to have a sensitivity limit of one to five ng protein per

band for IEF and native-PAGE and 0.3 to 0.5 ng protein per band for SDS-PAGE. For each of these techniques, staining was carried out as detailed by the manufacturer. The basic steps were: the fixation and removal of buffer ions by washing the gel in trichloroacetic acid (IEF and native-Page only), fixation and sensitization of proteins using glutaraldehyde, reaction with silver nitrate, development in formaldehyde, stopping the development in acetic acid and finally washing in glycerol to prevent the gels cracking upon drying.

6.3.3 Results.

a) Preparation and initial characterization.

1. U/V absorbance spectrum of the supernatant obtained from the centrifugation of the colleterial fluid extract contained a major peak at 265.6 nm (see Figure 6.1a). This was then freeze-dried, resuspended in DDH_2O and a new absorption spectrum obtained which showed a major peak at 259.2 nm (see Figure 6.1c). The spectrum of the wash from the pellet obtained after the second centrifuge was also obtained and this showed an absence of any major absorption in the region of 260 nm (see Figure 6.1b).

2. Measurements of pH.

Crude extract, after sonication, before centrifuge			pH 5.9
After first centrifuge	Supernatant pH 6.3	Pellet	pH 6.7
Repeat of above	Supernatant pH 5.4	Pellet	pH 6.3
repeat of above	Supernatant pH 5.6	Pellet	pH 6.5

3. Estimation of protein concentration.

Point 2 on Figure 6.1a = absorbance at 280 nm = 1.336

Point 1 on Figure 6.1a = absorbance at 260 nm = 1.493

Estimation of protein concentration = (1.55 x 1.336) - (0.76 x 1.493)

= 0.93612 mg/ml

corrected for dilution of sample = 4.1806 mg/60 sacs.

contents of one gland = 0.07 mg

volume of one gland= 0.50 mm^3 (see Appendix Eight)

Therefore protein concentration = approximately 140 mg/ml

Protein concentration by the method of Warburg and Christian (Dawson et al., 1986: 541-542).

absorbance at 260 nm = 1.569

absorbance at 280 nm = 1.585

ratio of A280/A260 = 1.010

factor from Dawson et al. (1986: 542) = 0.80

Estimation of protein concentration = 1.268 mg/ml

= approximately 189 mg/ml

4. Estimation of mass of dried supernatant.

Mass of freeze-dried material from 60 sacs = 7.2 mg.

Therefore mass of freeze-dried material from one gland = 0.12 mg.

b) Comparison of colleterial fluid extract with frass and gall tissue.

In the region of 250 nm to 350 nm, the spectrum of the frass contained a major peak at 288.1 nm and a smaller one at 317.7 nm. The U/V spectrum of the gall tissue contained major peaks at 281.5 nm and 322.4 nm (see Figure 6.2a and b).

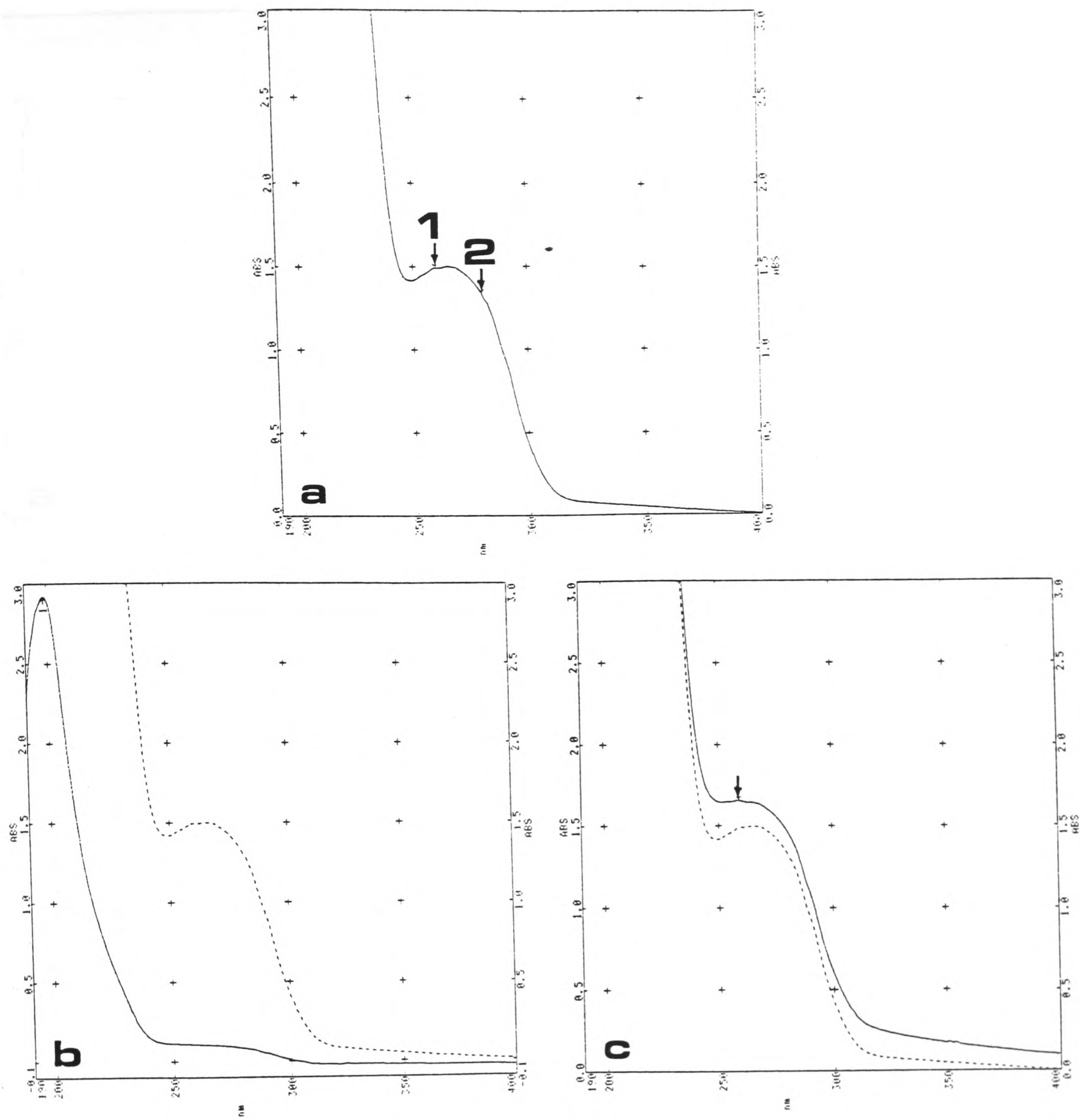


Figure 6.1. U/V absorption spectra of (a) supernatant obtained from the colleterial fluid (1 = absorbance at 260 nm; 2 = absorbance at 280 nm), (b) pellet (dotted line = spectrum 6.1a), (c) resuspended freeze-dried supernatant (dotted line = spectrum 6.1a). Arrows on 6.1b and 6.1c indicate absorption peaks (see text for details).

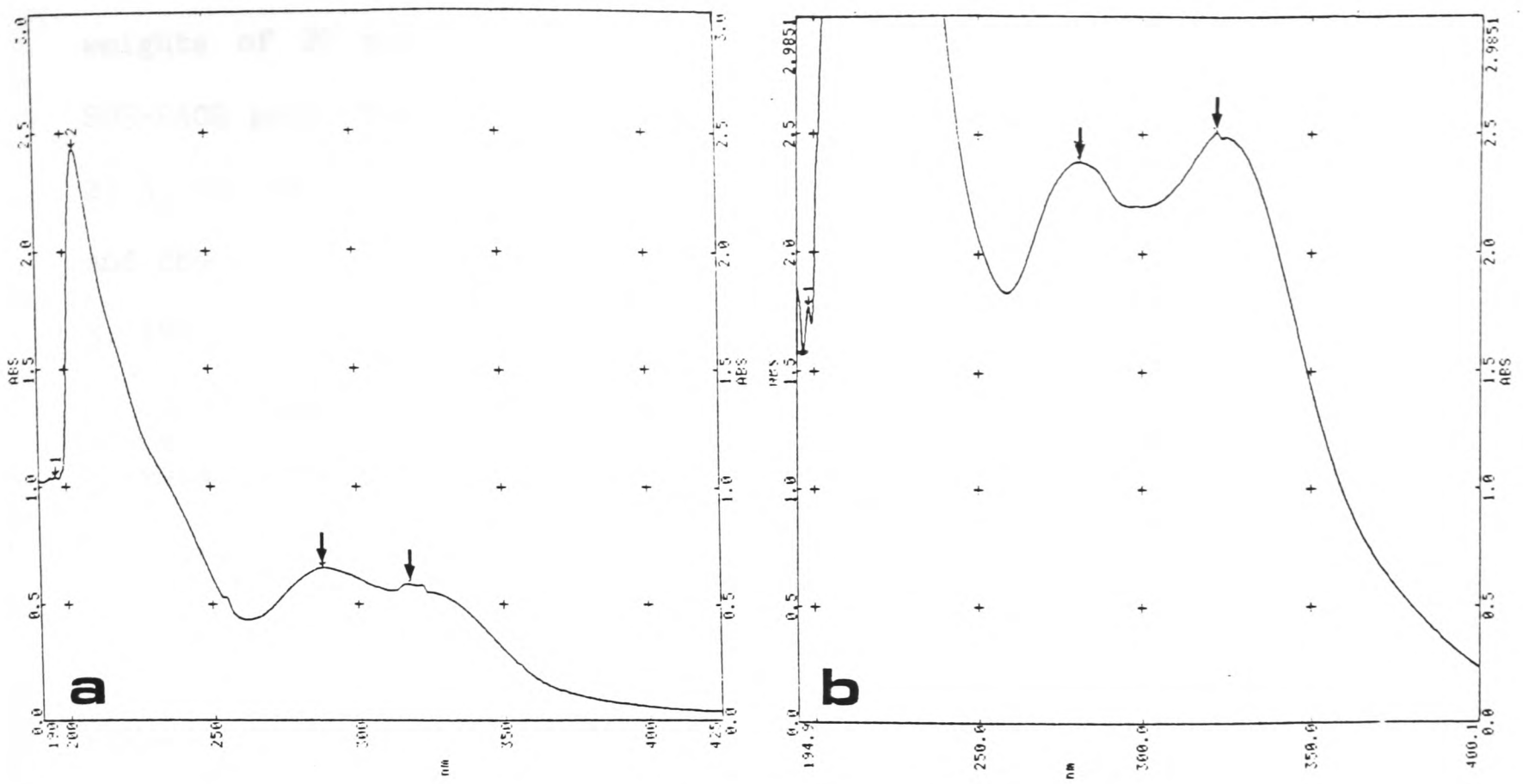


Figure 6.2. Absorption spectra for (a) frass obtained from section galls, (b) tissue from the inner layer of the gall. Arrows indicate absorption peaks (see text for details).

c) Polyacrylamide gel electrophoresis.

1. Native-PAGE.

The results obtained from native-PAGE showed poor separation (see Plate 6.6), a number of runs were repeated using higher concentrations of DTT in an attempt to obtain good band separation. The addition of DTT had little effect on the resolution of native proteins within the colleterial fluid. Any attempt to produce measurements of protein molecular weight from these gels would have been unrealistic.

2. SDS-PAGE.

The results obtained using this technique were much better. Good separation of the polypeptides was achieved (see Plate 6.7). Figure 6.3a shows the calibration curve obtained from the molecular weight marker. Using this graph is was possible to measure the molecular

weights of 20 polypeptide units that were consistently separated on SDS-PAGE gels. The mass of these units (kDa) was 11.9, 13.5, 16.4, 19, 21.3, 24, 29, 33, 34, 38, 42, 65, 71, 76, 84, 108, 125, 175, 184, 222, and 289.

3. IEF.

Good separation was achieved over the whole of the pH gradient with 37 plus bands being visible covering a range of pH values of 3.55 to 9.30 (see Plate 6.8 and Figure 6.3b).

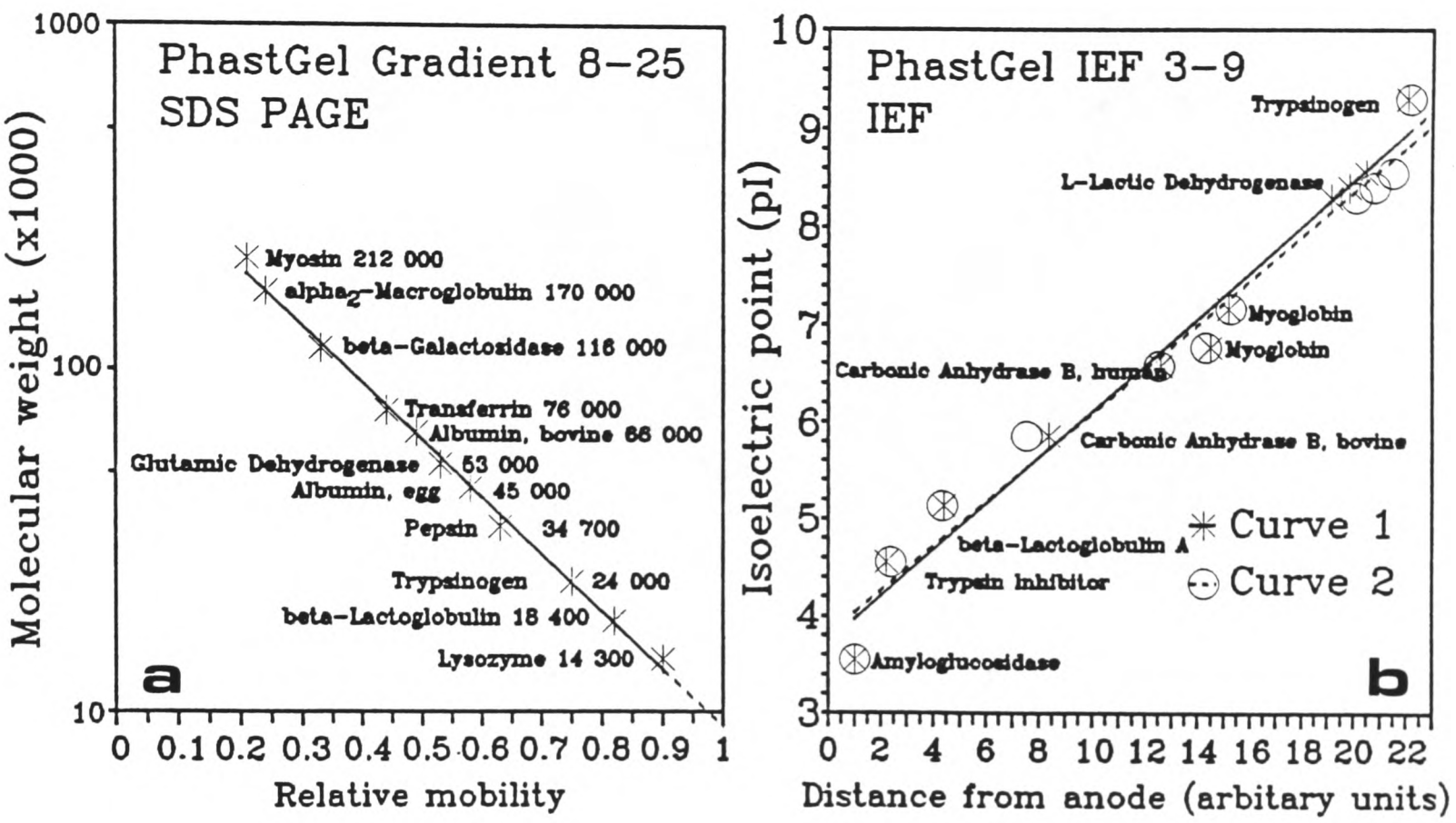


Figure 6.3. Calibration curves for (a) SDS-PAGE and (b) IEF. Details given in the text.

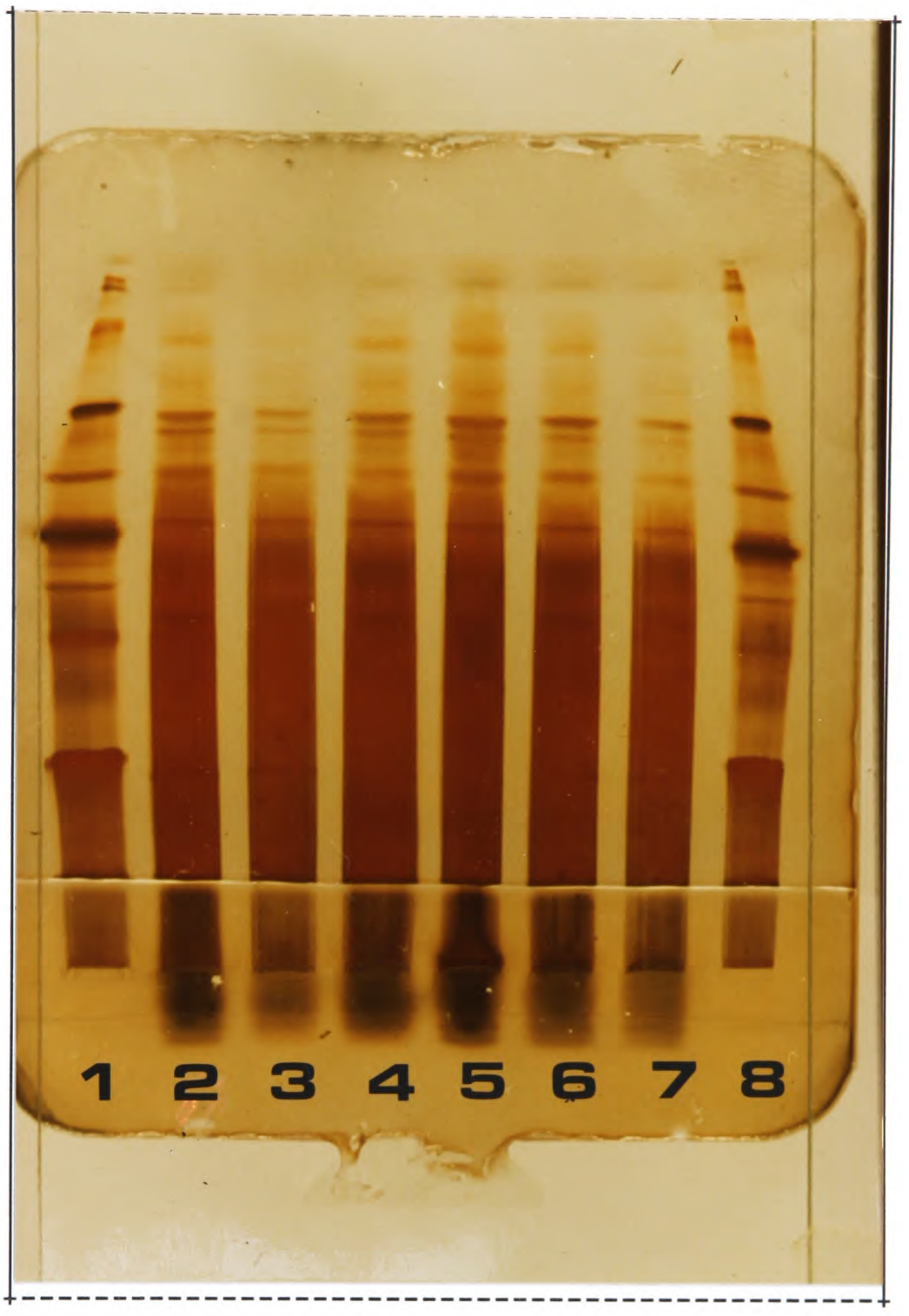


Plate 6.6. Native-PAGE. Lanes: (1) and (8) molecular weight markers. (2) colleterial fluid. (3) colleterial fluid, dilution 1+1. (4) colleterial fluid, dilution 1+2. (5) colleterial fluid. (6) colleterial fluid, dilution 1+1. (7) colleterial fluid, dilution 1+2. Silver staining.

Plate 6.7. SDS-PAGE. Lanes: (1) and (8) molecular weight markers. (2) colleterial fluid. (3) colleterial fluid, dilution 1+1. (4) colleterial fluid, dilution 1+3. (5) colleterial fluid (6) colleterial fluid, dilution, 1+1. (7) colleterial fluid, 1+3. Silver staining.

Plate 6.8. IEF. Lanes: (1) and (8) isoelectric point markers. (2) colleterial fluid. (3) colleterial fluid, dilution 1+1. (4) colleterial fluid, dilution 1+2. (5) colleterial fluid, dilution 1+3. (6) colleterial fluid, dilution 1+2. (7) colleterial fluid. Silver staining.

6.3.4 Discussion.

The results given in this Section are the first for an initial analysis of the colleterial fluid of Pontania proxima. The pH of the initial extract was shown to be slightly acid as were the supernatant and the pellet. Little stress, however, should be given to these figure as Scopes (1978: 3-4) maintained that the process of extraction and maceration could change the pH from that found in the intact sac.

The absorption spectrum shown in Figure 6.1a was almost identical to that obtained by McCalla et al. (1962), with a major peak in the region of 260 nm. The use of this spectrum, as an indicator of possible constituents of the colleterial fluid, is unrealistic, as many biologically active molecules absorb strongly in this region of the U/V spectrum. The spectra obtained, however, did demonstrate that the extraction methods employed did lead to a partial purification of the material and that freeze-drying would be a method that could be used without adversely affecting the spectrum.

The absorption at 260 nm and 280 nm allowed an estimation of the protein concentration of the extract to be made and, by using two methods of estimation, values of between 140 and 189 mg/ml were obtained. This may be an over-estimation, since both RNA and DNA also absorb strongly at the 280 nm wavelength. If the results obtained are reliable, then it would seem that the protein content of the freeze dried material was between 58 and 60%.

Studies using PAGE techniques demonstrated that the colleterial fluid contained a complex mixture of protein. Work in the next Section will demonstrate that the cecidogen was probably not a protein, at least, not of a type separated by these techniques. Therefore, no further work was carried out on the protein constituent of the

colleterial fluid. There were many more bands present on the IEF gel, which would suggest that similar polypeptides from different proteins were migrating to the same bands on the SDS gel. Of the bands separated by SDS-PAGE and IEF one had the same molecular weight and another the same isoelectric point as trypsinogen. If these were of the same protein and trypsinogen is present in the colleterial fluid, then this may explain how the colleterial fluid moves out of the cavity of the gall into the surrounding plant tissues (as suggested in the previous Chapter). In its natural state the colleterial fluid is viscid and on contact with air soon hardens; however, if this does not occur within the confines of the plant tissues, trypsinogen could function to digest the proteins of the colleterial fluid, making it less viscid and able to pass into the surrounding tissues to exert its effect. There has not been any work conducted to establish the feasibility of this hypothesis; however, there are parallels to be drawn e.g. the function of fibrolytic enzyme in the liquefaction of the seminal fluids of mammals. (The Open University, 1974: 17).

6.4 **Further separation.**

6.4.1 Introduction.

With so few data available on the composition of the colleterial fluid of Pontania proxima it was considered impossible to identify the cecidogen in the time available. It was decided, therefore, to test the following hypotheses:-

a) That the cecidogen was a molecule of low molecular weight. McCalla et al. (1962) found low molecular weight compounds with growth-promoting properties in the colleterial fluid of Pontania pacifica.

b) That there may be some link between the cecidogen and ecdysone (see Section 3.3.4).

c) That as the gall of Pontania proxima contained higher quantities of cytokinin N^6-(isopent-2-enyl)adenosine (Leitch, personal communication, see also Section 3.2.1), this or other plant growth regulators may be present in the colleterial fluid.

When the maintenance of biological activity was essential, separation of the colleterial fluid was achieved using ultrafiltration and tested for using the bioassay developed in Section 6.2. For further separation and comparison with known standards, high pressure liquid chromatography (HPLC) was employed.

Lafont et al. (1981) described the use of reverse phase HPLC as the most widely used procedure for the analysis of ecdysteroids. Unlike straight phase chromatography, this form involved the use of a non-polar stationary phase and a polar mobile phase. This resulted in the elution of the most polar compounds at the start of the run. Lindsay (1987: 98) lists the disadvantages of reverse phase bonded columns as being:-

a. Silica bonded columns are unstable at pH's above 8.0, when the silica begins to dissolve.

b. The presence of unreacted silanol groups often causes poor peak shape and non-reproducibility between columns (this last point was illustrated by Lafont et al. (1981)).

c. The mechanism for peak separation is not properly understood.

Despite these disadvantages, the use of reverse phase bonded columns, has allowed for fast, cheap and easy separation of biological substances across a wide range of polarities. The most common and widely used bonded phase is the ODS or C-18 bonded phase (Lindsay,

1987: 88). Here octadecylsilane chains are linked to the silica of the column by siloxane bonds. Lafont et al. (1982) evaluated a variety of C-18 bonded columns for the analysis of ecdysteroids, and recommended the use of columns filled with spherical particles five microns in size. Larger 10 micron particles were not as efficient in separating the ecdysteroids from all interfering substances.

With respect to the mobile phase, Lafont et al. (1982) rejected the use of an acetonitrile-water mixture as this led to poor results with certain columns. Better results with more symmetrical peaks were obtained using acetonitrile-buffer systems.

The methods chosen for the identification of insect hormones reflect as far as is possible the comments of Lafont et al. (1982), which included off-line purification as part of the sample preparation procedure. It was not possible to follow their recommendations exactly, e.g. they advised a running temperature of 50° C and this was not possible with the equipment available.

6.4.2 Methods.

a) High pressure liquid chromatography.

Unless otherwise stated all HPLC equipment was obtained from Pharmacia LKB. The equipment used consisted of a 2156 Solvent Conditioner, a 2151 Variable Wavelength Monitor, which after measurements of U/V absorption spectra was set at 247 nm (for the detection of insect hormones) and 265 nm (for the detection of plant growth regulators), a 2152 HPLC Controller, a 2150 HPLC Pump, a 11300 Ultrograd Mixer Driver, a 2240 Alphaplot Recorder and a 20 μl injection loop (Rheodyne, Cotati, California, USA). The column used was a C-18, Spherisorb-ODS2, five microns, 4x100 mm. Solvent system 1: 17%

acetonitrile in 20 mM Tris-$HClO_4$ buffer, pH 8.5. Solvent system 2: A = H_2O, B = CH_3OH; 0% B -> 100% B, 30 minutes. Flow-rate: 0.5 ml/min. Temperature: ambient. All chemicals for HPLC were purchased from BDH and were of HPLC grade.

Since the column had been stored in methanol before use, both the equipment and column were flushed through with methanol at a flow-rate of 0.2 ml/min. Solvent system 1 was prepared by dissolving 2.42 g of Tris(hydroxymethyl)-methylamine in 900 ml of DDH_2O. The pH was adjusted to 8.5 using perchloric acid. The solvent was then made up to one litre and filtered through a 0.2 micron filter (Millipore) before being connected to a helium source for degassing. After degassing for 10 minutes the solvent was passed into the HPLC apparatus at a rate of 0.5 ml/min, from a concentration of 0% to 100% over five minutes. The flow rate was then reduced to 0.1 ml/min and left overnight. Solvent system 2 was prepared as above, but DDH_2O was used instead of the acetonitrile-Tris buffer.

b). Extraction of biological material.

Initial extraction and purification was carried out as detailed in Section 6.3.2. For some experiments, both colleterial sacs and ovaries were dissected out and kept separately.

1. Extraction for insect hormones involved, firstly the removal of lipids, proteins and compounds less polar the ecdysteroids; each sample of both colleterial fluid and ovary homogenate was added to five millilitres of chloroform and mixed for one minute. The resulting emulsion was added to a centrifuge tube and x6000 rpm for 20 minutes to separate the emulsion. Secondly, the aqueous layer was then decanted and retained, and the chloroform layer was rewashed with a further five

millilitres of DDH_2O, centrifuged and the aqueous layer combined with the first.

Off-line purification was performed using a SEP-PAK C-18 cartridge (Waters, Harrow, Middlesex, HA1 2YH); this was prepared by first flushing with five millilitres of methanol, then five millilitres of DDH_2O (Robinson et al. 1987). Then five millilitres of the aqueous extract was added to the SEP-PAK cartridge, flushed with five millilitres of 25% methanol (to collect the polar products of ecdysteroids if present). The cartridge was then flushed with five millilitres of 60% methanol (to collect the ecdysteroids if present). All liquids were added at a rate of approximately two ml/min (Lafont et al. 1982).

At each stage the U/V spectrum of the collected fraction was measured using a Philips PU 8700 Series UV/Vis. Spectrophotometer. Each fraction was then reduced to dryness under vacuum at 50° C and the residue was redissolved in 100 μl of Tris-buffer. After flushing the HPLC sample loader with solvent, 40 μl of sample was injected, so that it was completely full. When the sample loading valve was turned, 20 μl of sample was transferred to the HPLC column. The chromatograms obtained were compared with those obtained from fractions containing alpha-ecdysone, 20-hydroxyecdysone and makisterone A (Sigma), all extracted by the same methods as detailed above.

2. Separation by ultrafiltration was carried out only on the colleterial fluid. This was achieved using Centricon microconcentrators (Amicon Ltd., Stonehouse, Gloucestershire. GL10 2BJ). Three types of concentrators were used: each had a different molecular weight cutoff i.e. 30 kDa, 10 kDa and 3 kDa. Two millilitres of extracted colleterial fluid were placed in the sample reservoir of a 30 kDa concentrator and spun at 5000 x g for 40 minutes: the filtrate was collected, the pellet

resuspended and the procedure repeated, after which the filtrates were combined and freeze-dried. This residue was then resuspended in two millilitres of distilled water and centrifuged at 5000 x g in a 10 kDa concentrator for one hour; the pellet was washed and the filtrates freeze-dried. Finally, the freeze-dried filtrate from the 10 kDa concentrator was resuspended in one millilitre of distilled water and centrifuged at 6500 x g for four hours. Samples of all filtrates were run on an SDS-PAGE gel, using the methods detailed in Section 6.3.2: 20 μl samples from the 3 kDa concentrator were passed through the HPLC using Solvent system 2 (see above) and samples from the 10 kDa and 3 kDa were injected into Salix leaves as detailed in Section 6.2.2.

6.4.3 Results.

a) Insect hormones.

The U/V absorption spectra for the ovaries and colleterial fluid were similar, with a major peak in the 260 nm region. Extraction by chloroform did not change their overall shape. Even without lipids and proteins the spectrum for colleterial fluid was almost identical with that shown in Figure 6.1a, with an absorption peak at 266 nm. However, for both the ovaries and colleterial fluid this peak was absent in the 60% methanol wash faction i.e. the one that should contain ecdysterone or related compounds. It was also absent in the spectrum of the 100% methanol was from the SEP-PAK cartridge. From an inspection of spectra of the liquid that passed through the cartridge when the extract was first added and from the 25% wash it would seem that the material responsible for the 260 nm peak had been lost in these washes (see Table 6.4).

Although the HPLC chromatograms demonstrated good peaks for the ecdysteroids using both Solvent system 1 and 2, there were no such similar peaks for any of the factions obtained from the SEP-PAKs for any concentration of methanol flush. Therefore, either the concentrations of ecdysterone or 20-hydroxysterone were too low to be detected or more likely they were absent.

Fraction	Ovaries	Colleterial fluid
aqueous layer after chloroform extraction	258.9	266.0
waste from SEP-PAK	259.4	272.0
25% methanol wash	260	258.4
60% methanol wash	absent	absent
100% methanol wash	absent	absent

Table 6.4. Absorption peaks in the 240 to 260 nm region of fractions collected during off-line purification (nm).

The results obtained for the ultrafiltration demonstrated biological activity of both the 10 kDa filtrate and the 3 kDa filtrate. When samples of these filtrates were injected into Salix leaves (using the techniques detailed in Section 6.2.1), callus tissue developed over and above that visible in control experiments using distilled water. Ten trials of each filtrate were conducted and all the results obtained were consistent; with the 10 kDa and 3 kDa filtrates forming more callus material than the control experiments (see Plates 6.8 to 6.10). SDS-PAGE of the 30 kDa, 10 kDa and 3 kDa filtrates showed an absence of all proteins in the 3 kDa fraction (see Plate 6.12); this meant that the 3 kDa filtrate could be passed through the HPLC column without fear of contaminating the column packing. The chromatogram obtained using

Solvent system 2 is shown in Figure 6.4. Chromatograms were also obtained of the major natural and synthetic plant growth regulators, using Solvent system 2, peaks were obtained for abscisic acid, benzylaminopurine, gibberellic acid, indoleacetic acid, kinetin, zeatin and N^6-(isopent-2-enyl)adenosine, with the U/V detector set at 265 nm. None of these compounds, produced any peak that could be matched to any in Figure 6.4. The U/V spectra of the filtrates and the retentates, measured under the same conditions as shown above, demonstrated for the filtrates, strong absorption at a slightly lower wave length. Whereas before this had been in the 260 nm region, it was now for all filtrates in the 246 nm region. The 30 kDa was the only retentate to show a peak in 260 nm region; even after repeated washing this peak was still retained. All other retentates showed no major peaks in the 240 to 260 region.

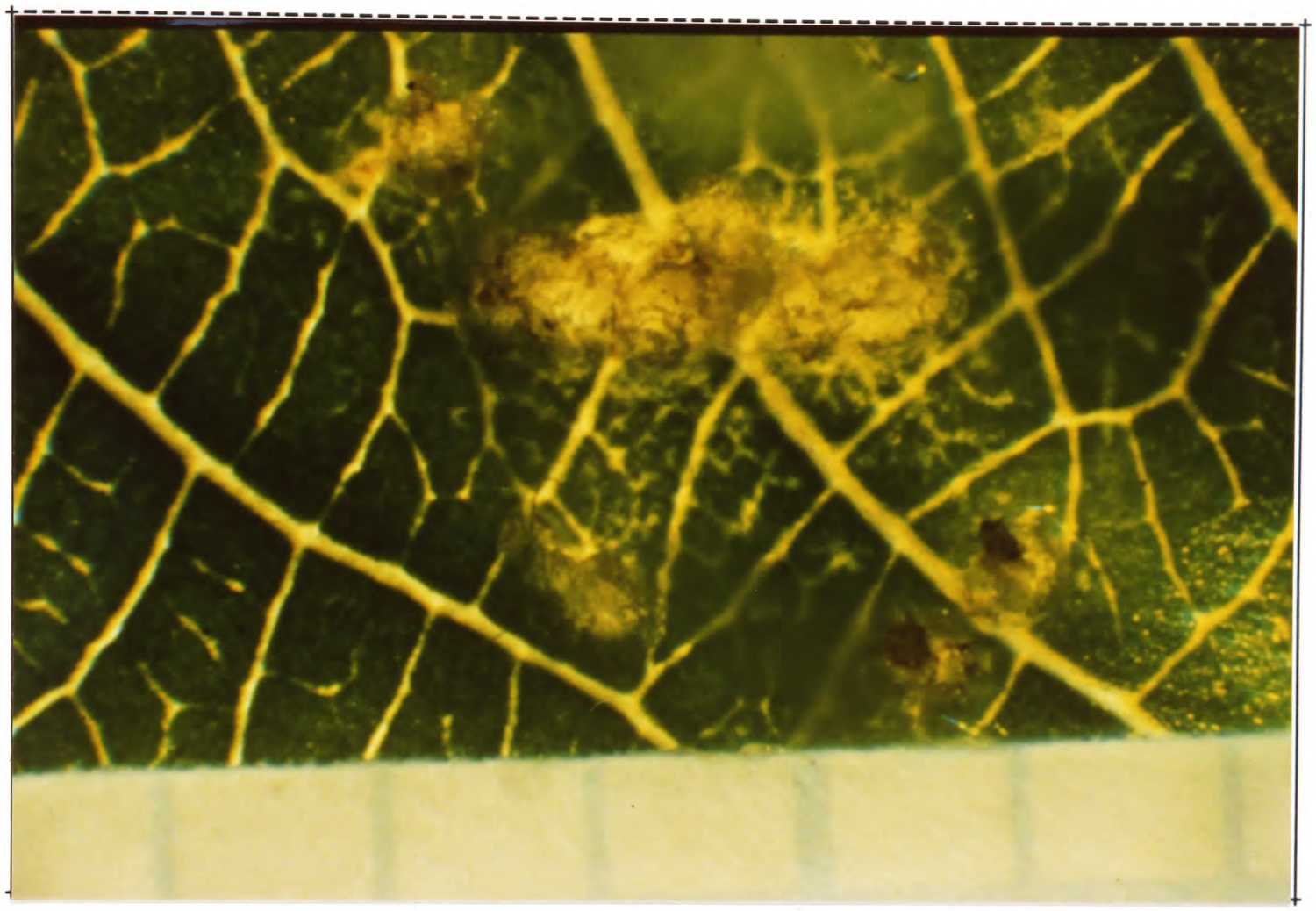


Plate 6.9. Callus tissue induced by the injection of the 10 kDa filtrate into Salix tissue. Nine days after inoculation (scale divisions mm).

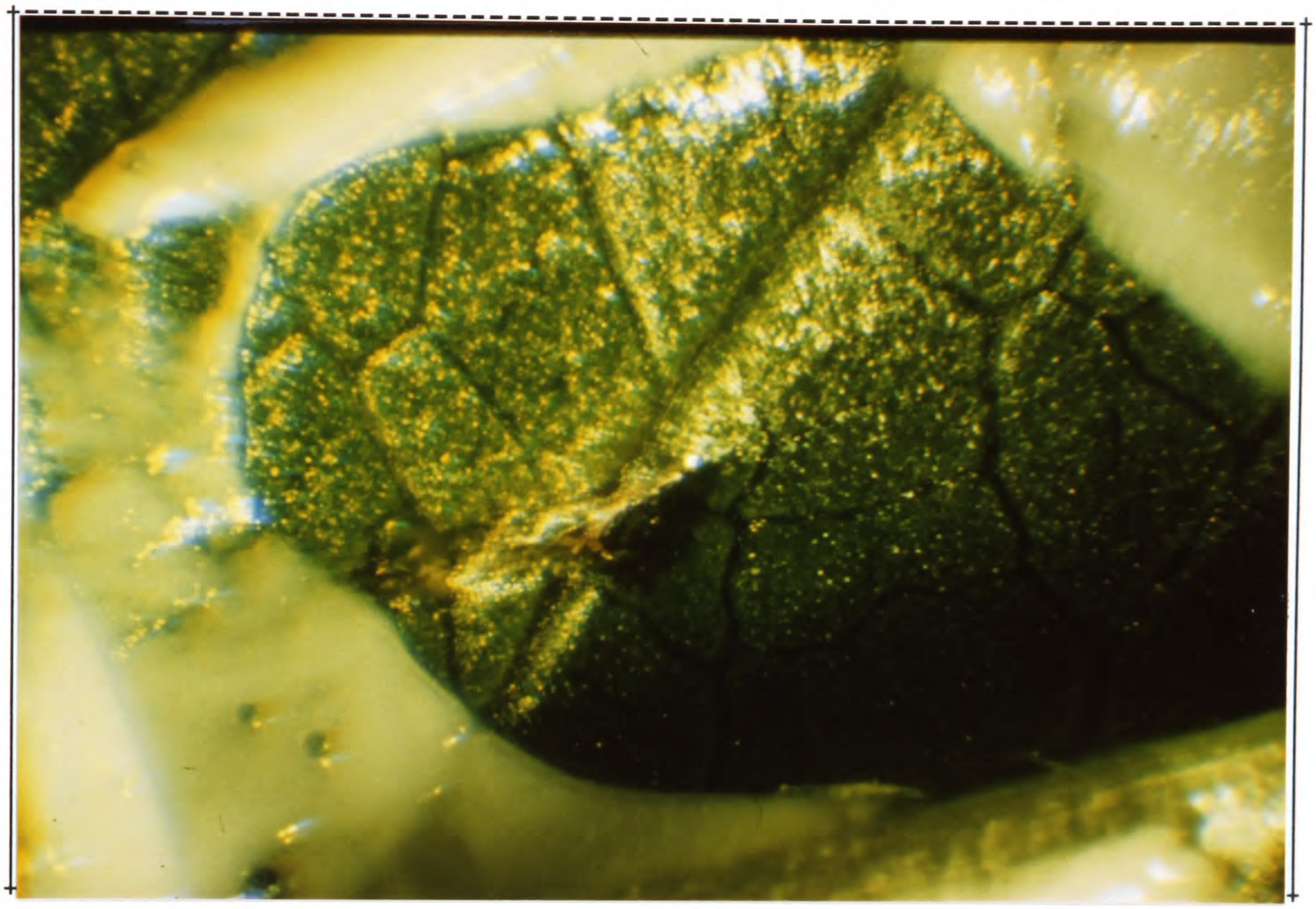


Plate 6.10. Callus tissue induced by the injection of the 3 kDa filtrate into Salix leaf tissue. Eight days after injection. Same magnification as Plate 6.9.

Plate 6.11. Necrotic issue produced by injecting distilled water into Salix leaf tissue. Eight days after inoculation (scale divisions mm).

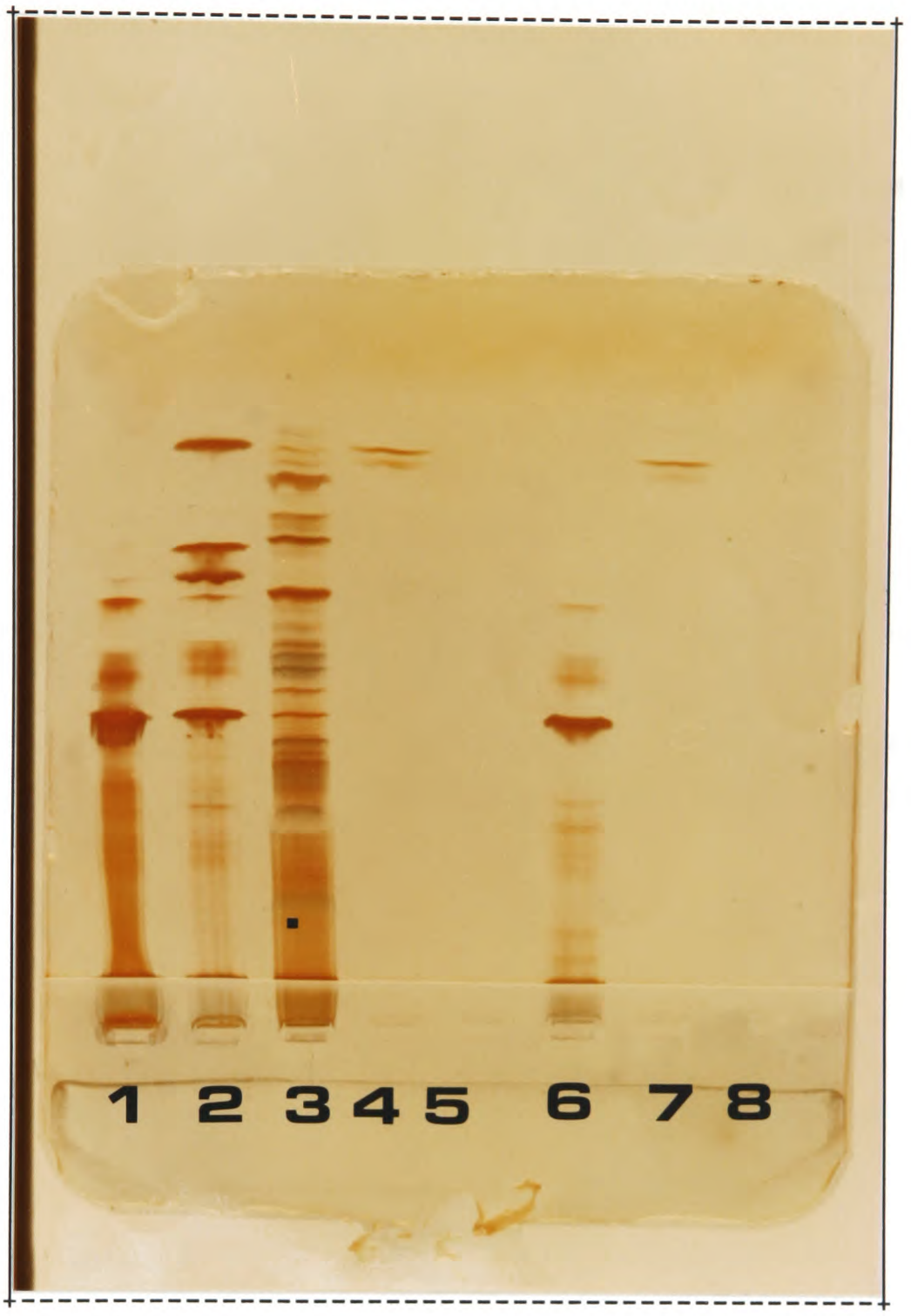


Plate 6.12. SDS-PAGE gel of filtrates produced by ultrafiltration. Lanes: (1) and (2) LMW molecular weight markers, (3) and (4) 30 kDa filtrate, (4) and (7) 10 kDa filtrate, (5) and (8) 3 kDa filtrate. Methods used as in Section 6.3.2. Silver staining. LANE (6) HMW markers.

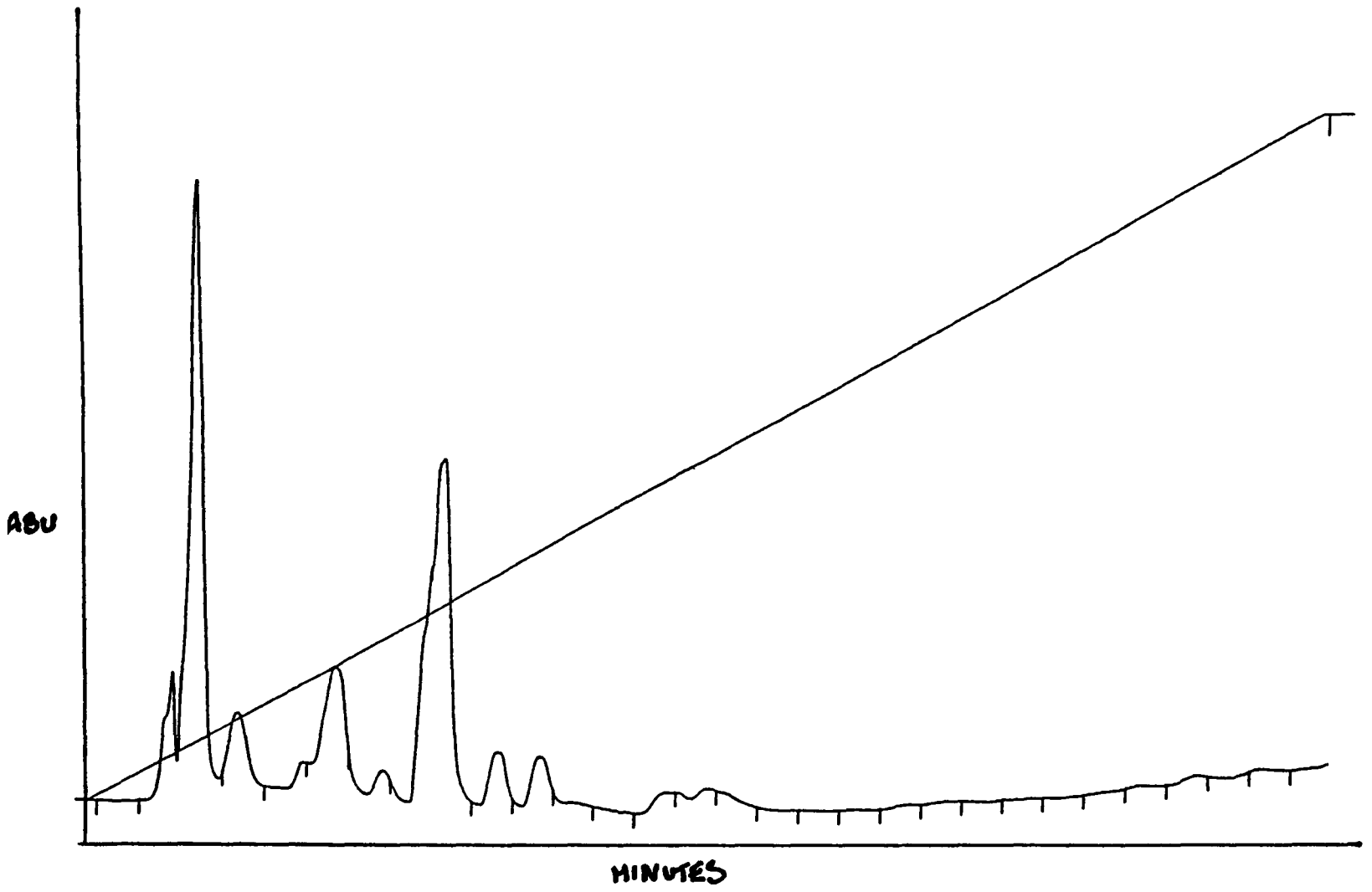


Figure 6.4. HPLC chromatogram of the 3 kDa filtrate. 0.08 ABU's, 265 nm, Solvent system 2. 10 μl sample. Chart speed 10 mm/min.

Fraction	Filtrate	Retentate
concentrator 30 kDa	245.6	264.8
concentrator 10 kDa	247.2	absent
concentrator 3 kDa	246.4	absent

Figure 6.5. Absorption peaks in the 240 to 260 nm region of the fractions collected by ultrafiltration (nm).

6.4.4 Discussion.

The results described in this section would seem to suggest that the cecidogen is a molecule of low molecular weight i.e. less than 3 kDa. If is of the same type as that isolated by McCalla et al. (1962), the work in this section has demonstrated that the compound involved is not only a growth-promoter, but is also a growth initiator - the cecidogen, at least within the condition of the bioassay.

The U/V spectra of the filtrates from the concentrators showed a shift from 260 nm to 247 nm. Although PAGE suggests that a protein was not present in the 3 kDa filtrate, the cecidogen could be a peptide of <25-30 amino acid residues; the reduced absorption at 280 nm would indicate no aromatic amino acids e.g. trytophan or tyrosine.

The retentate of the 30 kDa concentrator retained the 260 nm absorption pattern after repeated resuspension and recentrifugation. The filtrates of both the 10 kDa and 3 kDa concentrator demonstrated absorption at 247 nm, with their respective retentates showing no absorption in this region. The wavelength of 247 nm is that of the absorption for ecdysteroids, but neither of the naturally occuring ecdysteroids (ecdysone and 20 hydroxyecdysone) produced peaks on the HPLC chromatography to match any of those of the 3 kDa.

Due to lack of both insect material and time this aspect of the work had to stop. However, the possibility of another ecdysteroid, precursor or product of the ecdysteroid metabolic pathways being involved in gall formation remains an attractive suggestion and would be well work pursuing (see Section 3.3.4).

It is acknowledged that much of the work in this Chapter could be

improved upon and developed further. The season nature of the insects, prevented this from being possible in the three years allotted to this studentship. On a personal note, I believe that the techniques developed in this thesis provide a framework for the eventual identification of an insect cecidogen. Attempts were made to identify or at least classify the compound obtained in the HPLC chromatogram of the 3 kDa filtrate. The filtrate was, after derivatization passed through a GC-MS column, but only the mono-, di- and tri-trimethlysilyl derivatives of glycerol. This could have been a contaminant from the concentrators, although they were flushed through before use, or it could have been from the insect (see Section 4.3.2), there was no time to establish this.

CHAPTER SEVEN

DISCUSSION

> "The galls arising in plant tissues through the presence of parasitic insects and fungi are of peculiar interest and significance, and offer a most attractive field of investigation, abounding in problems awaiting elucidation".
>
> (Swanton, 1912: xi).

7.1 Introduction.

A plant gall is an example of a localized abnormal growth, induced by an external agent. Nearly all the major classes of plants and fungi contain examples of galling, but the highest percentage (98%) of all galls is found in the Angiosperms, with no part of the plant being exempt from the influence of a gall-former.

During gall development, plant cells are released from morphogenetic controls such that normal patterns of organization are disturbed, suppressed or abolished; previous work suggests that these effects are moderated via plant growth regulators. Insect-induced galls demonstrate morphological and anatomical specificity, but little is known of the initiating stimulus or of its mode of action. Reasons for the lack of mechanistic investigations into this field of study are

probably due, not only to the requirement for workers to be thoroughly familiar with the disciplines of both botany and entomology, but also to the seasonal nature of gall-inducers themselves.

Galling represents a utilization and depletion of a host's resources. Not only do gall-formers cause damage to staple foods e.g. species of Cecidomyiidae, such as Pachydiplosis oryzea (Wood Mason), gall the stems of species of Oryza L. (Gramineae), while Contarinia tritici (Kirby), Sitodiplosis mosellana (Géhin) and Haplodiplosis marginata (von Roser) gall other cereal crops, but galling insects also adversely affect many fruits and ornamentals. Conversely, gall-inducing insects have also been used as biological control agents e.g. Trichilogaster acaciaelongifoliae (Froggatt) (Eurytomidae) to control the weed Acacia longifolia (Andr.) Willdenow (Leguminosae), and Urophora affinis (Frauenfeld) biological control of diffuse and spotted knapweed (Centaurea diffusa Lamarck and Centaurea maculosa Lamarck, Compositae).

7.2 **Gall-induction by insects.**

A knowledge of how gall-formers induce dysfunction presents a model to aid the understanding of the mode of action of plant growth regulators and how these are mediated through the genome of the plant. Galling represents a highly specific link between organisms of different kingdoms, to such an extent that some authors view the gall as an extension of the insect's phenotype i.e. although the gall is plant tissue, the insect codes for the stimulus and the plant for the response. Thus, the gall is considered a product of two genomes.

The diverse nature of gall-formers and hosts suggests that there is no common causal mechanism. However, one example of the benefits ensuing from this type of work is that studies of crown-gall disease have yielded benefits for the plant geneticist and the Ti plasmid is now used as a vector for genetic material. Work undertaken for this thesis suggests that a mutualistic association is not responsible for gall-formation in at least one species of insect gall-former.

7.3 **Research undertaken.**

Most of the discussion concerned with the investigations carried out as part of this thesis, is dealt with in the relevant Sections. Briefly, investigations have been conducted using the Pontania proxima (Lepeletier) (Tenthredinidae)-Salix fragilis L. (Salicaceae) system, since supplies of both host and parasite were readily available in the field and in controlled conditions. The phenology and ecology of Pontania have been reviewed and methods of rearing, which resulted in an extension of its flight period, the sawfly have been devised.

The usefulness of this system is that gall initiation and growth occurs before the egg hatches. This work has shown that the stimulus for gall-induction (cecidogen) is a colleterial secretion injected by the female into the leaf during oviposition, which results in the formation of a procecidium before the larva hatches. The colleterial secretion is produced by the accessory glands, which are a common feature of Hymenoptera. Thus, a single event is the initiator, the continuous presence of a larva not being required. The development of a bioassay which involves micro-injection of colleterial fluid into leaf mesophyll tissue has meant that, for the first time, for this system,

it was possible to begin an investigation into gall-induction mechanisms.

This has been essentially pioneering work as there are few data on the composition of this fluid within the family Tenthredinidae (Hymenoptera: Symphyta). This thesis also contains the first detailed examination of the ultrastructure of the female reproductive system for any member of this family.

7.4 **Experimental limitations.**

This project has covered many aspects of gall biology from ecology, and morphology to biochemistry. Not only have techniques had to be learned, but limitations of time and biological material have meant that many of methods employed have not been optimized e.g. extraction, separation and HPLC. All the results contained in this thesis are of more than one trial, but refinement and more objective assessments are needed e.g. of the bioassay.

In the course of this study, more than 20 000 galls were collected - a time consuming task, but even so there were not enough specimens of Pontania proxima available for further analysis of the colleterial fluid by other methods e.g. GC-MS.

Tissue culture techniques would appear to be of use in the study of insect-plant interaction. Unfortunately, Salix tissue was more recalcitrant than expected and work on the development of conditions for callus formation, micro-propagation and the fusion of protoplast material was not achieved in the time available. Although this work was not completed it yielded useful information, that provides a basis for future studies

7.5 **Future research.**

The broad aim of further research should be the separation, isolation and identification of the cecidogen. Within Britain there are approximately 400 species of Tenthredinidae, with the highest number in the tribe Nematini. Within this tribe there are examples of galling and non-galling insects e.g. species of Pontania O. Costa which induce leaf-galls on species of Salix L., while the larvae of Pristiphora pallipes Lepeletier and Nematus ribesii (Scopoli) do not inhabit galls, but are notorious pests of species of Ribes L. (Grossulariaceae), while the larvae of Hoplocampa flava (L.) attack the fruits of Prunus spinosa L. (Rosaceae). Since all these species are plentiful and easily obtainable so that breeding would be a low-cost undertaking, a comparative study of both the ultrastructure of the female reproductive system and the colleterial fluid of gallers and non-gallers would be a feasible extension of the work. It would also deepen our understanding of plant/insect relationships at both ecological and biochemical levels.

7.6 **Methodology.**

The identification of the cecidogen within the colleterial fluid of a single species of gall-former would be a complex and time consuming task. However, by comparing the colleterial fluids from non-galling species, with those of gall-forming species, this problem would be circumvented by the elimination of common compounds within these closely-allied organisms. This approach should also provide valuable data relevant to the possible control of serious insect-pests by more

selective means. This would be the first time that such a comparative analysis would have been undertaken.

The methods employed would be those classically used for the extraction and separation of biologically active material. Many hypotheses exist to help explain the phenomenon of gall induction. Cytokinins, polar auxin transport, gibberellins, amino acids, abscisic acid, ecdysteroids and viral mutualism have all been implicated at one time or another. The described in this thesis has established that the cecidogen is probably a low molecular weight compound suitable for separation by ultrafiltration, solid phase extraction and HPLC. When these methods have been optimized and linked to the bioassay, it will be possible, for the first time, to isolate the cecidogen from a gall-forming insect. Once the type of compound involved has been identified, monoclonal, GC-MS and NMR facilities will be useful in extending this aspect of the work,

7.7 **Conclusions.**

Over two millennia after Hippocrates and Theophrastus commented on the attributes of insect-galls, Malpighi established the link between insects and galls, but only now would it seem that there is a real possibility of finally establishing (for at least one species pair) the mechanism whereby insects are able to manipulate the meristematic tissue of their host. This is long overdue but the background work has now been completed and the quotation heading this Chapter is as relevant today as it was in 1912.

REFERENCES

ABRAHAMSON, W. G. & K. D. McCREA, (1986). The impacts of galls and gallmakers on plants. Proceedings of the Entomological Society of Washington 88(2): 364-367.

ADLER, H., (1894). Alternating generations. A biological study of oak galls and gall flies. (trans. and ed. STRATON, C. R., [of 1881 edition]). Pp. xl + 198. Clarendon Press, Oxford.

AHUJA, M. R., (1983). Somatic cell differentiation and rapid clonal propagation of aspen. Silvae Genetica 32: 131-135.

AHUJA, M. R., (1987). In vitro propagation of poplar and aspen. Pp. 207-223, in BONGA, J. M. & D. J. DURZAN (eds.), Cell and tissue culture in forestry. Volume Three. Case histories: gymnosperms, angiosperms and palms. Pp. xviii + 416. Martinus Nijhoff, Dordrecht, Netherlands.

ALDERSON, P. G., (1986). Micropropagation of woody plants. Pp. 37-52, in ALDERSON, P. G. & W. M. DULLFORCE (eds.), Micropropragation in horticulture: practice and commercial problems. Proceedings of the Institute of Horticulture Symposium, University of Nottingham School of Agriculture, 24-26 March 1986. Pp. 216. Institute of Horticulture, London.

ALLARD, R.W., (1975). The mating system and microevolution. Genetics 77 (supplement): 115-126.

AMERSON, H. V. & R. L. MOTT, (1982). Phytopathology and tissue culture alliances. Pp. 208-230, in BONGA, J. M. & D. J. DURZAN (eds.), Tissue culture in forestry. Pp. xi + 420.

Martinus Nijhoff/Junk, The Hague.

ANANTHAKRISHNAN, T. N., (1984). Adaptive strategies in cecidogenous insects. Pp. 1-9, in ANANTHAKRISHNAN, T. N. (ed.), Biology of gall insects. Pp. xii + 362. Arnold, London.

ANDERS, F., (1961). Untersuchungen über des cecidogene Princip der Reblaus Viteus (=Phylloxera) vitifolii (Shimer). III. Biochemische Untersuchungen über das galleninduzierende Agenz. Biologisches Zentralblatt 80: 199-233.

ANGRISH, R. & K. K. NANDA, (1982). Dormancy and flowering process in reproductive buds of Salix babylonica cultured in vitro. Zeitschrift für Pflanzenphysiologie 106: 263-269.

ASKEW, R. R., (1984). The biology of gall wasps. Pp. 223-272, in ANANTHAKRISHNAN, T. N. (ed.), Biology of gall insects. Pp. xii + 362. Arnold, London.

ATKINS, E. D. T., N. E. FLOWER & W. KENCHINGTON, (1966). Studies on the oöthecal protein of the tortoise beetle, Apsidomorpha. Journal of the Royal Microscopical Society 86: 123-135.

BAILEY, N. T., (1981). Statistical methods in biology. 2nd. Ed. Pp. viii + 216. Arnold, London.

BALL, E. & P. C. JOSHI, (1965). Divisions in isolated cells of palisade parenchyma of Arachis hypogaea. Nature (London) 207: 213-214.

BAIRD, A. B., (1921). Some notes on the female reproductive organs in the Hymenoptera. Proceedings of the Acadian Entomological Society, Truro, Nova Scotia 7: 73-88.

BARNES, H. F., (1931). Further results of an investigation into the resistance of basket willows to button gall formation. Annals of Applied Biology 18: 75-83.

BARROS, R. S. & S. J. NEIL, (1987). Shoot growth in willow (Salix viminalis) in relation to abscisic acid, plant water status and photoperiod. Physiologia Plantarum 70(4): 708-712.

BASTIN, H., (1921). Vegetable galls. Journal of Bath and West and Southern Counties Society. 5th. series 15: 30-56.

BENES, K., (1968). Galls and larvae of the European species of genera Phyllocolpa and Pontania (Hymenoptera: Tenthredinidae). Acta Entomologica Bohemoslovaca 65: 112-137.

BENSON, R. B., (1940). Further sawflies of the genus Pontania Costa (Hymenoptera: Symphyta) in Britain. The Entomologist's Monthly Magazine 76: 88-94

BENSON, R. B., (1941). On some Pontania species, with a revision of the proxima and herbaceae groups (Hymenoptera: Symphyta). Proceedings of the Royal Entomological Society of London. Series B 10: 131-136.

BENSON, R. B., (1943). Collecting sawflies. The Amateur Entomologist 7(40): 36-42.

BENSON, R. B., (1950). An introduction to the natural history of British sawflies. Transactions of the Society for British Entomology 10(2): 45-142.

BENSON, R. B., (1954). British sawfly galls of the genus Nematus [Pontania] on Salix. (Hymenoptera: Tenthredinidae). Journal of the Society for British Entomology 4(a): 206-211.

BENSON, R. B., (1958). Handbook for the identification of British insects. Volume Six, part 2(c). Hymenoptera. Symphyta. Pp. vi. + 252. Royal Entomological Society of London.

BENSON, R. B., (1960a). A new genus for the leaf-edge-rolling Pontania (Hymenoptera: Tenthredinidae). The Entomologist's Monthly Magazine 96: 59-60.

BENSON, R. B., (1960b). Studies in Pontania (Hymenoptera: Tenthredinidae). Bulletin of the British Museum (Natural History). Entomology 8(9): 369-384.

BERGMAN, L., S. von ARNOLD & T. ERIKSON, (1985). Effects of N^6-benzyladenine on shoots of five willow clones (Salix spp.) cultured in vitro. Plant Cell, Tissue and Organ Culture 4: 135-144.

BERGMAN, L., G. SANDBERG, S. von ARNOLD & T. ERIKSON, (1986). Indole-3-acetic acid content in buds of five willow genotypes. Journal of Plant Physiology 125(5): 485-489.

BEYERINCK, M. W., (1887). De la cecide produite par le Nematus capreae sur le Salix amygdalina. Archives Néerlandaises des Sciences Exactes et Naturelles 21: 475-492.

BEYERINCK, M. W., (1888). Ueber das Cecidium von Nematus capreae auf Salix amygdalina. Botanische Zeitung 46: 1-11; 17-28.

BHOJWANI, S. S., (1980). Micropropagation method for a hybrid willow. (Salix matsudana X alba NZ-1002). New Zealand Journal of Botany 18: 209-214.

BIRCH, M. L., (1974). [Studies on gall formation in Viola odorata by Dasyneura affinis Kieffer (Diptera: Cecidomyiidae), with emphasis on the experimental modification of this process.] Unpublished Ph.D. thesis, University of Reading.

BISSETT, J. & A. BORKENT, (1988). Ambrosia galls: the significance of fungal nutrition in the evolution of the Cecidomyiidae

(Diptera). Pp. 203-225, In PIROZYNSKI, K. A. & D. L. HAWKESWORTH (eds.), Coevolution of fungi with plants and animals. pp. xii + 285. Academic Press, London.

BLUNDEN, G. & S. B. CHALLEN, (1965). Red pigmant in leaf galls of Salix fragilis L. Nature (London) 208(5008): 388-389.

BÖHNER, K., (1935). Geschichte der Cecidologie. II Teil: Botanik und Entomologie. Pp. vii + 710. Nemayer, Mittelwald, (Bayern).

BONGA, J. M. & J. CLARK, (1965). The effect of beta-inhibitor on histogenesis of balsam fir bark cultured in vitro. Forest Science 11(3): 271-278.

BOYSEN JENSEN, P., (1948). Formation of galls by Mikiola fagi. Physiologia Plantarum 1: 95-108.

BRAAM, J. & R. W. DAVIS, (1990). Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. Cell 60: 357-364.

BRONNER, R., (1977). Contribution a l'étude histochimique des tissus nourriciers des zoocecidies. Marcellia 40: 1-134.

BRONNER, R.,(1981). Observations on cynipid galls modified by inquilines. Cecidologia Internationale 2: 53-61.

BRONNER, R., (1983). Adaption insect-plant in cynipid galls. Pp. 61-68, in MARGARIS, N.S., M. ARIANOUTSOU-FARAGGITAKI & R.J. REITER (eds.), Adaptions to terrestrial environments. Plenum Press, London.

BROWN, C. L. & H. E. SOMMER, (1982). Vegetative propagation of dicotyledonous trees. Pp. 109-149, in BONGA, J. M. & D. J. DURZAN (eds.), Tissue culture in forestry. Pp xi + 420. Martinus Nijhoff/Junk, The Hague.

BULTMAN. T. L. & S. H. FAETH, (1985). Patterns of intra- and interspecific association in leaf-mining insects on three oak species. Ecological Entomology 10: 121-129.

BUTT, A. D., (1985). A general method for the high-yield isolation of mesophyll protoplasts from deciduous tree species. Plant Science. Irish Republic 42: 55-59.

BYCHENKOVA, É. A. & A. DAVID, (1987). Callus formation and organogenesis in tissues of leaves of Populus balsamifera L. cultivated in vitro. Soviet Plant Physiology 25: 216-222.

BYERS, J. A., J. W. BREWER & D. W. DENNA, (1976). Plant growth hormones in pinyon insect galls. Marcellia 39: 125-134.

CAIN, A. L., (1957). Logic and memory in Linnaeus's system of taxonomy. Proceedings of the Linnean Society of London 169: 144-163.

CALTAGIRONE, L. E., (1964). Notes on the biology, parasites and inquilines of Pontania pacifica, a leaf gall incitant on Salix lasiolepis. Annals of the Entomological Society of America 57: 279-291.

CAMERON, P., (1885). A monograph of the British phytophagous Hymenoptera. Volume Two. Pp. vi. + 233. Ray Society, London.

CARLETON, M., (1939). The biology of Pontania proxima (Lep.) the bean gall sawfly of willows. Journal of the Linnean Society of London (Zoology) 40(275): 575-624.

CARLISLE, D. B., D. J. OSBORNE, P. E. ELLIS & J. E. MOORHOUSE, (1963). Reciprocal effects of insect and plant-growth substances. Nature (London) 200: 1230.

CARTER, W., (1962). Insects in relation to plant disease. Pp. xiv + 705.

Interscience Publishers, London.

CHALUPA, V., (1983). In vitro propagation of willows (Salix spp.), European mountain-ash (Sorbus aucuparia L.) and black locust (Robinia pseudoacacia L.). Biologia Plantarum 25(4): 305-307.

CHALUPA, V., (1987). European hardwoods. Pp. 224-246, in BONGA, J. M. & D. J. DURZAN (eds.), Cell and tissue culture in forestry. Volume Three. Case histories: gymnosperms, angiosperms and palms. Pp. xviii + 416. Martinus Nijhoff Publishers, Dordrecht, Netherlands.

CHAPMAN, R. F., (1982). The insects. Structure and function. 3rd. Ed. Pp. xiv + 917. Hodder and Stoughton, London.

CHEEMA, G. S., (1989). Somatic embryogenesis and plant regeneration from cell suspension and tissue cultures of mature Himilayan poplar (Populus ciliata). Plant Cell Reports 8: 124-127.

CHMELAR, J., (1974). Propagation of willows by cuttings. New Zealand Journal of Forestry Science 4: 185-190.

CLANCY, K. M. & P. W. PRICE, (1987). Rapid herbivore growth enhances enemy attack: sublethal plant defences remain a paradox. Ecology 68(3): 733-737.

CLANCY, K. M., P. W. PRICE & T. P. CRAIG, (1986). Life history and natural enemies of an undescribed sawfly near Pontania pacifica (Hymenoptera: Tenthredinidae) that forms leaf galls on arroyo willow, Salix lasiolepis. Annals of the Entomological Society of America 79: 884-892.

CLAPHAM, A. R., T. G. TUTIN & E. F. WARBURG, (1981). Excursion flora of the British Isles. 3rd. Ed. Pp. xxxiii + 499. Cambridge University Press, London.

COCKERELL, T. D. A., (1890). Galls. Nature (London) 41: 344.

COMRIE, L. C., (1938). Biological and cytological observations on tenthredinid parthenogenesis. Nature (London) 142: 877-878.

CONNOLD, E. T., (1901). British vegetable galls. An introduction to their study. Pp. xii + 312. Hutchinson, London.

COPLAND, M. J. W. & P. E. KING, (1971). The structure and possible function of the reproductive system in some Eulophidae and Tetracampidae. Entomologist 104: 4-28

CORNELL, H. V., (1983). The secondary chemistry and complex morphology of galls formed by the Cynipinae (Hymenoptera): why and how? American Midland Naturalist 110(2): 225-232.

CORNER, E. J. H., (1963). A dipterocarp clue to the biochemistry of Durianology. Annals of Botany. New Series 27: 339-341.

CORNER, E. J. H., (1981). Angiosperm classification and phylogeny: a critism. Botanical Journal of the Linnean Society 82: 81-87.

COSENS, A., (1912). A contribution to the morphology and biology of insect galls. Transactions of the Canadian Institute 9: 297-387.

COSENS, A., (1916). The founding of the science of cecidology. 46th. Annual Report of the Entomological Society. Ontario for 1915: 88-93.

COSENS, A. & T. A. SINCLAIR, (1916). Aeriferous tissue in willow galls. The Botanical Gazette 62: 210-225.

CRAIG, T. P., P. W. PRICE, K. M. CLANCY, G. L. WARING & C. F. SACHI, (1988). Forces preventing coevolution in the three-trophic-level system: willow, a gall-forming

herbivore, and parasitoid. Pp. 57-80, in SPENCER, K. C. (ed.), Chemical Mediation of Coevolution. Pp. 1000. Academic Press, San Diego, California.

CRAIG, T. P.., P. W. PRICE & J. K. ITAMI, (1986). Resource regulation by a stem-galling sawfly on arroyo willow. Ecology 67: 419-425.

CRAM, W. H. & C. H. LINDQUIST, (1982). Refrigerated storage for hardwood cuttings of willow and poplar. Tree Planters' Notes 33(4): 3-5.

CRONQUIST, A., (1981). An integrated system of classification of flowering plants. Pp. xviii + 1262. Columbia University Press, New York.

CZECZUGA, B., (1977). Carotenoids in leaves and their galls. Marcellia 40: 177-180.

DARLINGTON, A., (1968). The pocket encyclopedia of plant galls. Pp. 191. Blandford Press, London.

DARWIN, C., (1906). The origin of species by means of natural selection or the preservation of favoured races in the struggle for life. 6th. Ed. Pp. xxxi + 703. Murray, London.

DAGUIN, F. & R. LETOUZÉ, (1985). Relations entre hypolignification et état vitreux chez Salix babylonica en culture in vitro. Rôle de la nutrition ammonicale. Canadian Journal of Botany 63(2): 324-326.

DAGUIN, F. & R. LETOUZÉ, (1986). Ammonium-induced vitrification in cultured tissues (of Salix babylonica). Physiologia Plantarum 66(1): 94-98.

DAWKINS. R., (1989). The extended phenotype. Pp. viii + 307. Paperback edition with corrections. Oxford University Press,

Oxford.

DAWSON, R. M., D. C. ELLIOT, W. M. ELLIOT & K. M. JONES, (1986). Data for biochemical research. 3rd. Ed. Pp. xii + 580. Oxford University Press, Oxford.

DAWSON, W. M., (1988). Production of biomass from short-rotation coppice willow in Northern Ireland 1974-1987. Metsäntutkimuslaitoksen Tiedonantoja 304: 91-100.

DENNILL, G. B., (1988). Why a gall former can be a good biocontrol agent: the gall wasp Trichilogaster acaciaelongifoliae and the weed Acacia longifolia. Ecological Entomology 13: 1-19.

DETHIOUX, M. & P. van DAMME, (1987). Essai de stabilisation de berges au moyen de végétaux ligneux. Cas d'un bouturage le long du Geer. Notes Techniques du Centre d'Ecologie Forestière et Rurale Belgium No. 52. Pp. 16. Fac. des Sciences Agronomiques, Gembloux, Belgium.

DIELEMAN, F. L., (1969). Effects of gall midge infestation on plant growth and growth regulating substances. Entomologia Experimentalis et Applicata 12: 745-749.

DODDS, J. H. & T. BENGOCHEA, (1983). Principles of plant genetic engineering. Outlook on Agriculture 12: 16-21.

DODDS, J. H. & L. W. ROBERTS, (1985). Experiments in plant tissue culture. 2nd. Ed. Pp. xvii + 232. Cambridge University Press, Cambridge.

DOUGALL, D. K., (1980). The use of tissue cultures in studies of metabolism. Pp. 627-642, in STUMPF, P. K. & E. E. CONN (eds.-in-chief). The biochemistry of plants: a comprehensive treatise. Volume Two. Metabolism and respiration. DAVIES, D. D. (ed.). Pp. xvi + 687.

Academic Press, London.

DOUGLAS, G. C., (1985). Formation of adventitious buds in stem internodes of Populus hybrid TT32 cultured in vitro: effects of sucrose, zeatin, IAA and ABA. Journal of Plant Physiology 121(3): 225-231.

DUDA, U. & A. KACPERSKA, (1983). Frost tolerance estimation in callus derived from poplar (Populus nigra) and winter rape plants using three different methods. Zeitschrift für Pflanzenphysiologie 111: 69-73.

DUDITS, D., G. Y. HADLACZKY, G. Y. BAJSZAR, C. S. KONCZ, G. LáZáR HORVáRTH, (1979). Plant regeneration from intergeneric cell hybrids. Plant Science Letters 15: 101-112.

DURZAN, D. J., S. C. CHAFE & S. M. LOPUSHANSKI, (1973). Effects of environmental changes on sugars, tannins and organized growth in cell suspension cultures of white spruce. Planta (Berlin) 113: 241-249.

EDSON, K. M., S. B. VINSON, D. B. STOLTZ & M. D. SUMMERS, (1981). Virus in a parasitoid wasp: suppression of the cellular immune response in the parasitoid's host. Science 211: 582-583.

ELZEN, G. W., (1983). Cytokinins and insect galls. Comparative Biochemistry and Physiology 76A(1): 17-19.

ENGELBRECHT, L., U. ORBAN & W. HEESE, (1969). Leaf-miner caterpillars and cytokinins in the "green islands" of autumn leaves. Nature (London) 223: 319-321.

ENGELBRECHT, L., (1971). Cytokinin activity in larval infected leaves. Biochemie und Physiologie der Pflanzen 162: 9-27.

ESTABLE-PUIG, J. F., W. C. BAUER & J. M. BLUMBERG, (1965). Technical note. Paraphenylenediamine staining of osmium-fixed, plastic

embedded tissue for light and phase microscopy. Journal of Neuropathology and Experimental Neurology 24: 531-535.

EVANS, H. F. & P. F. ENTWISTLE, (1987). Viral diseases. Pp. 257-322, in FUXA, J. R. & Y. TANADA (eds.), Epizootiology of insect diseases. Wiley, New York.

EVANS, P. K. & E. C. COCKING, (1977). Isolated plant protoplasts. Pp. 103-135, in STREET, H. E. (ed.), Plant Tissue and Cell Culture. Botanical monographs. Volume Eleven. 2nd. Ed. Pp. x + 614. Blackwell Scientific, Oxford.

EVERS, P. W., J. DONKERS, A. PRAT & E. VERMEER, (1988). Micropropagation of forest trees through tissue culture. Pp. vii + 84. Pudoc, Wageningen, Netherlands.

FAGIN, M. M., (1918). The use of insect galls. The American Naturalist 52: 155-176.

FEHRNSTRÖM, H. & U. MOBERG, (1977). SDS and conventional polyacrylamide gel electrophoresis with LKB 2117 Multiphor. Application Note 306. LKB-Produkter AB Bromma, Sweden.

FERNANDES, G. W. & P. W. PRICE, (1988). Biogeographical gradients in galling species richness. Oecologia 76: 161-167.

FEUTRY, A. & R. LETOUZÉ (1984). Properties of hydroxycinnamate: CoA ligase from stem of Salix babylonica cultured in vitro. Phytochemistry 23(8): 1557-1559.

FOLKARD, R., (1892). Plant lore, legends and lyrics. 2nd. Ed. Pp. xxiv + 610. Sampson Low Marston, London

FRISCH, J. L., (1766). Beschreibung von allerlen Insecten in Deutschland. Underer Theil (1753): 22-24. pl.iv. Ricolai, Berlin.

FRITZ, R. S., W. S. GAUD, C. F. SACCHI & P. W. PRICE, (1987). Patterns of

intra- and interspecific association of gall-forming sawflies in relation to shoot size on their willow host plant. Oecologia (Berlin) 73: 159-169.

FRITZ, R. S. & P. W. PRICE, (1988). Genetic variation among plant and insect community structure: willows and sawflies. Ecology 69(3): 845-856.

FRITZ, R. S., C. F. SACCHI & P. W. PRICE, (1986). Competition verses host plant phenotype in species composition: willow sawflies. Ecology 67(6): 1608-1618.

GALTON, A. W. & P. J. DAVIES, (1970). Control mechanisms in plant development. Foundations of developmental biology series. Pp. 184. Prentice-Hall, New Jersey.

GASPAR. Th., C. KEVERS, P. DEBERGH, L. MAENE, M. PAQUES & Ph. BOXUS, (1987). Vitrification: morphology, physiological, and ecological aspects. Pp 152-166, in BONGA, J. M. & D. J. DURZAN (eds.), Cell and Tissue Culture in Forestry. Volume One. General Principles and Biotechnology. Pp. xiv + 422. Martinus Nijhoff Publishers, Dordrecht, Netherlands.

GAULD, I. & B. BOLTON, (1988). The Hymenoptera. Pp. xi + 332. British Museum (Natural History) and Oxford University Press, Oxford.

GEORGE, E. F., D. J. M. PUTTOCK & H. J. GEORGE, (1987). Plant culture media. Volume One. Formulations and uses. Pp. vi + 567 Exegetics Ltd, Wiltshire.

GEUNS, J. M. C., (1978). Steroid hormones and plant growth and development. Phytochemistry 17: 1-14.

GLASS, B., (1968). The germination of the idea of biological species. Pp.

30-48, in BLASS, B., O. TEMKIN & W. L. STRAUS (eds.), Forerunners of Darwin 1745-1859. Pp. xx + 471. Paperback edition of 1959 edition. Johns Hopkins Press, Baltimore.

GORDON, A. H., (1971). Electrophoresis of proteins in polyacrylamide and starch gels. WORK, T. S. & E. WORK (eds.), Laboratory Techniques in Biochemistry and Molecular Biology. Pp. 149. North-Holland Publishing Company, London.

GREENE, E. L., (1983). Landmarks of Botanical History. Volumes One & Two. EGERTON, F. N. (ed.). Pp. x + 505, pp. 506-1139. Stanford University Press, California.

GROUT, B. W. W., J. H. M. WILLISON & E.C. COCKING, (1973). Interactions at the surface of plant protoplasts, an electrophoretic and freeze-etch study. Journal of Bioenergetics 4: 311-328.

HAGEDORN, H. H., (1974). The control of vitellogenesis in the mosquito Aedes aegypti. American Zoologist 14: 1207-1217.

HARBORNE, J. B., (1984). Phytochemical methods. A guide to modern techniques of plant analysis. 2nd. Ed. Pp. xii + 288. Chapman and Hall, London.

HARBORNE, J. B., (1988). Introduction to ecological biochemistry. 3rd. Ed. Pp. xv + 356. Academic Press, London.

HELLER, R., (1953). Recherches sur la nutrition minérale des tissues végétaux cultivés in vitro. Annales des Sciences Naturelles Botanique et Biologie Vegetale, 11^{e} série 14: 1-223.

HENDRIX, S. D. & R. L. JONES, (1972). The activity of beta-ecdysone in four gibberellin bioassays. Plant Physiology 50: 199-200.

HESLOP HARRISON, J. W., (1927). Experiments on the egg-laying instincts of the sawfly Pontania salicis Christ., and their bearing on the inheritance of acquired characters; with some remarks on a new principle in evolution. Proceeding of the Royal Society of London. Series B: Biological Sciences 101:115-126.

HIGTON, R. N., (1989). Historical perspectives in the development of cecidology. Cecidology 4(3): 5-9.

HILDEBRAND T. A. C., A. J. RIKER & B. M. DUGGAR, (1946). The influence of the compositions of the medium on growth in vitro of excised tobacco and sunflower tissue cultures. American Journal of Botany 33: 591-597.

HOFFMAN, A., A. R. MILLER & W. L. PENGELLY, (1985). Characterization of polyphenols in cell walls of cultured Populus trichocarpa tissues. Phytochemistry 24(11): 2685-2687.

HOLLIS, W. A., (1889). Galls. Nature (London) 41: 131.

HOLLIS, W. A., (1890). Galls. Nature (London) 41: 272.

HORI, K. & P. MILES, (1977). Multiple plant growth-promoting factors in the salivary glands of plant bugs. Marcellia 39: 399-400.

HORN, D. H. S. & R. BERGAMASCO, (1985). Chemistry of ecdysteroids. Pp. 185-248, in KERKUT, G. A. & L. I. GILBERT (eds.), Comprehensive insect physiology, biochemistry and pharmacology. Volume Seven. Pp. xvi + 564. Pergamon Press, Oxford.

HOVANITZ, W., (1959). Insects and plant galls. Scientific American 201(5): 151-162.

HUTCHINSON, H. P. & H. G. H. KEARNS, (1930). Insect pests of willows.

Nature (London) 125: 201.

IHERING, H. von., (1907). Die Cecropien und ihre Schutzameisen. Botanische Jahrbücher 39: 666-714.

INGRAM, D. S., (1973). Growth of plant parasites in tissue culture. Pp. 392-421, in STREET, H. E. (ed.), Plant cell and tissue culture. Pp viii + 503. Blackwell Scientific Publications, Oxford.

ITAI, C. & Y. VAADIA, (1965). Kinetin-like activity in root exudate of water stressed sunflower plants. Physiologia Plantarum 18: 941-944.

IWATA, K., (1958). The comparative anatomy of the ovary in Hymenoptera. Part II. Symphyta. Mushi 31(5): 46-60.

JACOBS, M. & P. H. RUBERY, (1988). Naturally occurring auxin transport regulators. Science 241: 346-349.

JACOBS, W. P. & H. B. SUTHERS, (1971). The culture of apical buds of Xanthium and their use as a bioassay for flowering activity of ecdysterone. American Journal of Botany 58(9): 836-843.

JOLIVET, P., (1986). Insects and plants. Parallel evolution and adaptations. Flora and Fauna Handbook No. 2. Pp. xii + 197. E. J. Brill/Flora and Fauna Publications, New York.

JOSHI, P. C. & E. BALL, (1968a). Growth of isolated palisade cells of Arachis hypogaea in vitro. Developmental Biology 17: 308-325.

JOSHI, P. C. & E. BALL, (1968b). Growth values and regenerative potentiality in mesophyll cultures of Arachis hypogaea. Zeitschrift für Pflanzenphysiologie 59: 109-123.

JUNIPER, B. E., R. J. ROBINS & D. M. JOEL, (1989). The carnivorous plants.

Pp. xxii + 353. Academic Press, London.

JUNTILLA, O., (1982). Gibberellin-like activity in shoots of Salix pentandra as related to the elongation growth. Canadian Journal of Botany 60(7): 1231-1234.

KAY, M. K., (1980). Pontania proxima (Lepeletier) (Hymenoptera: Tenthredinidae). Willow gall sawfly. Forest and Timber Insects in New Zealand. No. 45. Pp 4. Forest Research Institute, New Zealand Forest Service, New Zealand.

KIM, J. H., E. NOH & J. I. PARK, (1983). Haploid plantlet formation through anther culture of Populus glandulosa. Research Report of the Institute of Forest Genetics, Korea Republic 19: 93-98.

KING, P. E. & N. A. RATCLIFFE, (1969). The structure and possible mode of functioning of the female reproductive system in Nasonia vitripennis (Hymenoptera: Pteromalidae). Journal of Zoology 157: 319-344.

KLOET, G. S. & W. D. HINCKS, (1978). Handbook for the identification of British insects. Volume Eleven, part 4. A checklist of British insects. Hymenoptera. 2nd. Ed. Pp. ix + 159. Royal Entomological Society of London.

KOPELKE, J.-P., (1985a). Biologie und Parasiten der gallenbilden Blattwespe Pontania proxima (Lepeletier 1823). (Insecta: Hymenoptera: Tenthredinidae). Senckenbergiana Biologica 65(3/6): 215-239.

KOPELKE, J.-P., (1985b). Über die Biologie und Parasiten der gallenbildenden Blattwespenarten Pontania dolichura (Thoms. 1871), P. vesicator (Bremi 1849) und P. viminalis (L. 1758) (Hymenoptera: Tenthredinidae).

Faunistisch-Ökologische Mitteilungen 5: 331-344.

KOPELKE, J.-P., (1986). Zur Taxonomie und Biologie neuer Pontania-Arten der dolichura-Gruppe. (Insecta: Hymenoptera: Tenthredinidae). Senckenbergiana Biologica 67: 51-71.

KOPELKE, J.-P., (1988). Zur Biologie und Ökologie der Arten des Brutparasiten-Parasitoiden-Komplexes von gallenbildenden Blattwespen der Gattung Pontania (Hymenoptera: Tenthredinidae: Nematinae). Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie 6: 150-155.

LAFONT, R., P. BETDON, C. BLAIS, M. GARCIA, F. LACHAISE, F. RIERA, G. SOMME & J. P. GIRAULT, (1986). Ecdysteroid metabolism a comparative study. Insect Biochemistry 16(1): 11-16.

LAFONT, R., P. BEYDON, B. MAUCHAMP, G. SOMME-MARTIN, M. ANDRIANJAFINTRIMO & P. KRIEN, (1981). Recent progress in ecdysteroid analytical methods. Pp. 125-144, in SEHNAL, F., A. ZABZA & B. CYMBOROWSKI (eds.) Regulation of insect development and behaviour. Technical University of Wroclow Press, Poland.

LAFONT, R., J.-L. PENNETIER, M. ANDRIANJAFINTRIMO, J. CLARET, J.-F. MODDE & C. BLAIS, (1982). Sample processing for High-Performance Liquid Chromatography of ecdysteroids. Journal of Chromatography 236: 137-147.

LAZARENA, A. I., V. I. OGORODNIKOVA & A. G. TRUSEVICH, (1986). [The willow bean-gall sawfly.] In Russian. Zashchita Rastenii 9: 33-34.

LAREW, H. G., (1986). The fossil record: A brief summary. Proceedings of the Entomological Society of Washington 88(2): 385-388.

LEACH, C. K. & N. MORRIS, (1990). Phenylalanine: ammonium lyase of oak marble galls and leaves of Quercus robur. Cecidology 5(1): 8-16.

LEACH, C. K. & G. MASON, (1990). Cynipid update 1990. Part 1. The "uncommon" spangle gall. PEARSON, J. A. (ed.). BPGS Occasional Paper 1: 1-5.

LEATHERDALE, D., (1955). Plant hyperplasia induced with a cell-free insect extract. Nature (London) 175: 553.

LEDINGHAM, J. M. & F. O. SIMPSON, (1970). Intensification of osmium staining by p-phenylenediamine: paraffin and epon embedding: lipid granules in renal medulla. Stain Technology 45(6): 255-260.

LEDINGHAM, J. M. & F. O. SIMPSON, (1972). The use of p-phenylenediamine in the block to enhance osmium staining for electron microscopy. Stain Technology 47(5): 239-243.

LEUWENHOECK, A, van, (1701). Concerning excrescencies growing on willow leaves, &c. Philosophical Transactions of the Royal Society of London 22: 786-792.

LEWIS, I. F. & L. WALTON, (1964). Gall-formation on leaves of Celtis occidentalis L. resulting from material injected by Pachypsylla sp. Transactions of the American Microscopical Society 83: 62-78.

LIN, J. F., DONG, M. S. & Q. C. HUANG, (1983). Tissue culture of Populus adenopoda and P. grandidentata. Plant Physiology Communications 2: 37.

LIN, Y. -L. & C. -H. LIN, (1990). Involvement of tRNA bound cytokinin on the gall formation in Zizania. Journal of Experimental Botany 41(224): 277-281.

LINDSAY, S., (1987). High Pressure Liquid Chromatography. Analytical chemistry by open learning series. KEALEY, D. (ed.). Pp. xx + 244. Wiley, Chichester.

LINNAEUS, C., (1761). Fauna Svecica. Second Edition. Pp. 578. Salvius, Stockholm.

LINTON, E. F., (1913). A monograph of the British willows. Supplement to Journal of Botany 1913. Pp. 92. West, Newman, London.

LIVINGSTONE, D., (1978). Phytosuccivorous bugs and cecidogenesis. Journal of the Indian Academy of Wood Science 9(1): 39-45.

LLOYD, G. & B. H. McCOWN, (1981). Commercially-feasible micropropagation of Mountain laurel, Kalmia latifolia, by the use of shoot tip culture. Combined Proceedings. International Plant Propagators Society 30: 421-427.

MAGNUS, W., (1914). Die Entstehung der Pflanzengallen verursacht durch Hymenopteren. Pp. 160. + 4 pls. Fischer, Jena.

MANI, M. S., (1964). The ecology of plant galls. Pp. xii + 434. Junk, The Hague.

MAPES, C. C. & P. J. DAVIES, (1984). Auxin involvement in ball gall development on Solidago altissima. Plant Physiology 75 (Supplement): 27.

MARCHESE-RAGONA, S. P. & S. P. S. JOHNSON, (1982).A simple method for the progressive infiltration of resin into a dehydrated biological sample. Proceedings of the Royal Microscopical Society 17(6): 311-312.

MATRENINA, R. M. & L. A. GUSTELEVA, (1984). [Epiphytic microorganisms of the larch bud gall midge as producers of auxins.] In Russian. Izvestiya Sibirskogo Otdeleniya Akademii Nauk

SSSR, Biologicheskikh Nauk 18(393): 80-85.

MATTHEWS, J. D., (1989). Silvicultural Systems. Pp. xii + 284. Clarendon Press, Oxford.

McCALLA, D, R., M. K. GENTHE & W. HOVANITZ, (1962). Chemical nature of an insect gall growth-factor. Plant Physiology 37: 98-103.

McCOWN B. H. & J. A. RUSSELL, (1987). Protoplast culture of hardwoods. Pp. 16-30, in BONGA, J. M. & D. J. DURZAN (eds.), Cell and tissue culture in forestry. Volume Two. specific principles and methods: growth and development. Pp. xvi + 447 Martinus Nijhoff Publishers, Dordrecht, Netherlands.

McLACHLAN, R., (1889). Galls. Nature (London) 41: 131.

MEIKLE, R. D., (1984) Willows and poplars of Great Britain and Ireland. B.S.B.I. Handbook No. 4. Pp. 198. B.S.B.I. c/o British Museum (Natural History), London.

MERCER, E. H. & P. C. J. BRUNET, (1959). The electron microscopy of the left colleterial gland of the cockroach. The Journal of Biophysical and Biochemical Cytology 5: 257-261

MEYER, J., (1987). Plant galls and gall inducers. Pp. viii + 291. Borntraeger, Berlin.

MIALL, L. C., (1912). The early naturalists their lives and work. (1530-1789). Pp. xi + 396. Macmillan, London.

MILES, H. W., I. THOMAS & G. L. HEY, (1933). On Hoplocampa flava L., the plum sawfly. Annals of Applied Biology 20: 722-730.

MILES, P. W., (1968a). Insect secretions in plants. Annual Review of Phytopathology 6: 137-164.

MILES, P. W., (1968b). Studies on the salivary physiology of plant-bugs: experimental induction of galls. Journal of Insect

Physiology 14: 97-106.

MILLER, L.K. & R. WERNER, (1987). Extreme supercooling as an overwintering strategy in three species of willow gall insects from interior Alaska. Oikos 49: 253-260.

MITCHELL, C. P., (1988) The world perspective. Pp 235-255, in HUMMEL, F. C., W. PALZ & G. GRASSI (eds.), Biomass forestry in Europe: a strategy for the future. Pp. vii + 600. Elsevier Applied Science, London.

MIVART, S. G., (1889a). Prof. Weismann's "Essays". Nature (London) 41: 38-41.

MIVART, S. G., (1889b). Galls. Nature (London) 41: 174-175.

MONAD, T. & C. SCHMITT, (1968). Contribution à l'étude des pseudo-galles formicaires chez quelque acacias Africains. Bulletin de l'Institut Français d'Afrique Noire 30: 953-1012.

MÜLLER, A., (1872). Nematus vallisnierii, Hartig, ovipositing under difficulties. The Entomologist's Monthly Magazine 8: 109.

MURASHIGE, T. & F. SKOOG., (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15: 473-497.

MURPHY, I. S., (1929). The oviposition of Pontania gallicola Steph. (the bean gall of willows). The Entomologist's Monthly Magazine 65(787): 270-272.

MYERS, J. H. & P. HARRIS, (1980). Distribution of Urophora galls in flower heads of diffuse and spotted knapweed in British Columbia. Journal of Applied Ecology 17: 359-367.

NARENDRAN, T. C., (1984). Chalcids and sawflies associated with plant galls. Pp. 273-303, in ANANTHAKRISHNAN, T. N. (ed.),

Biology of gall insects. Pp. xii + 362. Arnold, London.

O'BRIEN, T. P. & M. E. McCULLY, (1981). The study of plant structure. Principles and selected methods. Pp. x + 318. Termarcarphi Pty, Melbourne.

OSBORNE, D. J., (1972). Mutual regulations of growth and development in plants and insects. Pp. 33-42, in EMDEN, H. F. van (ed.), Insect/plant relations. Symposia of the Royal Entomological Society of London. No. 6. Blackwell Scientific Publications, London.

PARK, Y. G. & S. H. SON, (1988) Regeneration of plantlets from cell suspension culture derived callus of white poplar (Populus alba L.). Plant Cell Reports 7: 567-570.

PEACOCK, A. D., (1928). On rearing larvae from eggs dissected out of a sawfly. Proceedings of the Royal Physical Society of Edinburgh 21: 171-174.

PEACOCK, A. D. & A. R. SANDERSON, (1939). The cytology of the thelytokously parthenogenetic saw-fly Thrinax macula Kl. Transactions of the Royal Society of Edinburgh 59(3): 647-660.

PEARCE, R. B. & J. P. RIDE, (1982). Chitin and related compounds as elicitors of the lignification response in wounded wheat leaves. Physiological Plant Pathology 20: 119-123.

PELET, F., A. C. HILDEBRANDT, A. J. RIKER & F. SKOOG, (1960). Growth in vitro of tissues isolated from normal stems and insect galls. American Journal of Botany 47: 186-195.

PIERIK, R. L. M., (1987). In vitro culture of higher plants. Pp. v + 344. Martinus Nijhoff Publishers, Netherlands.

PLUMB, G. H., (1953). The formation and development of the Norway Spruce

gall caused by Adelges abietis L. The Connecticut Agricultural Experiment Station. New Haven. Bulletin 566: 1-77.

POWER, J. B. & E. C. COCKING, (1971). Fusion of plant protoplasts. Science Progress, Oxford 59: 181-198.

PRESZLER, R. W. & P. W. PRICE, (1988). Host quality and sawfly populations: a new approach to life table analysis. Ecology 69(6): 2012-2020.

PRICE, P. W., (1988). Inversely density-dependent parasitism: the role of plant refuges for hosts. Journal of Animal Ecology 57: 89-96.

PRICE, P. W. & K. M. CLANCY, (1986a). Interactions among three trophic levels: gall size and parasitoid attack. Ecology 67(6): 1593-1600.

PRICE, P. W. & K. M. CLANCY, (1986b). Multiple effects of precipitation on Salix lasiolepis and populations of the stem-galling sawfly, Euura lasiolepis. Ecological Research 1: 1-14.

PRICE, P. W. & T. P. CRAIG, (1984). Life history, phenology, and survivorship of a stem-galling sawfly, Euura lasiolepis (Hymenoptera: Tenthredinidae), on the arroyo willow, Salix lasiolepis, in northern Arizona. Annals of the Entomological Society of America 77(6): 712-719.

PRICE, P. W., W. G. FERNANDES & G. L. WARING, (1987). Adaptive nature of insect galls. Environmental Entomology 16: 15-24.

PRICE, P. W. & H. PSCHORN-WALCHER, (1988). Are galling insects better protected against parasitoids than exposed feeders?: a test using tenthredinid sawflies. Ecological Entomology 13(2): 195-205.

PRICE, P. W., H. ROININEN & J. TAHVANAINEN, (1987a). Plant age and attack by the bud galler, Euura macronata. Oecologia (Berlin) 73: 334-337.

PRICE, P. W., H. ROININEN & J. TAHVANAINEN, (1987b). Why does the bud-galling sawfly, Euura mucronata, attack long shoots? Oecologia (Berlin) 74: 1-6.

RATCLIFFE, R. A. S., (1989). Review of winter 1988-89 in the northern hemisphere. Weather 44(5): 226-228.

RATCLIFFE, R. A. S., (1990). Review of winter 1989-90 in the northern hemisphere. Weather 45(5): 193-194.

RAVEN, J. A., (1987). The role of vacuoles. New Phytologist 106: 357-422.

REAM, L. W. & M. P. GORDON, (1982). Crown gall disease and prospects for genetic manipulation of plants. Science 218: 854-859.

RÉAUMUR, R. A. F. de, (1737). Mémoires pour servir à l'histoire des insectes. Tome III. Mémoire XII. Imprimérie Royale, Paris.

REDI, F., (1688). Esperienze intorno alla generazione degl'insetti. Quinta impressione. Pp. 177 + 29 pl. Matini, Firenze.

REID, N., (1975). Ultramicrotomy. Practical methods in electron microscopy. Volume Three, part 2. GLAUERT, A. M. (ed.). Pp. 140. Elsevier/North-Holland Biomedical Press, Amsterdam.

REINERT, J. & M. M. YEOMAN, (1982). Plant cell and tissue culture. A laboratory manual. Pp. 83. Springer-Verlag, Berlin.

REY, L., (1967). Les premiers stades de développement de la galle de Pontania proxima Lep. Bulletin. Société Botanique de France 114: 80-95.

REY, L., (1968). La galle de Pontania proxima Lep.: stades ultérieurs du

développement. Bulletin. Société Botanique de France 115: 413-424.

REY, L., (1972). Sur l'évolution particulière des chloroplasts dans la galle de Pontania proxima Lep. sur la feuille de Salix triandra L. Journal de Microscopie (Paris) 14(3): 87a.

REY, L., (1973). Ultrastructure des chloroplasts au cours de leur évolution pathologique dans le tissu central de la jeune galle de Pontania proxima Lep. Comptes rendus hebdomadaires des séances de l'Académie des sciences. Série D 276(7): 1157-1160.

REY, L., (1974). Modifications ultrastructurales pathologiques présentées par les chloroplastes de la galle de Pontania proxima Lep. en fin de croissance. Comptes rendus hebdomadaires des séances de l'Académie des sciences. Série D 278(10): 1345-1348.

RICHARDS, O. W. & R. G. DAVIES, (1977). Imms' general textbook of entomology. 10th. Ed. Volume Two: classification and biology. Pp. vii + 421-1354. Chapman and Hall, London.

RIDDIFORD, L. M. & J. W. TRUMAN, (1978). Biochemistry of insect hormones and insect growth regulators. Pp. 307-357, in ROCKSTEIN, M. (ed.), Biochemistry of Insects. Pp. xiv + 649. Academic Press, London.

ROBINSON, P. D., E. D. MORGAN, I. D. WILSON & R. LAFONT, (1987). The metabolism of ingested and injected [^{3}H]ecdysone by final instar larvae of Heliothis armigera. Physiological Entomology 12: 321-330.

ROHFRITSCH, O. & J. D. SHORTHOUSE, (1982). Insect Galls. Pp. 131-152, in KAHL, G. & J. SCHELL (eds.), Molecular biology of plant

tumors. Pp. 617. Academic Press, New York.

ROLAND, J. C., (1978). General preparation and staining of thin sections. Pp. 1-62, in HALL, J. L. (ed.), Electron microscopy and cytochemistry of plant cells. Elsevier/North-Holland Biomedical Press, Amsterdam.

ROMANES, G. J., (1889a). Galls. Nature (London) 41: 80.

ROMANES, G. J., (1889b). Galls. Nature (London) 41: 174.

ROMANES, G. J., (1890). Galls. Nature (London) 41: 369.

ROSEN, H. R., (1916). The development of the Phylloxera vastatrix leaf gall. American Journal of Botany 3: 337-360.

ROSS, H. H., (1945). Sawfly genitalia: terminology and study techniques. Entomological News 56: 261-268.

ROSS, H. H., (1951). Family Tenthredinidae. Subfamily Nematinae. Pp. 31-55, in MUESEBECK, C. . W., K. V. KROMBEIN & H. K. TOWNES (eds.), Hymenoptera of America north of Mexico. Synoptic Catalog. U.S. Department of Agriculture. Agriculture Monograph No. 2. Pp. 1420. Government Printing Office, Washington.

ROTHSCHILD, M., Y. SCHLEIN & S. ITO., (1986). A coloured atlas of insect tissues via the flea. Pp. 184. Wolfe, London.

RUDNYANSKAYA, E. I., (1987). [Pollen yield of floodplain forests in Volgograd Province.] In Russian. Rastitel'nye Resursy 23: 616-621.

RUSSELL, J. A. & B. H. McCOWN, (1986a). Techniques for the enhanced release of leaf protoplasts in Populus. Plant Cell Reports 5: 284-287.

RUSSELL, J. A. & B. H. McCOWN, (1986b). Culture and regeneration of Populus leaf protoplasts isolated from non-seedling

tissue. Plant Science. Irish Republic 46(2): 133-142.

RUSSELL, J. A. & B. H. McCOWN, (1988). Recovery of plants from leaf protoplasts of hybrid-poplar and aspen clones. Plant Cell Reports 7(1): 59-62.

RUTLEDGE, C. B. & G. C. DOUGLAS, (1988). Culture of meristem tips and micropropagation of 12 commercial clones of poplar in vitro. Physiologia Plantarum 72(2): 367-373.

RYAN, B. F., B. L. JOINER & T. A. RYAN, (1985). Minitab Handbook. 2nd. Ed. Pp. x + 397. Duxbury Press, Boston.

SACCHI, C. F., P. W. PRICE, T. P. CRAIG & J. K. ITAMI, (1988). Impact of shoot gall attack on sexual reproduction in the arroyo willow. Ecology 69(6): 2021-2030.

SACHS, T., (1975). Plant tumors resulting from unregulated hormone synthesis. Journal of Theoretical Biology 55: 445-453.

SAITO, A., (1980). Effects of Inorganic Elements in the Medium on Shoot Differentiation from Populus Callus. Journal of the Japanese Forestry Society 62(4): 147-149.

SAITO, A., Y. HOSOI, K. ISHII & T. SATO, (1987). Callus formation from protoplasts of mesophyll cells of Populus plantlets. Journal of the Japanese Forestry Society 69(12): 472-477.

SANDLANT, G. R., (1979). Arthropod successori inhabiting willow galls during Autumn in Christchurch, New Zealand. Mauri Ora 7: 83-93.

SCHÄLLER, G. von., (1965). Untersuchungen über den beta-indolylessigsäure-Gehalt des Speichels von Aphidenarten mit unterschiedlicher Phytopathogenität. Zoologische Jahrbücher. Allgemeine Zoologie und

Physiologie der Tiere 71: 385-392.

SCHÄLLER, G., (1969). Untersuchungen zur erzeugung küstlicher Pflanzengallen. Marcellia 35: 131-153.

SCHMITZ, R. Y. & F. SKOOG, (1970). The use of dimethylsulfoxide as a solvent in the tobacco bioassay for cytokinins. Plant Physiology 45: 537-538.

SCHLUTER, U., (1985). Untersuchungen uber das Bewurzelungsvermogen ingenieurbiologisch wichtiger Weidenarten wahrend der Vegetationsperiode. Zeitschrift fur Vegetationstechnik im Landschafts und Sportstattenbau 8: 123-129.

SCOPES, R. K., (1978). B101-Techniques for protein purification. In KORNBERG, H. L., J. C. METCALFE, D. H. NORTHCOTE, C. I. POGSON & K. F. TIPTON (eds.), Techniques in the Life Sciences. Biochemistry-Volume B1/1. Techniques in Protein and Enzyme Biochemistry-Part 1. Pp. 42. Elsevier/North-Holland Biomedical Press. Ireland.

SENN, G., (1941). Oak galls in the Historia Plantarum of Theophrastus. Transactions of the Royal Society of Edinburgh 60: 343-354.

SEVERIN, H. C. & H. H. P. SEVERIN, (1908). Anatomical and histological studies of the female reproductive organs of the American saw-fly, Climex america Leach. Annals of the Entomological Society of America 1(2): 87-101.

SHANAHAN, P., (1957). The distribution of the bean gall sawfly Pontania proxima (Lep.) (Hymenoptera: Tenthredinidae) on Salix fragilis L. The Entomologist's Monthly Magazine 93: 182-183.

SHEPARD, J. F., D. BIDNEY & E. SHAHIN, (1980). Potato protoplasts in crop improvement. Science 208: 17-24.

SHORTHOUSE, J. D. & R. G. LALONDE, (1988). Role of Urophora cardui (L.) (Diptera, Tephritidae) in growth and development of its gall on stems of Canada thistle. The Canadian Entomologist 120(7): 639-646.

SINGER, C., (1959). A history of biology to about the year 1900. A general introduction to the study of living things. 3rd. and revised Ed. Pp. xxxvi + 580. Abelard-Schuman, London.

SKOOG, F. & M. O. MILLER, (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Pp. 118-131, in PORTER, H. K. (ed.), The biological action of growth substances. Symposia of the Society for Experimental Biology. Number 11. Pp. vi + 344. University Press, Cambridge.

SLEPYAN, E. I., (1962). Effect of Pontania proxima Lep. (Tenthredinidae) on growth, photosynthesis, and chlorophyll and carotenoid contents of leaf laminae in Salix fragilis L. pathogenicity of gall formers. Doklady (Proceedings) of the Academy of Sciences of the U.S.S.R. Biological Sciences Sections 147(1-6): 1432-1435.

SLEPYAN, E. I. & N. I. GABARAYEVA, (1976). Morphology of the tracheal system in larvae of the sawfly Pontania proxima (Lepel.) (Hymenoptera: Tenthredinidae) in relation to their mode of life. Entomological Review, Washington 55(3): 30-36.

SLEPYAN, E. I. & N. I. GABARAYEVA, (1978). Morphological and histological features of the gut, malphigian tubes and

salivary glands of Pontania proxima larvae (Hymenoptera: Tenthredinidae) in connection with their mode of life. Entomological Review, Washington 57(3) :337-338.

SLEPYAN, E. I. & N. I. GABARAYEVA, (1981). Structure and development of the gall formed by the larvae of the sawfly Pontania proxima (Lepel.) (Hymenoptera, Tenthredinidae) on the leaves of willow Salix fragilis L. Entomological Review, Washington 60(3): 55-63.

SLEPYAN, E. I. & L. S. GUREVICH, (1976). [Dynamics of organic acids in leaf tissues of Salix fragilis L. during the process of gall genesis, provoked by Pontania proxima (Lepel.).] in Russian. Biologicheskie Nauki 8: 87-90.

SMITH, D. R., (1979). Suborder Symphyta. Pp 3-137, in KROMBEIN, K. V., HURD, P. D., SMITH, D. R. & BURKS, B. D. (eds.), Catalogue of Hymenoptera in America north of Mexico. Volume One. Smithsonian Institution Press, Washington, D. C.

SMITH, E.L., (1970). Biosystematics and morphology of Symphyta II. Biology of gall-making Nematine sawflies in the California region. Annals of the Entomological Society of America 63: 36-51.

SMITH, F., (1876). Note on Nematus gallicola Steph. Proceedings of the Royal Entomological Society of London 1876: xxii-xxiv.

SMITH, F. F., R. E. WEBB, G. W. ARGUS, J. A. DICKINSON & H. W. EVERETT, (1983). Willow beaked-gall midge, Mayetiola vigidae (Osten Sacken), (Diptera: Cecidomyiidae): differential susceptibility of willows. Environmental Entomology 12(4): 1175-1181.

SMITH, K. M., (1951). A textbook of agricultural entomology. Pp. xiii + 289. Cambridge University Press, Cambridge.

STADEN, J. van & J. E. DAVEY, (1978). Endogenous cytokinins in the larvae and galls of Erythrina latissima leaves. Botanical Gazette 139: 36-41.

STADEN, J. van, J. E. DAVEY & A. R. A. NOEL, (1977). Gall formation in Erythrina latissima. Zeitschrift für Pflanzenphysiologie 84: 283-294.

STÄDLER, E. & H.-R. BUSER, (1984). Defense chemicals in leaf surface wax synergistically stimulate oviposition by a phytophagous insect. Experimentia 40: 1157-1159.

STEARN, W. T., (1976). From Theophrastus and Dioscorides to Sibthorp and Smith: the background and origin of the Flora Graeca. Biological Journal of the Linnean Society 8: 285-298.

STEENIS, C. G. G. J. van., (1976). Autonomous evolution in plants. Differences in plant and animal evolution. The Gardeners' Bulletin, Singapore 29: 103-126.

STOLTZ, D. B., D. GUZO & D. COOK, (1986). Studies on polydnavirus transmission. Virology 115: 120-130.

STOLTZ, D. B. & S. B. VINSON, (1979). Viruses and parasitism in insects. Advances in Virus Research 24: 125-171.

STRAND, M. R., S. M. MEOLA & S. B. VINSON, (1986). Correlating pathological symptoms in Heliothis virescens eggs with development of the parasitoid Telenomus heliothidis. Journal of Insect Physiology 32(4): 389-402.

STRONG, D. R., J. H. LAWTON & T. R. E. SOUTHWOOD, (1984). Insects on plants. Community patterns and mechanisms. Pp. vi + 313. Blackwell Scientific Publications, Oxford.

SUMIYA, A., T. SUNAKAWA, T. ISHIMOTO & Z. KASAI, (1988). Plant regeneration from long-term cultured callus of poplar (Populus nigra). Mokuzai Gakkaishi (Journal of the Japan Wood Research Society) 34(4): 354-358.

SWAMMERDAM, J., (1758). The book of nature; or, the history of insects. FLLOYD, T. (trans.). Revised and improved by notes from Réaumur & others by John Hill. Pt. 2. Pp. 75-87. Reprint Ed. (1978). Arno Press, USA.

SWANTON, E. W., (1912). British plant galls. Pp. xv + 287. Methuen, London.

TAFT, J. B. & D. R. BISSING, (1988). Developmental anatomy of the horned oak gall induced by Callirhytis cornigera on Quercus palustris (pin oak). American Journal of Botany 75: 26-36.

TAIT, N. N., (1962). The anatomy of the sawfly Perga affinis affinis Kirby (Hymenoptera: Symphyta). Australian Journal of Zoology 10: 652-683.

THE OPEN UNIVERSITY, (1974). Physiology of cells and organisms. Unit 8. Gametes and the physiology of early gestation. HOLMES, R. (ed.). The open University Press, Milton Keynes.

THEOPHRASTUS, (1916). Enquiry into plants and minor works on odours and weather signs. Volume One. (trans. HORT, A.). Pp. xxviii + 475. Heinemann, London

TOGASHI, I., (1970). The comparative morphology of the internal reproductive organs of the Symphyta (Hymenoptera). Mushi 43 (Supplement): 1-114.

TORMALA, T. & E. SAARIKKO, (1985). Micropropagation of willows (Salix spp.). Pp. 427-429, in PLAZ, W., J. COOMBS & D. O. HALLS (eds.), Energy from biomass: 3rd E.C. conference, held

in Venice 25-29 March, 1985. Pp. xxv + 1211. Elsevier Applied Science Publishers, London.

TSOUPRAS, G. & B. LUU, (1983). A cytokinin (isopentenyl-adenosyl-mononucleotide) linked to ecdysone in newly laid eggs of Locusta migratoria. Science 220: 507-509.

URITANI, I. & T. ASAHI, (1980). Respiration and related metabolic activity in wounded and infected tissues. Pp. 463-485, in STUMPF, P. & E. E. CONN (eds.-in-chief). The biochemistry of plants: a comprehensive treatise. Volume Two. Metabolism and respiration. DAVIES, D. D. (ed.). Pp. xvi + 687. Academic Press, London.

WAN, Z. S., Z. W. YIN, Y. Y. MENG, F. T. CHAO, J. B. LI & Y. K. CAO. (1986). Researches on species selection for establishing a fuel stand of willows and the technique of tree planting. Forest Science and Technology 8: 5-8.

WARICK, R. P. & A. C. HILDEBRANDT, (1966). Free amino acid contents of stem and Phylloxera gall tissue cultures of grape. Plant Physiology 41: 573-578.

WARING, G., (1986). Galls in harsh environments. Proceedings of the Entomological Society of Washington 88(2): 376-380.

WEILER, E. M., (1981). Dynamics of endogenous growth regulators during the growth cycle of a hormone-autotrophic plant cell culture. Naturwissenschaften 68: 377-378.

WEIS, A. E. & W. G. ABRAHAMSON, (1986). Evolution of host-plant manipulation by gall-makers: ecological and genetic factors in the Solidago-Eurosta system. The American Naturalist 127(5): 681-695.

WEIS, A. E., W. G. ABRAHANSON & K. D. McCREA, (1985). Host gall size and oviposition success by the parasitoid Eurytoma gigantea. Ecological Entomology 10: 341-348.

WEIS, A. E., R. WALTON & C. L. CREGO, (1988). Reactive plant tissue sites and the population biology of gall makers. Annual Review of Entomology 33: 467-486.

WENT, F. W., (1970). Plants and the chemical environment. Pp. 72-81, in SONDHEIMER, E. & J. B. SIMEON (eds.), Chemical ecology. Pp. xv + 336. Academic Press, London.

WESTPHAL E. & P. MICHLER, (1975). Mise en évidence de particules bacilliformes dans le malformations du type "Wirrzoph" apparaissant sur certains Saules. Compte rendu de l'Académie des sciences Paris 281: 403-405.

WETTERHAN, D., (1889) Galls. Nature (London) 41: 131.

WHITE, M. J. D., (1954). Animal cytology and evolution. Pp. xii + 454. Cambridge University Press, Cambridge.

WILLIAMS, A. G. & T. G. WHITHAM, (1986). Premature leaf abscission: an induced plant defense against gall aphids. Ecology 67(6) 1619-1627.

WILLS, R. B. H. and B. D. PATTERSON, (1970). Ethylene, a plant hormone from fluorescent lighting. Nature (London) 225: 199.

WINTON, L. L., (1968). Plantlets from aspen tissue cultures. Science 160: 1234-1235.

WINTON, L. L., (1970). Shoot and tree production from aspen tissue cultures. Americal Journal of Botany 57: 904-909.

WINTON, L. (1971). Tissue culture propagation of European aspen. Forest Science 17: 348-350.

WOLTER, K. E., (1968). Root and shoot initiation in aspen callus

cultures. Nature (London) 219: 509-510.

WOLTER, K. E. & F. SKOOG, (1966). Nutritional requirements of Fraxinus callus cultures. American Journal of Botany 53: 263-269.

YERMAKOV, I. P., (1965). [Cytochemical investigations of proteins and nucleic acids in the process of gall development on the leaves of willow.] In Russian. Nauchnye doklady Vyssheishkoly Biologicheskie nauki 1: 151-169.

YOUN, Y., S.J. LEE & S. K. LEE, (1985). Isolation and culture of protoplasts from suspension-cultured cells of Populus alba X P. glandulosa F_1. Research Report of the Institute of Forest Genetics, Korea Republic 21: 109-113.

ZAERR J. B. & M. O. MAPES, (1982). Action of growth regulators. Pp. 231-255, in BONGA, J. M. & D. J. DURZAN (eds.), Tissue Culture in Forestry. Pp. xi + 420. Martinus Nijhoff/Junk, The Hague.

ZIMMERMAN, M., (1979). Oviposition behaviour and the existence of an oviposition-deterring pheromone in Hylemya. Environmental Entomology 8: 277-279.

ZIMMERMAN, R. H., (1985). Application of tissue culture propagation to woody plants. Pp 165-177, in HENKE, R. R., K. W. HUGHES, M. J. CONSTANTIN & A. HOLLAENDER (eds.), Tissue culture in forestry and agriculture. Pp. xvii + 390. Plenum Press, New York.

APPENDIX ONE

Tissue culture.

> "In studies of the interaction between insects and plants, tissue culture techniques are likely to be of most value for the investigation of gall induction and formation".
>
> (Ingram, 1973)

A1.1 Introduction.

A1.1.1 The rôle of tissue culture techniques in the study of insect-gall induction.

Dougall (1980) listed various advantages of the use of tissue cultures over the use of whole plants. Two of these are especially relevant to the work in this section. These are:-

1) A rapid exponential growth with population doubling times of one to four days.

2) A greater range of environmental and nutritional manipulations are possible.

Following on from the quotation shown above, Ingram (1973) recognized that work on insect-gall initiation and formation, involving the use of tissue culture techniques, was minimal and cited only five references. Indeed, Amerson and Mott (1982) concluded that "judging from the paucity of literature in the area, it appears that insect-plant dual cultures are little used in plant pathology".

The main objective of this work was the use of tissue culture as a model for development and morphogenesis, both of normal and gall tissues. Differences in growth regulator requirements may have given some indication of patterns of development. However, caution needed to be used in the interpretation of any results as Zaerr and Mapes (1982), stressed that observations of in vitro development cannot be related directly to in vivo development, i.e. growth regulators may have reacted differently in intact plants than in culture systems.

Within this objective, the main aims were:-

a) Induction and maintain calli from normal and gall tissue. If this proved to be possible then an assessment could be made of whether gall tissue can be maintained in tissue culture, or whether without an interaction from the insect, it would revert to normal callus tissue.

b) Micropropagation of plantlets from both types of callus and tissue explants.

c) Production of insect-plant dual cultures.

d) Use of protoplast isolation and fusion techniques to create normal/gall somatic hybrids.

To achieve effective culturing there was a need to create a balance of growth regulators within the chosen tissue culture medium. The "hormone balance" of Skoog and Miller (1957) appears now to be a fundamental of tissue culture techniques - "that quantitative interactions between growth factors, especially between IAA and kinetin (auxins and kinins) and between these and other factors, provide a common mechanism for the regulation of all types of growth investigated from cell enlargement to organ formation" (Skoog and Miller, 1957). Few studies have dealt with the mechanisms behind the action of growth

regulators in tissue culture, most of the "hormone balance(s)" being derived empirically (Zaerr and Mapes, 1982).

A search of the literature reveals that the tissue culture of Salix species is not well documented (Bergman et al., 1985). Being easy to propagate vegetatively (Brown and Sommer, 1982; Bergman et al., 1986), this is understandable, although Evers et al. (1988: 10) present a good case for micropropagation, with Salix alba being now a realistic source of energy production in Scandinavian countries (see Section 4.2.1). Examples include organculture of buds (Angrish and Nanda, 1982), biochemical studies (Feutry and Letouzé, 1984; Daguin and Letouzé, 1985 and 1986) and micropropagation (Bhojwani, 1980; Chalupa, 1983; Tormala and Saarikko, 1985). From these references and others given in Tables A1.3 and A1.4, guidelines on the types and ratios of growth regulators that could be tried were obtained, but Bergman et al. (1985) noted genotypic differences in growth and differentiation between tissue cultured Salix clones. So, as yet no single technique can be applied to all clones/species. The initial experiments in this section deal with the derivation of a suitable "hormone balance" for callus formation.

A1.1.2 Methods used throughout.

a) General procedures

1. Unless otherwise stated, all chemicals and reagents were purchased from Sigma Chemical Company. Reagent grade water (DDH_2O) was obtained using a MILL-Q UF water system (Millipore, S A 67 120, Molsheim, France) and sterilization by autoclaving was achieved using a Gallenkamp autoclave Model AUX-750-010V (Fisons, Crawley, Sussex. RH10 2UL). Autoclaving was carried out at a pressure of 1.06 kg/cm^2 giving a steam temperature of 121° C, for 15 minutes.

2. A small draught-free room was used. At least two hours before use the room was illuminated with an ultraviolet bactericidal lamp. Immediately before use, surfaces were swabbed down with 95% industrial methanol. During all work a bunsen burner was kept burning.

3. All apparatus and media had been sterilized by autoclaving, or were of the sterile disposable type, or filter sterilized as appropriate.

4. Stainless steel instruments were used and were kept in 95% industrial methanol. They were flamed before use.

5. Once prepared, 25 ml of tissue culture medium was dispensed into 9 cm diameter sterile petri dishes (Sterilin, Feltham, Middlesex. TW14 8QS), or 10 ml were dispensed into 30 ml Universal sterile tubes (Alpha Laboratories, Eastleigh, Hampshire. SO5 4NU).

b) Preparation of stock solutions.

1. Cytokinin stock solution. 10 g of cytokinin was dissolved in a few drops of 0.1 N sodium hydroxide (Pierik, 1987: 68); a little DDH_2O was added and the mixture transferred to a 100 ml volumetric flask; more DDH_2O was added, until the final volume was reached. The solution was stored in the dark as it is unstable in light (Pierik, 1987: 68), and under refrigeration. Aliquots were then added to the media during preparation.

2. Auxin stock solutions. Due to the toxicity of 2,4-D, this preparation was carried out in a fume-cupboard. The auxin was dissolved in ethanol, to give a concentration of 10 mg/ml. Aliquots were then added to the media during preparation. Dodds and Roberts (1985: 45) recommend the use of dimethyl sulphoxide (DMSO) as a solvent for 2,4-D. Schmitz and Skoog (1970), however, while acknowledging the useful rôle DMSO can play as a solvent in tobacco bioassays for cytokinins, they do

not recommend its use as routine in nutrient media. For this reason ethanol was used as an alternative.

3. Vitamin stock solution. Either a pre-prepared vitamin solution, or tissue culture grade vitamins were used (see Tables A1.1 and A1.2). This was added to the media after it had been autoclaved. A sterile syringe and a 0.20 micron syringe filter were used to add the vitamin mixture to a concentration of 1 ml/l.

c) Preparation of media.

Pre-prepared basal salt mixtures or tissue culture grade chemicals were used throughout (see Tables A1.1 and A1.2). These were dissolved in 900 ml DDH_2O and, except where stated otherwise, the following added (mg/l): agar, 7 000; sucrose, 20 000; casein hydrolysate, 100; cytokinins and auxins as required (see individual reports). While stirring, the pH was adjusted using 1 N sodium hydroxide or 1 N hydrochloric acid. This was measured using a newly calibrated Pye Model 293 pH meter fitted with a Pye Unichem Ingold pH probe. Additional DDH_2O was added to give a final volume of one litre. The medium was then boiled slowly to dissolve the agar, mixed thoroughly and dispensed into four 250 ml flasks. After being sealed with heavy duty aluminium foil these were autoclaved. After cooling, but before setting, 0.25 ml of vitamin stock solution was added to each flask and mixed thoroughly.

d) Surface sterilization.

All explants were surface sterilized. Various techniques were assessed, as explants were found to possess differing levels of contamination. Gall material was especially difficult to surface sterilize; this was due, possibly, to the uneven surface of the gall and to the internal contamination of the leaf during oviposition. The first technique to be tried was on buds (see Plate 4.8) removed from willow cuttings,

originally collected from Little Wittenham (see Appendix Two) and rooted and grown in a plant growth cabinet (see Section 4.2.2). These were the type of leaves chosen by Pontania proxima for oviposition (see Section 4.4). Surface sterilization should be easier to accomplish with only two to four leaves being exposed; this process was also aided by the plants being grown in a cabinet (Tormala and Saarikko, 1985). The leaves were separated from each other and washed in warm soapy tap water. They were then rinsed three times in 200 ml of DDH_2O. Sterilization was achieved by placing the leaves in a solution of 10% hydrogen peroxide for nine minutes (Bychenkova and David, 1978), followed by three rinses in DDH_2O to remove all traces of the peroxide. The use of hydrogen peroxide, however, did not prove to be as satisfactory as a 10 minute immersion in 10% sodium hypochlorite, pH 6.0 (Dodds and Roberts, 1985: 26). Gall material or older leaves were not amenable to either of these treatments as cultures of these explants soon exhibited high levels of contamination. Finally, the following protocol was adopted for all explant material. The explants were immersed in 70% ethanol for one minute; this was followed by three washes in DDH_2O, each wash lasting three minutes. Sterilization was achieved by immersion for 12 minutes in 100 ml of 10% sodium hypochlorite, pH 6.0, containing two drops of TWEEN 20 (polyoxyethylenesorbitan). The explants were finally washed three times in DDH_2O, three minutes each wash.

	Media used mg/1				
	I	II	III	IV	
Inorganic salts.					
Ammonium chloride				5.35	NH_4Cl
Ammonium nitrate	1650.0	400.0	1650.0		NH_4NO_3
Boric acid	6.2	6.2	6.2	0.056	H_3BO_3
Calcium chloride (anhydrous)	332.2	72.5	332.2		$CaCl_2$
Calcium chloride.$2H_2O$				75.0	$CaCl_2.2H_2O$
Calcium nitrate.$4H_2O$		386.0			$Ca(NO_3)_2.4H_2O$
Cobalt (II) chloride $6H_2O$	0.025		0.025	0.02	$CoCl_2.6H_2O$
Copper (II) chloride.$2H_2O$				0.054	$CuCl_2.2H_2O$
Copper (II) sulphate $5H_2O$	0.025	0.25	0.025		$CuSO_4.5H_2O$
Ethylenediaminetetraacetic acid (Disodium salt)	37.26	37.26	37.26	0.8	
Iron (III) chloride.6H20				0.5	$FeCl_3.6H_2O$
Iron (II) sulphate	27.8	27.8	27.8		$FeSO_4.7H_2O$
Magnesium sulphate	180.7	180.7	180.7	112.1	$MgSO_4$
Manganese (II) chloride $4H_2O$				0.036	$MnCl_2.4H_2O$
Manganese (II) sulphate (anhydrous)		22.3			$MnSO_4$
Manganese (II) sulphate	16.9		16.9		$MnSO_4$
Molybdic acid (sodium salt).$2H_2O$	0.25	0.25	0.25	0.025	$NaMoO_2.2H_2O$
Potassium chloride				750.0	KCl
Potassium iodide	0.83		0.83		KI
Potassium nitrate	1900.0		1900.0		KNO_3
Potassium phosphate	170.0	170.0	170.0		KH_2PO_4

Table A1.1. Composition of media used in the isolation of protoplasts (I), micropropagation of plantlets (II), induction and maintenance of calli (III) and for the culture of mesophyll cells (IV). (Continued in Table A1.2. See text for full details).

	Media used mg/l				
	I	II	III	IV	
Inorganic salts continued from Table A1.1.					
Potassium sulphate		990.0			K_2SO_4
Sodium phosphate (anhydrous)				108.75	NaH_2PO_4
Sodium nitrate				600.0	$NaNO_3$
Zinc chloride				0.15	$ZnCl_2$
Zinc sulphate.$7H_2O$	8.6	8.6	8.6		$ZnSO_4.7H_2O$
Organics.					
Mannitol	144160.0				
Sucrose		20000.0	20000.0	20000.0	
Ascorbic acid				0.1	
Biotin				0.01	
Calcium pantothenate				0.1	
Choline chloride				10.0	
Myo-inositol	100.0	100.0	100.0	100.0	
Nicotinic acid	0.5	0.5	0.5	0.5	
Pyridoxine HCl	0.5	0.5	0.5	0.1	
Riboflavin				0.1	
Sorbitol				50.0	
Thiamine HCl	1.0	1.0	0.1	0.1	
Glycine	2.0	2.0	2.0		
Casein hydrolysate	100.0	100.0	100.0	400.0	
Growth Regulators.					
Kn			0.5	0.1	
2,4-D			0 to 4	1.0	
BAP	0.023	0 to 2			
NAA	0.187	0 to 0.5			
Agar	7500.0	7000.0	7000.0		
pH	5.6	5.6	5.9	5.9	

Table A1.2. Composition of media used in the isolation of protoplasts (I), micropropagation of plantlets (II), induction and maintenance of calli (III) and for the culture of mesophyll cells (IV). (Continuation of Table A1.1. See text for full details).

A1.2 Induction and maintainance of calli.

A1.2.1 Introduction.

AUTHOR	BASAL MEDIUM PLUS SUPPLEMENTS	SPECIES	EXPLANT	RESULT
Hovanitz, (1959).	?	Salix	?	callus?
Pelet et al., (1960).	modified White's (1942)	Salix	leaf galls normal leaves	callus? callus?
Bychenkova and David, (1978).	MS + 3 mg/l 2,4-D + 0.5 mg/l Kn	Populus	leaf	callus
Kim, Noh and Park, (1983).	WS + 1 ppm 2,4-D + 1 ppm NAA	Populus	anther	callus
Hoffman, Miller and Pengelly, (1985).	MS + 5 μM 2,4-D. (NAA + BAP for xylem production)	Populus	young branches	callus
Youn, Lee and Lee, (1985).	MS + 0.01 ppm BAP + 5.0 ppm NAA	Populus	shoots from *in vitro* plantlets	callus
Saito, Hosoi, Ishii and Sato, (1987).	WS + 0.02 ppm BAP + 0.20 ppm NAA Subcultured on MS + 0.02 ppm BAP + 0.02 ppm NAA	Populus	cambium from branch	callus
Sumiya, Sunakawa, Ishimoto and Kasai, (1988).	modified SM + 0.4 mg/l 2,4-D + 0.5 0.5 mg/l Kn	Populus	petioles	callus

Table A1.3. Summary of media and explants used in the production of callus tissue of species of Populus and Salix by earlier workers.

Tissue culture of species of Populus has attracted more attention than that for species of Salix, not only in terms of micropropagation (Lin et al., 1983; Russell and McCown, 1986a, 1986b and 1988; Rutledge and Douglas, 1988), but also with respect to calli formation for both physiological and biochemical experiments (see Table A1.3; Duda and Kacperska, 1983; Douglas, 1985). Table A1.3 summarizes details of papers concerned with the tissue culture of Salix, Salix galls and Populus that resulted in callus formation rather than in propagation or organogenesis.

From the selection of references in Table A1.3, the methods of Bychenkova and David (1978) and Sumiya et al. (1988) were adopted for callus induction (see Tables A1.1 and A1.2, tissue culture medium I). Bychenkova and David (1978) used leaf tissue in particular, which for the purpose of the study of Pontania galls is essential, together with Murashige and Skoog based media (MS). This medium was designed to minimize response to inorganic salts, sugars etc. and so reflect "the presence in the medium of growth promoting organic substances" (Murashige and Skoog, 1962); it is also thought that for many woody plants, MS inhibits shoot growth and subsequent rooting (Alderson, 1986). The use of 2,4-D is effective in inducing and proliferating callus in many species (Zaerr and Mapes, 1982), but may induce mutations (Zaerr and Mapes, 1982; Pierik, 1987: 69). Because of this, various dilutions of 2,4-D were tested, so as to keep this chemical minimal, but still reflect the work of Bychenkova and David (1978) and Sumiya et al. (1988), who developed techniques for the regeneration of Populus from callus material.

The culture of Salix galls has only been attempted in a few cases. Hovanitz (1959) mentions the tissue culture of Salix tissue, but gives

few details of the methodology he employed. More details are given by Pelet et al. (1960) who compared the in vitro growth and nutrition of callus tissue, derived from insect-induced galls and normal plant tissue. Although willow was used to some extent, their main conclusions were based on the galls induced by Phylloxera vastatrix (= Viteus vitifolii) and a species of midge (Cecidomyia Meigen, Cecidmyiidae) on Vitis riparia Michaux (Vitaceae; =Vitis vulpina L.?). Their results suggested that there was nutritional variation between calli derived from galls and calli derived from normal tissue. These variations also varied with the time of cultivation in vitro. Warick and Hildebrandt (1966) recognized these nutritional differences, but stated that they only existed when first isolated; later, both normal and gall-derived tissue grew well on the same tissue culture medium. Pelet et al. (1960) also concluded that the induction of gall tissue did not result in a permanent change in growth regulator requirements. All these galls are of the simple cataplasmic type; indeed Pelet et al. (1960) note that attempts to grow the highly organized oak apple failed.

A second method of callus induction was also attempted. This was based on the culture of palisade mesophyll cells. Much of the tissues of the galls of Pontania species is derived from the mesophyll layers; if successful, callus derived from these cells may create a better approximation of the tissues used for gall formation.

The culture of mesophyll cells has been achieved by Joshi and Ball (1968a, 1968b), who developed this technique during the study of the division in isolated cells of palisade parenchyma of Arachis hypogaea L. (Leguminosae; Ball and Joshi, 1965). Shake cultures were obtained from mature palisade cells (those from immature palisade and spongy mesophyll failed), which did not lose their chloroplasts during

dedifferentiation. Joshi and Ball (1968a) evaluated different media and their effect on the growth of different species, none of which was related to Salix. From their observations the most effective tissue culture medium was based on the macronutrients of Heller (1953) and a mixture of micronutrients, vitamins and other compounds shown in Tables A1.1 and A1.2. The inclusion of casein hydrolysate and a small amount of ammonium chloride was found to produce a high growth value. Vitamins and inositol were also included in the first evaluation of this technique although Joshi and Ball (1968b) found that with Arachis hypogaea they were unnecessary. Joshi and Ball (1968a, 1968b) used small inocula (average mass 0.60 mg). These resulted in the growth of cellular masses on both the walls of the culture flasks (125 ml Erlenmeyer flasks with 40 ml of tissue culture medium) and of large spheres of tissue (1-2 cm in diameter). The surface of these spheres was composed of large cells, under which were smaller, more densely staining cells, it was believed by Joshi and Ball (1968a) that these cells were meristematic. The next inner layer was a wide zone of small cells containing occasional groups of tracheids. Finally, the centre of the sphere was occupied by large thin-walled cells. In a very superficial way this bears some similarity to the structure of a typical Pontania gall (see Section 5.3.2).

A1.2.2 Methods.

a) Use of explants.

Efforts to induce callus formation was carried out on tissue culture medium I (see Tables A1.1 and A1.2). Levels of growth regulators were 0.5 mg/l Kn together with either four, two, one or 0.5 mg/l 2,4-D.

These were chosen to reflect the various concentrations listed in Table A1.3.

A variety of explant material was used; this included leaf and gall material of all ages. Leaf material ranged from bud-leaves to mature leaves and gall material from newly implanted eggs to mature galls. Leaf disc explants were obtained using a size 2 cork borer. This resulted in circular leaf discs, each with a diameter of five millimetres. The discs were either removed from the centre portion of the leaf and thus was transected by the midrib, or from the lamina itself. All explants were placed with their abaxial surface in contact with the growth media. Alternatively, leaves were cut into strips each four millimetres in length. These lengths were either laid with their abaxial surface in contact with the tissue culture medium, or they were pushed to 1/3-1/2 of their length into the tissue culture medium. Gall explants were removed from the leaf in a similar fashion, or if they were not small enough they were cut from the leaf using a sterile scalpel. Galls were either placed on the tissue culture medium as they were or the lower surface of the gall was removed, so bringing the mesophyll cells into direct contact with the medium. Various sections through gall material were also tried.

All the petri dishes were then sealed with tape and they were incubated in the dark (Dodds and Roberts, 1985: 63) at a temperature of 27° C (Murashige and Skoog, 1962).

b) The culture of mesophyll cells.

The tissue culture medium used (see Table A1.1 and A1.2, medium IV) was filter sterilized (Joshi and Ball, 1968a) and aliquots of 50 ml were placed in 150 ml Erlenmeyer flasks. It was not possible to obtain the inocula in the same way as detailed by Joshi and Ball (1968b), as

leaves of Salix fragilis were not amenable to being torn across to expose the mesophyll. Instead, after being washed and surface-sterilized, the leaves were carefully scraped on their abaxial surface with a small scalpel. This resulted in the lower layers of the leaf being removed. It was then possible to scrape out a small amount of palisade mesophyll. This technique produced inocula of approximately 0.5 to one milligram (fresh weight).

Once inoculated, the tissue culture medium was then placed in a shaker set at 80 rpm. The cultures were placed in a growth cabinet, under light at a temperature of 27° C.

A1.2.3. Results.

a) Explants.

The first indications of callus formation from leaf-bud explants were observed between day seven and 14; by day 19 all material had produced callus. At this stage, the leaf discs were the most effective at producing callus, and did so primarily from cut surfaces at leaf veins, and from the midrib. All tissue cultures contained Kn at 0.5 mg/l with varying amounts of 2,4-D. Of these, quality of growth by day 33 was least in the tissues grown on 0.5 mg/l 2,4-D and most in those grown on four mg/l 2,4-D. Those grown on one or two mg/l 2,4-D were good with not much difference observable between them. After 47 days of growth, the best was observed in leaf discs grown on four mg/l, the worst from embedded leaf lengths on 0.5 mg/l 2,4-D.

Dodds and Roberts (1985: 58) suggested that passage time was somewhat variable, depending upon the growth rate of the callus, but should be within the region of four to six weeks. It was therefore

decided to subculture these tissues, 58 days after they were initiated. Choice of medium would be the same with the concentration of 2,4-D at one mg/l as this encouraged reasonable growth, while keeping the concentration of the auxin at a minimum. An estimation of the growth rate of these cultures is shown in Figure A1.1. This estimation was produced by measuring a set diameter of each callus at intervals and calculating the percentage change of this measurement over a period of approximately 100 days. Older leaves failed to produce as vigorous growth of callus, so were rejected.

Gall material failed to produce callus at all. Of the many explants of different ages that were tested on a variety of different concentrations of growth regulators, none produced any sign of callus formation.

b) The culture of mesophyll cells.

Although the cultures maintained healthy green cells, they failed to produce balls of cells. There were layers of cells on the side of the culture vessels, but no further development took place.

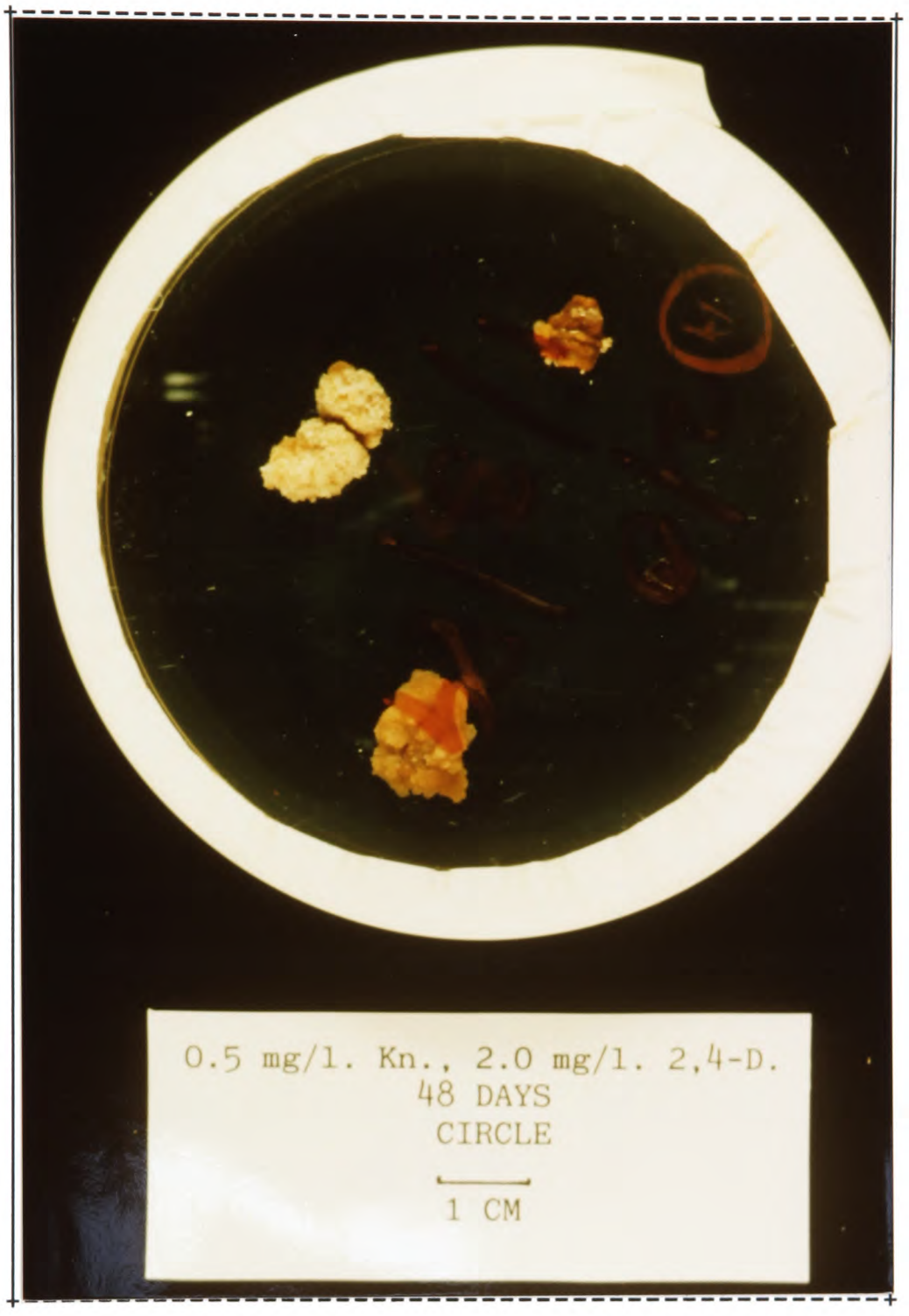


Plate A1.1. Calli developed from leaf-disc explants and grown on MS based media containing 0.5 mg/l Kn and 2.0 mg/l 2,4-D. Photograph taken after 48 days of development using 100D Professional Fuji film (used throughout).

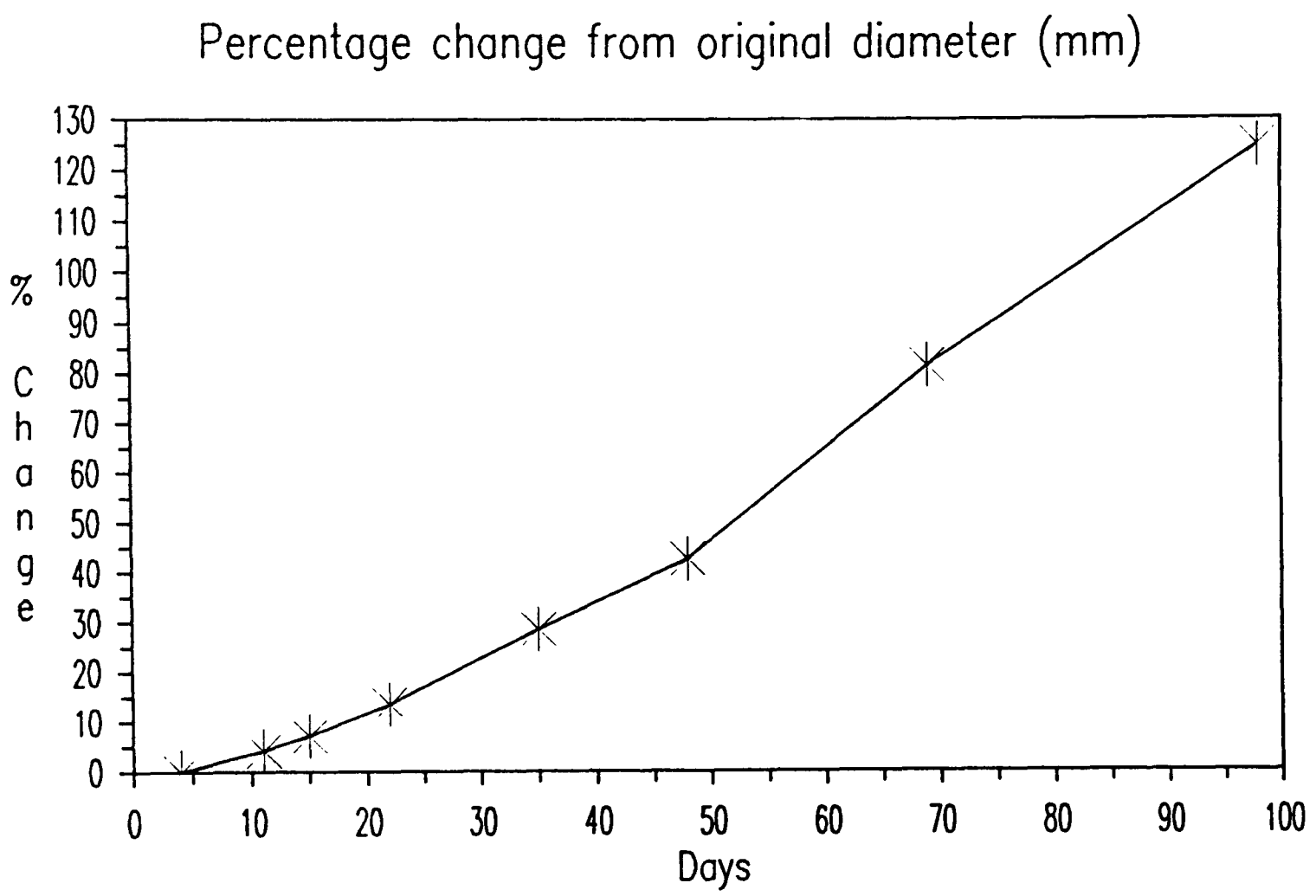


Figure A1.1. Growth rate of subculture. Percentage increase in diameter of callus from original diameter (n = 33). Kn = 0.5 mg/l; 2,4-D = 1 mg/l.

A1.3 **Micropropagation of plantlets.**

A1.3.1 Introduction.

Micropropagation of woody plants has been reviewed by Zimmerman (1985), Alderson (1986) and Chalupa (1987). Although this technique lags behind the propagation techniques used in floriculture (Evers et al., 1988: 1), by 1985 species in at least 50 genera of ornamentals, forest and fruit trees were commercially available (Zimmerman, 1985) using micropropagation. The main methods are either axillary shoot proliferation, organ culture, callus culture or embryogenesis. Leaf explants and callus cultures are not usually used in micropropagation work; questions have been raised regarding the genetic constitution of leaf cells (Ahuja, 1983) and problems with propagation from callus cultures include genetic variation of plantlets, contamination of tissues with bacteria, blackening of tissues and media, shoot-tip necrosis, vitrification, flushing of growth and phase change (Alderson, 1986).

Although much of the pre-1975 work concentrated on regeneration of trees from callus cultures, it was achieved in only a few hardwood species (Chalupa, 1987). Ahuja (1987) maintains that attempts to propagate from callus cultures should be avoided as there is a limited shoot production and increased genetic variation; more often these problems can be reduced using organ culture. This variation has been used by others for crop improvement studies (Shepard et al., 1980). Within this work, it is recognized that the danger of genetic variation exists, but overriding these considerations is the formulation of correct procedures for the comparison of callus derived from normal

leaf tissue and from gall tissue, with the regeneration of plantlets if possible.

BAP is recommended by Zaerr and Mapes (1982) for shoot formation and has been used by Bergman et al. (1985) with Salix and Wolter (1968) with Populus. Both Bergman et al. (1985) and Wolter (1968) subsequently used NAA for root production. Thus, the general trend is that cytokinins favour shoot development, auxins root differentiation (Skoog and Miller, 1957). In this way they act antagonistically, depending on the sensitivity of cells and levels of naturally occurring inhibitors and growth promotors (Brown and Sommer, 1982). They also warn that many earlier attempts to propagate whole plants from callus cultures e.g. Wolter (1968) and Winton (1968) gave doubts about normality and genetic stability. However, Winton (1970, 1971) did grow normal trees from shoots excised from callus.

A popular tissue culture medium for the regeneration of woody species is WPM, which contains lower concentrations of ammonium ions, high levels of which contribute to the possibility of vitrification i.e. hyperhydricity of tissues, which has widely been reported on MS substrates (Alderson, 1986; Daguin and Letouzé, 1986 and Gaspar et al., 1987). A summary of tissue culture media, used for production of plantlets from callus or explants, derived for species of both Populus and Salix, is given in Table A1.4. No examples could be found of this type of work on Salix leaves or calli. The medium chosen was WPM together with BAP as growth regulators (see Tables A1.1 and A1.2, tissue culture medium II).

AUTHOR	BASAL MEDIUM PLUS SUPPLEMENTS	SPECIES	EXPLANT	RESULT
Wolter, (1968).	WS + 0.14 mg/l BAP; WS + 0.5 mg/l. NAA	Populus	callus	shoot diff. root diff.
Winton, (1970).	modified WS + 0.05 mg/l BAP	Populus	callus	shoot and root diff.
Bychenkova and David, (1978).	DM + 2.25 mg/l BAP+5x10^{-9} mg/l NAA	Populus	callus	shoot and root diff.
Ahuja, (1983).	ACM + 0.5 mg/l BAP + 0.02 mg/l NAA	Populus	leaf discs	shoot diff.
Chalupa, (1983).	modified GD + low auxin	Salix	nodal	shoot growth and root diff.
Bergman et al., (1985).	WPM + 10^{-6} M BAP; WPM + 10^{-6} M NAA	Salix spp.	excised buds	shoot diff. and root diff.
Tormala and Saarikko, (1985).	WPM + 0.1 to 0 mg/l BAP; WPM + 0.1 mg/l NAA	Salix spp.	shoot tips or single node segments	shoot prolif. root diff.
Park and Son, (1988)	MS + 0.44 μM BAP + 4.52 μM NAA	Populus	callus	shoot regen.
Cheema, (1989)	MS + 0.4 mg/l BAP + 0.2 mg/l NAA	Populus	callus	shoot and root regen.

Table A1.4. Summary of media and explants used in the differentiation (diff.) of shoots and roots, regeneration (regen.) of shoots and roots, shoot proliferation (prolif.) and growth of species of Populus and Salix from explants and callus material by earlier workers.

A1.3.2 Methods.

WPM tissue culture medium was used together with BAP and NAA as growth regulators. The concentrations of BAP used were between 0 and two mg/1, those for NAA were 0 to 0.5 mg/1. These concentrations were chosen to reflect the work of Cheema (1989) for regeneration from callus tissue and that of Ahuja (1983) for micropropagation form leaf discs. Lysine at a concentration of 100 mg/1 was also added to some cultures. The usual protocols mentioned elsewhere in this Chapter were followed. The cultures were incubated at 25° C in dim light.

A1.3.3 Results.

The only successful regeneration was that of roots. Rhizogenesis occurred from both callus and leaf-disc cultures. Caulogenesis did not occur in either of these two type of culture, whether preceded by rhizogenesis or not.

Callus cultures previously maintained on MS produced roots from about 20 days onwards, when placed on WPM with BAP levels in the range of 0 to 0.5 mg/1 and NAA levels 0.1 to 0.2 mg/1. The cultures themselves developed green and red areas. Leaf-disc cultures produced small amounts of callus from which the roots arose. Growth regulator concentrations required for rhizogenesis were slightly different and BAP one to two mg/1 with NAA at 0.5 mg/1, or BAP 0 mg/1 and NAA 0.5 mg/1 were all effective.

From these initial experiments root formation from callus was achieved easily using a BAP concentration of 0 mg/1 and NAA concentration of 0.2 mg/1. For leaf-disc culture the levels used were BAP 0 mg/1 and NAA 0.5 mg/1. The addition of lysine did not seem to enhance rhizogenesis.

The roots formed in all cases were green when small and developed root hairs when approximately five millmetres in length. As the roots increased in length they became red in colour and developed laterals. When healthy rooting cultures were subcultured onto WPM containing different concentrations of regulators where NAA was reduced to encourage caulogenesis e.g. BAP 0.5 mg/l, NAA 0.02 mg/l, many roots became swollen at the tip and failed to continue development.

Plate A1.2. Rhizogenesis from callus material 34 days after subculture on WPM containing BAP 0 mg/l and NAA 0.2 mg/l (Mag. x9).

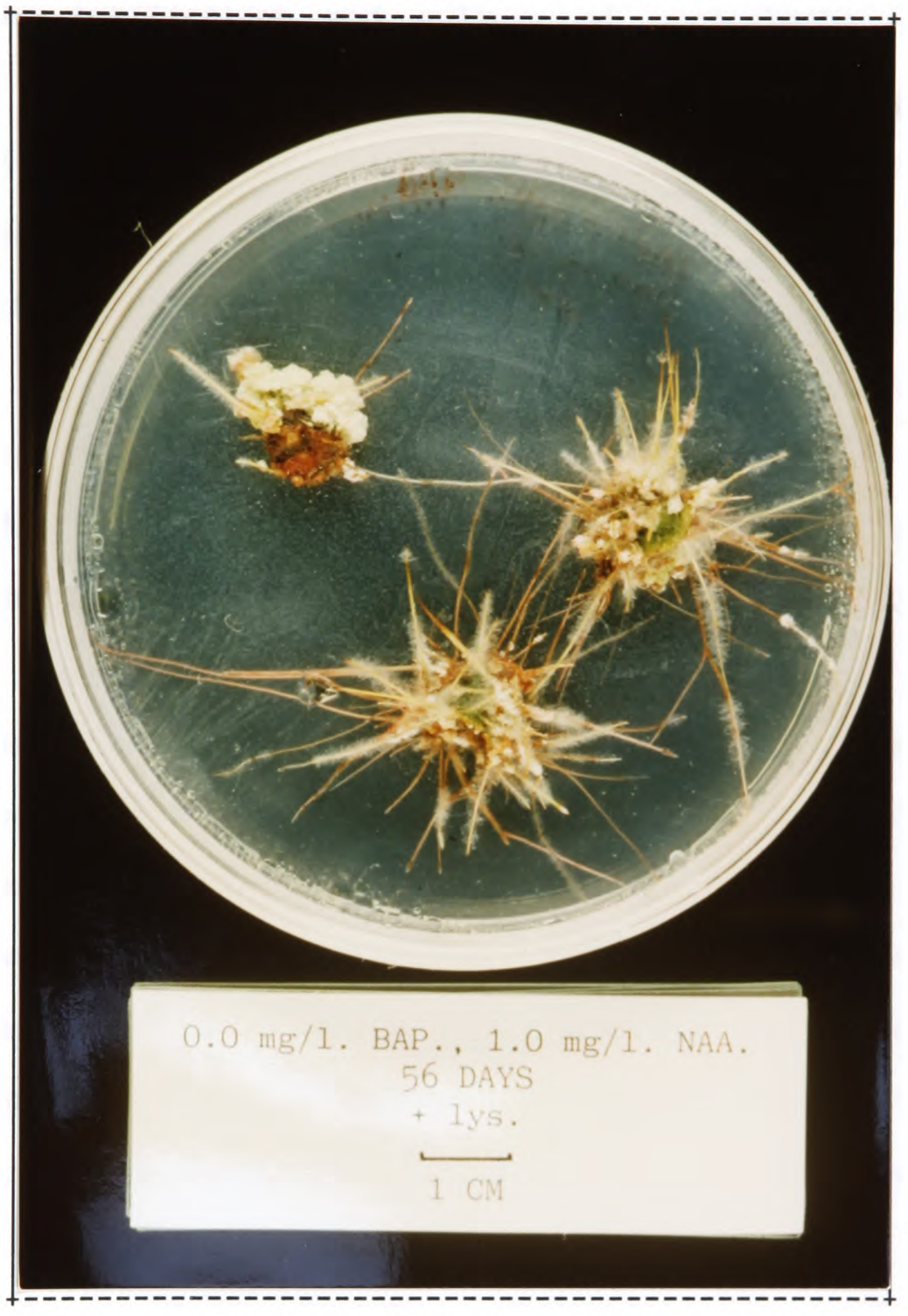


Plate A1.3. Rhizogenesis from leaf-disc 56 days after culture on WPM containing BAP 0 mg/l and NAA 1.0 mg/l with additional lysine 100 mg/l.

A1.4 **Protoplast extraction.**

A1.4.1 Introduction.

The isolation, purification and culture of protoplasts, has been recorded for a number of different species (George et al., 1987: 482-490; McCown and Russell, 1987: 16-30). However, information on woody species, especially Salix species, seems sparse and for gall tissue non-existent. Recovery of plants from leaf protoplasts has been reported for two clones of Populus species by Russell and McCown (1988), believed to be the first such for this genus.

Dodds and Roberts (1985: 133) reviewed general procedures, which included mechanical or enzymatic methods form the release of protoplasts; of these the enzymatic process is the more popular. Protoplasts are unusual in that the outer face of the plasmalemma is exposed and in direct contact with the external environment. This makes protoplasts amenable to fusion (Power and Cocking, 1971; Dudits et al., 1979), so that somatic cell fusion may be used to overcome genetic barriers. Protoplasts have also been used to study cell wall development, as cultured protoplasts rapidly regenerate a new cell wall (Grout et al., 1973). They have also been used to study endocytosis (Dodds and Bengochea, 1983). Evans and Cocking (1977) describe populations of protoplasts as having similarities to a single cell system, with their manipulation being similar to that of microorganisms.

These characteristics of protoplasts suggest that they may be a useful addition to the study of gall and non-galled tissue. Firstly, the production of protoplasts would be a ready supply of experimental material, to be used in the production of gall and non-galled cultures.

Secondly, the fusion of gall and non-galled tissues and the resulting heterokaryon may be useful in revealing the extent of the control of the genome over the characteristics of a gall cell, or even whether such characters are a permanent feature. Thirdly, protoplasts may aid the characterization of structural differences between galled and non-galled cells.

Numerous protocols were tried and modified in order to provide a basic procedure for the extraction and isolation of protoplast from both gall and normal tissue.

A1.4.2 Experiment to estimate optimum plasmolyticum for leaf and gall tissue.

1. Methods.

Leaf and gall tissue was shredded into strips approximately one to two mm in width. These were then washed in distilled water to remove debris (e.g. frass), and dried on tissue. Known masses were then placed for 30 minutes in solutions of mannitol ranging from 0.85 M to 0.5 M., after which they were dried on tissue and changes in mass calculated.

2. Results.

See Table A1.5 and Figure A1.2.

3. Discussion.

Before the cell wall is removed, it is necessary to bathe the cells in a suitable plasmolyticum to encourage plasmolysis, thus preventing subsequent osmotic lysis during the digestion of the cell wall and the escape of the protoplast from its remains. In this experiment both gall and normal leaf tissue from Salix fragilis are plasmolysed by a 0.8 M mannitol solution. Therefore, this concentration of mannitol was used through this work.

MOLARITY	Leaf tissue			Gall tissue		
	MASS AT START	MASS AT END	% CHANGE	MASS AT START	MASS AT END	% CHANGE
0.85	0.4635	0.4113	-11.2621	0.5130	0.4772	-6.97855
0.80	0.4724	0.4279	-9.4201	0.5291	0.4909	-7.21988
0.75	0.4723	0.4873	3.1760	0.5154	0.4956	-3.84171
0.70	0.5206	0.5619	7.9331	0.5252	0.5274	0.41891
0.65	0.4819	0.5435	12.7827	0.5179	0.5291	2.16257
0.60	0.4735	0.5480	15.7339	0.5214	0.5317	1.97557
0.55	0.4646	0.5690	22.4709	0.5172	0.5350	3.44149
0.50	0.4376	0.5791	32.3355	0.5186	0.5358	3.31653

Table A1.5. Percentage changes in mass of gall and leaf tissue after 30 minutes in mannitol solutions of differing molarity.

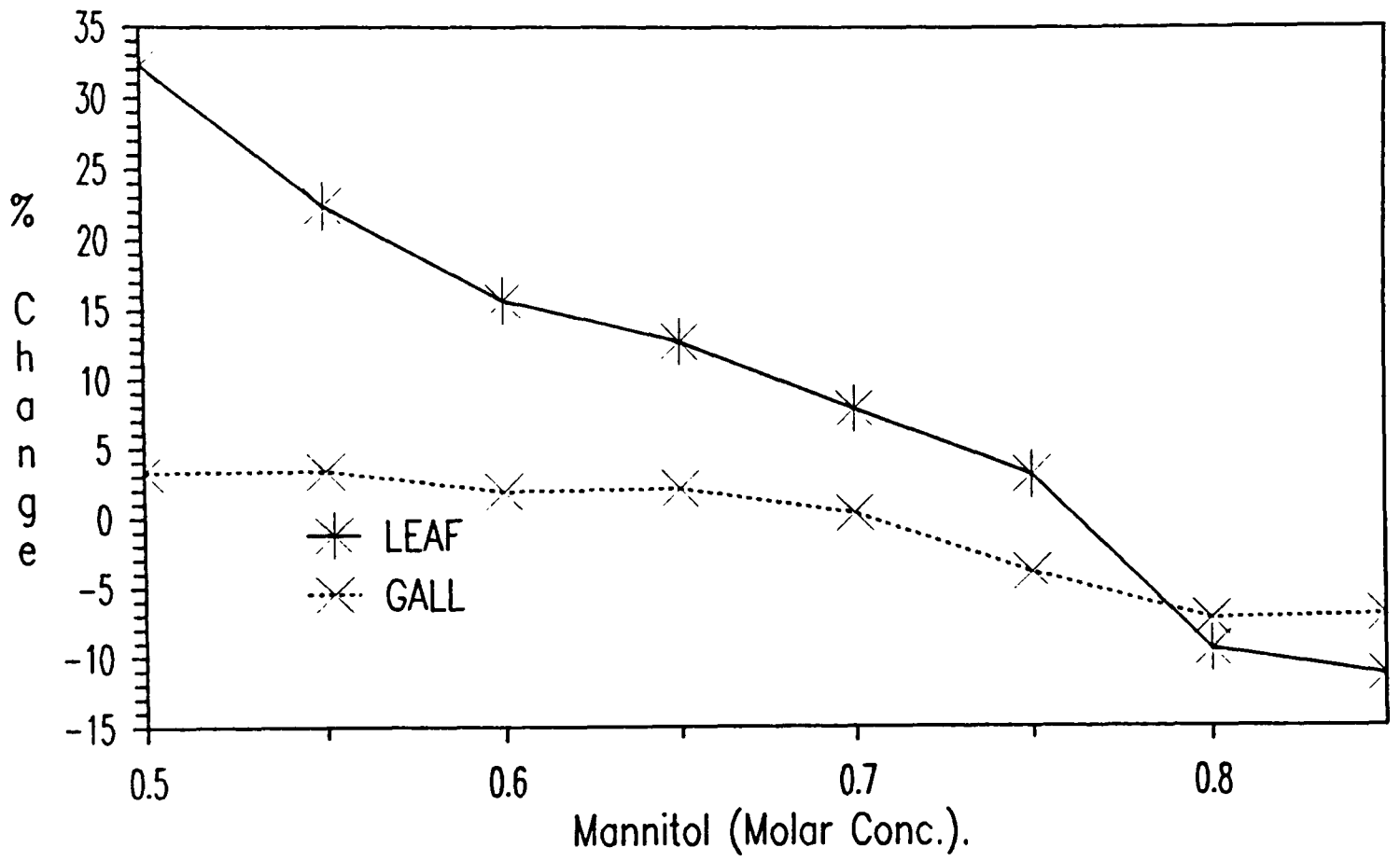


Figure A1.2. Percentage changes in mass of gall and leaf tissue after 30 minutes in mannitol solutions of differing molarity.

A1.4.3 Reagents used for protoplast extraction.

1. Prewash solution of 0.2 mM anhydrous calcium chloride in DDH_2O. This was used to wash the gall and leaf tissue prior to digestion. Each wash was for 30 minutes and was repeated three times (Butt, 1985), this removed the leachate that may inhibit digestion in woody species. Use of calcium chloride aided the preservation of membranes.

2. Plasmolysing solution. This was prepared from 0.8 M mannitol in complete MS. After sterilization of 100 ml a further 50 ml was added via filtration sterilization. This addition contained 0.0175 g bovine serum albumen, which acted as a substrate for excess proteases; 0.0116 g dithiothreitol, which maintained -SH groups in a reduced state; 0.00026 g phenylmethylsulfonyl fluoride (PMSF), which acted as a protease inhibitor and was prepared by dissolving 0.26 g in 100 ml of 2-propanol and adding 0.1 ml to the 50 ml for sterilization. Finally 0.15 ml of MS vitamin mixture was added. This gave the final concentration of 0.025% bovine serum albumen; 0.5 mM DTT; 10 M PMSF; vitamins.

3. Digest solution. To 50 ml of sterile plasmolysing solution was added 0.5 g Cellulysin; 0.25 g Hemicellulase and 0.25% Pectinase. This was filter sterilized and gave final concentrations of 2% Cellulysin; 1% Hemicellulase and 0.5% Pectinase. Before sterilization the pH was adjusted to 5.5.

4. Final wash solution. This consisted of 500 ml of 0.8 M mannitol in complete MS, containing 0.1 μM BAP and 1.0 μM NAA (Russell and McCown, 1988); 0.1 mM MES buffer (0.01 g/500 ml), adjusted to a pH of 6.5. After sterilization 0.5 ml of vitamin solution was added.

5. Plating medium. This had the same composition as the final wash solution. In addition agar was added to give a final concentration of

1.5%. When plated this medium was added to the wash in a 1:1 ratio, giving 0.75% agar concentration (Dodds and Roberts, 1985: 140-141).

A1.4.4. Methods.

Galls were chosen on the basis of their strong red colour (cyanidin-3-monoglucoside, Blunden and Challen, 1965), as it was hoped that the anthocyanin would be an easily identifiable marker for gall cells. From these, only galls that lacked an emergence hole for the insect were selected. This last criterion ensured that tissue within the gall had not been over-consumed and so reduce the high levels of contamination found in previous efforts to culture gall cells.

25 g of leaf or anthocyanin-rich gall tissue was chopped roughly and washed to remove debris. Sterilization was achieved by soaking the tissues in 70% alcohol for one minute, followed by a rinse for three minutes in DDH_2O, then immersion for 15 minutes in 10% sodium hypochlorite at pH 6.0. The tissues were then finely chopped and washed three times (30 minutes each wash) to remove the leachate. The tissues were then placed in the plasmolysing solution for 30 minutes. This was removed and digest solution added. The digest solution and tissue was incubated for seven hours at 27° C on a Mk. V shaker (L.H. Engineering Co., Stoke Poges), set at 40 rpm and one metre below two 75 watt natural light florescent tubes.

The digest solution was then filtered through a nylon mesh (mesh diameter, 100 microns). The filtrate was collected and spun at 100 x g for 10 minutes in a Super Minor Centrifuge (Measuring and Scientific Equipment Ltd., London). The supernatant was decanted off and the pellet resuspended in five cm^3 of wash solution. The resuspended pellet was spun, the supernatant discarded and the pellet resuspended

in 5 cm^3 of the wash solution. For assessment of protoplast concentrations, 50 μl of this suspension was diluted x4 and two microlitres of fluorescein diacetate (5% w/v in acetone) added. Isolated protoplasts were photographed on HP5 film by fluorescence and Nomaski interference contrast microscopy, using an Axiophot Photomicroscope (Carl Zeiss). Concentrations of protoplasts were determined using a haemocytometer. For the culture of protoplasts equal volumes of suspension and culture medium at a temperature of 40° C were carefully mixed and allowed to set. The petri dishes containing this mixture were incubated at 25° C in the presence of a dim white light.

A1.4.5 Results.

Protoplast extraction was possible using these techniques (see Plate A1.4), but yield was leaf protoplasts was low. Typically, yields of approximately 12 x 10^4 protoplasts per gram fresh weight were achieved for gall tissue, with that for leaf tissue being approximately 1 x 10^3. Culture of protoplasts was unsuccessful. Cultures of gall-derived protoplasts always showed signs of contamination within five days, even if treated with antibiotic nystin suspension (concentration equivalent to 6 ml/250 ml of culture medium), which is effective against yeasts and moulds; or antibiotic antimycotic solution effective against a range of bacteria, yeasts and moulds (concentration equivalent 2.5 ml/250 ml of diluted culture medium). Leaf tissue failed to regenerate.

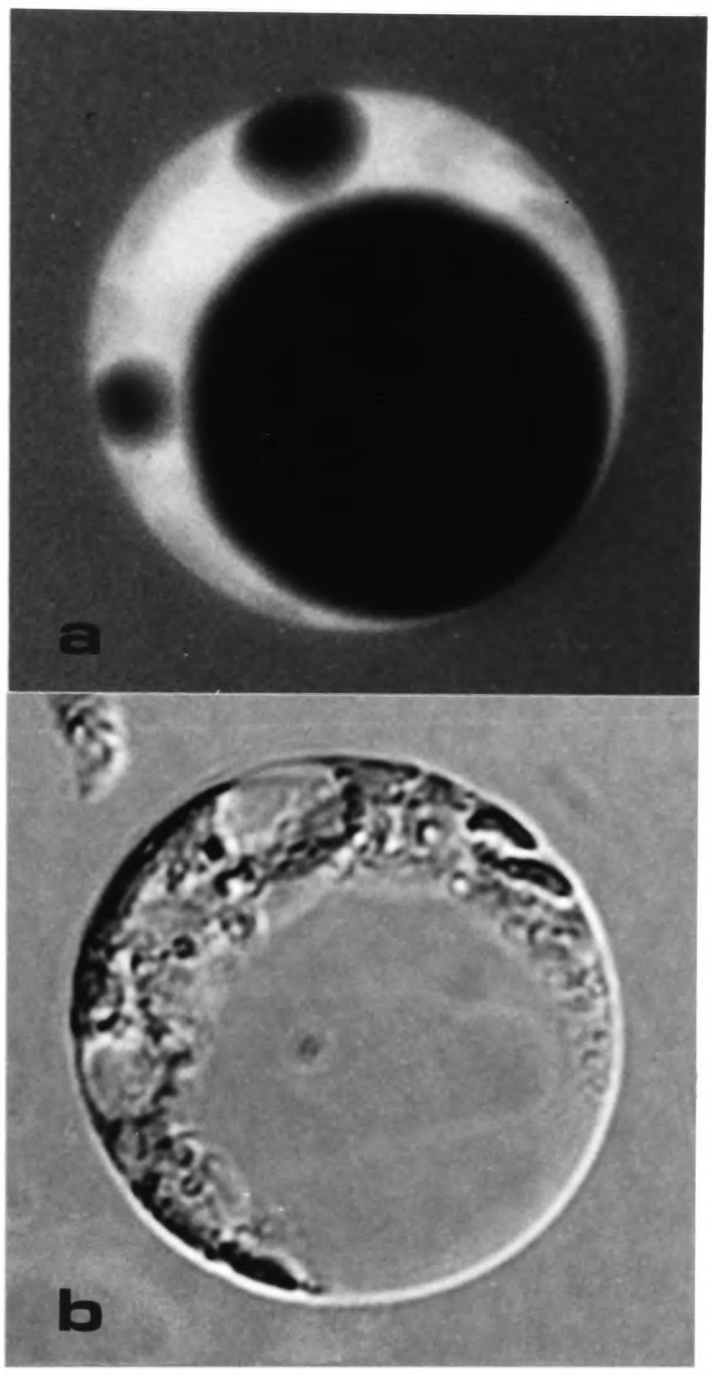


Plate A1.4. Gall protoplasts showing a large anthocyanin-containing vacuole, (a) fluorescence image, (b) Nomarski interference contrast image (Mag. x1300).

A1.5 Discussion.

Callus develops easily from 5 mm leaf discs cut from leaf-bud material and cultured on MS containing Kn at a concentration of 0.5 mg/l and 2,4-D at concentrations of one to four mg/l, the most extensive growth being on the latter.

Micropropagation from leaf material demonstrated differences in response to BAP and NAA between callus material and leaf disc explants. Rhizogenesis was achieved in both case using WPM with BAP and NAA; addition of lysine as suggested by Ahuja (1983) made no difference. Both types of explants produced roots over a range of concentrations of growth regulators but, in the absence of BAP, leaf tissue responded better to a high level of NAA (0.5 mg/l) than did callus material (0.1 to 0.2 mg/l). If BAP was included at levels of one to two mg/l then the reverse was true. Levels of NAA at 0.1 mg/l promoted rhizogenesis in leaf material, while a level of NAA at 0.5 mg/l acted in a similar manner for callus material.

It is difficult to find a reason for the failure of any culture to produce shoots; although a wide concentrations of BAP and NAA were tested, all failed. It could be that the cultures needed other environmental stimuli not tried, such as a different light regime or temperature. More work is needed in this area to bring it to a level similar to that of the culture techniques for species of Populus.

Perhaps the most disappointing, yet intriguing, observation was that gall tissue seemed immune to exogenous growth regulators. No gall culture developed in any way; galls all remained for weeks in the state that they were in when they were first introduced into culture. Reasons for this are unclear but, in the process of cecidogenesis, it could be

that cells become locked into a developmental pattern, a pattern which either does not allow external influences or in someway controls the regulatory metabolism of the gall tissue, with exogenous growth regulators being catabolized.

The initial aim of the protoplast work was to test the viability of methods of protoplast release when applied to gall tissue. The results obtained demonstrate that this initial aim has been achieved. Improvements to this technique should include the preparation of smaller ribbons, so exposing a greater surface area to the digesting enzymes, leading to the release of a larger number of protoplasts. The methods used by Russell and McCown (1986a), which involved a leaf-grinding technique may improve protoplast yields. They were not attempted in this work as access to the equipment required was not possible.

The contamination of gall-derived material is a serious problem. Although surface sterilization techniques were successful, the internal surfaces of the gall remain a difficult area to deal with. If callus tissues of gall-derived explants can be obtained, this may circumvent this problem. However, if this is impossible a number of different sterilizing solutions could be assessed e.g. hydrogen peroxide, calcium hypochlorite.

Protoplast fusion induced by polyethylene glycol (PEG; 20% w/v; molecular weight 3 000), using the methods of Reinert and Yeoman (1982: 44-45) was attempted, but not achieved, probably due to the low numbers of protoplasts available.

Overall the results demonstrated that Salix and Salix gall tissues were rather more recalcitrant than expected. Unfortunately, in the time available, it was not possible to develop basic techniques for the

culture of willow tissue for use in this project. However, advances have been made in the culture of Salix leaves and a start has been made in establishing the conditions required for micropropagation from callus and leaf tissue.

APPENDIX TWO

Locations of field sites used.

ABINGDON LOCK SU500969 50 m

Location: Footpath from bridge to just past lock (SU512969) and back across fields.

Species: Salix fragilis L.

Visited: 23.7.89; 26.6.90

Purpose: Collection of galls.

CLIFTON HAMPDEN SU549954 49 m

Location: 1. Line of trees running south-east to north-west on east bank of the river, 100 m north of the Barley Mow.

2. Riverside by B 4015, running south from Barley Mow.

Species: Salix fragilis L.

Visited: 11.8.88; 13.7.90.

Purpose: Collection of galls

CULHAM CUT SU505948 50 m

Location: 1. Island south of Culham Cut.

2. Path on north side of river, east of lock towards Clifton Hampden (SU509948).

Species: Salix fragilis L.

Visited: 27.7.90; 28.7.90.

Purpose: Collection of galls.

CUMNOR SP459038 105 m

Location: Path leading south by cricket pitch.

Species: Salix fragilis L.

Visited: 4.9.90; 5.9.90

Purpose: Collection of galls.

DRY SANDFORD. SU467996 75 m

Location: BBONT reserve. Side of Sandford Brook at edge of reserve.

Species: Salix fragilis L.

Visited: 9.8.88.

Purpose: Collection of galls.

GARSINGTON MANOR SP582019 95 m

Location: Garsington Village, Southend Road.

Species: Salix x rubens Schrank nothovar. basfordiana (Scaling ex Salter) Meikle forma basfordiana Meikle

Visited: 20.2.89; 22.5.89.

Purpose: Material for propagation; Herbarium specimens.

IFFLEY FIELDS SP522050 50 m

Location: Along east bank of the Isis.

Species: Salix fragilis L.

Visited: 17.7.90.

Purpose: Collection of galls.

LITTLE WITTENHAM SU568932 50 m

Location. Northmoor Nature Reserve on riverside. Trees pollarded 1987, the first time for 60 years. Usually an excellent source of galls. During the winter of 1989-1990, adjacent channel cleared and debris deposited on bank, some trees removed. This affected populations in 1990.

Species: Salix fragilis L.

Visited: 17.1.88; 14.9.88; 9.10.88; 16.10.88; 19.2.89; 22.5.89; 5.7.89; 27.7.89; 13.8.89; 4.9.89; 24.9.89; 8.10.89; 15.2.89; 19.5.90; 7.6.90; 26.6.90; 13.7.90; 23.7.90; 31.7.90.

Purpose: Collection of galls; Material for propagation; Herbarium material; Material for tissue culture; Material for protoplast extraction.

LOWER WOLVERCOTE SP484094 58 m

Location. 1. Field adjacent to A34(T), line of pollarded trees running north.

2. Lane to field 100 m east of Trout Inn (SP483093).

Species: Salix fragilis L.

Visited: 3.5.88; 9.8.88.

Purpose: Collection of galls; Material for propagation.

MEADOW LANE SP527042 55 m

Location. Lane off Donnington Bridge Road, running south by Sports Ground. Trees planted by Oxfordshire County Council.

Species: Salix alba var. caerulea (Sm.) Sm., supplied by Hilliers.

Visited: 24.7.89; 25.7.89; 5.9.89; 17.7.90.

Purpose: Collection of galls.

MESOPOTANIA SP523070 55 m

Location: South-east of University Parks, path opposite Great Meadow, passes across Cherwell and leads to Edgeway Road.

Species: Salix fragilis L.

Visited: 22.8.89; 18.10.89.

Purpose: Material for tissue culture; Material for protoplast extraction.

OCK BRIDGE SU489969 50 m

Location: 1. From bridge, along path towards centre of Abingdon.

2. From bridge along path towards Tesco.

Species: Salix fragilis L.

Visited: 18.7.90; 23.7.90.

Purpose: Collection of galls; Material for tissue culture.

OXFORD CANAL SP503074 55 m

Location: Along riverside between SP503070 and SP504079.

Species: Salix fragilis L.

Visited: 25.6.90; 16.7.90.

Purpose: Collection of galls.

PARK FARM SP524074 55 m

Location: Path by Hertford Sports Ground, leading from the end of Edgeway Road.

Species: Salix fragilis L.

Visited: 22.8.89.

Purpose: Collection of galls; Material for electron microscopy.

PORT MEADOW SP499073 60 m

Location: Opposite Fiddler's Island near Medley Boat Station.

Species: Salix fragilis L.

Visited: 10.1.88.

Purpose: Material for propagation.

RADLEY SU539988 55 m

Location: Towing path from boathouse to SU535977.

Species: Salix fragilis L.

Visited: 31.7.89; 24.7.90.

Purpose: Collection of galls.

SANDFORD SP531013 55 m

Location: 1. On island near lock.

2. Towing path to Radley.

Species: Salix fragilis L.

Visited: 22.7.89; 23.8.89; 4.6.90; 24.7.90; 21.8.90; 4.9.90.

Purpose: Collection of galls; Material for electron microscopy.

SHABBINGTON SU669066 60 m

Location: Bridge over Thames and surrounding area.

Species: Salix fragilis L.

Visited: 2.8.89; 21.7.90.

Purpose: Collection of galls.

STADHAMPTON SU597986 52 m

Location: Field north of small brook running east to west, north of B480, part of Lodge Stud Farm.

Species: Salix fragilis L.; Salix alba L.

Visited: 16.2.88; 7.5.88; 305.88; 10.8.88; 22.5.89.

Collection of galls; material for propagation; Herbarium material.

APPENDIX THREE

Weather observations for the local area.

All data supplied by the Radcliffe Meteorological Station.

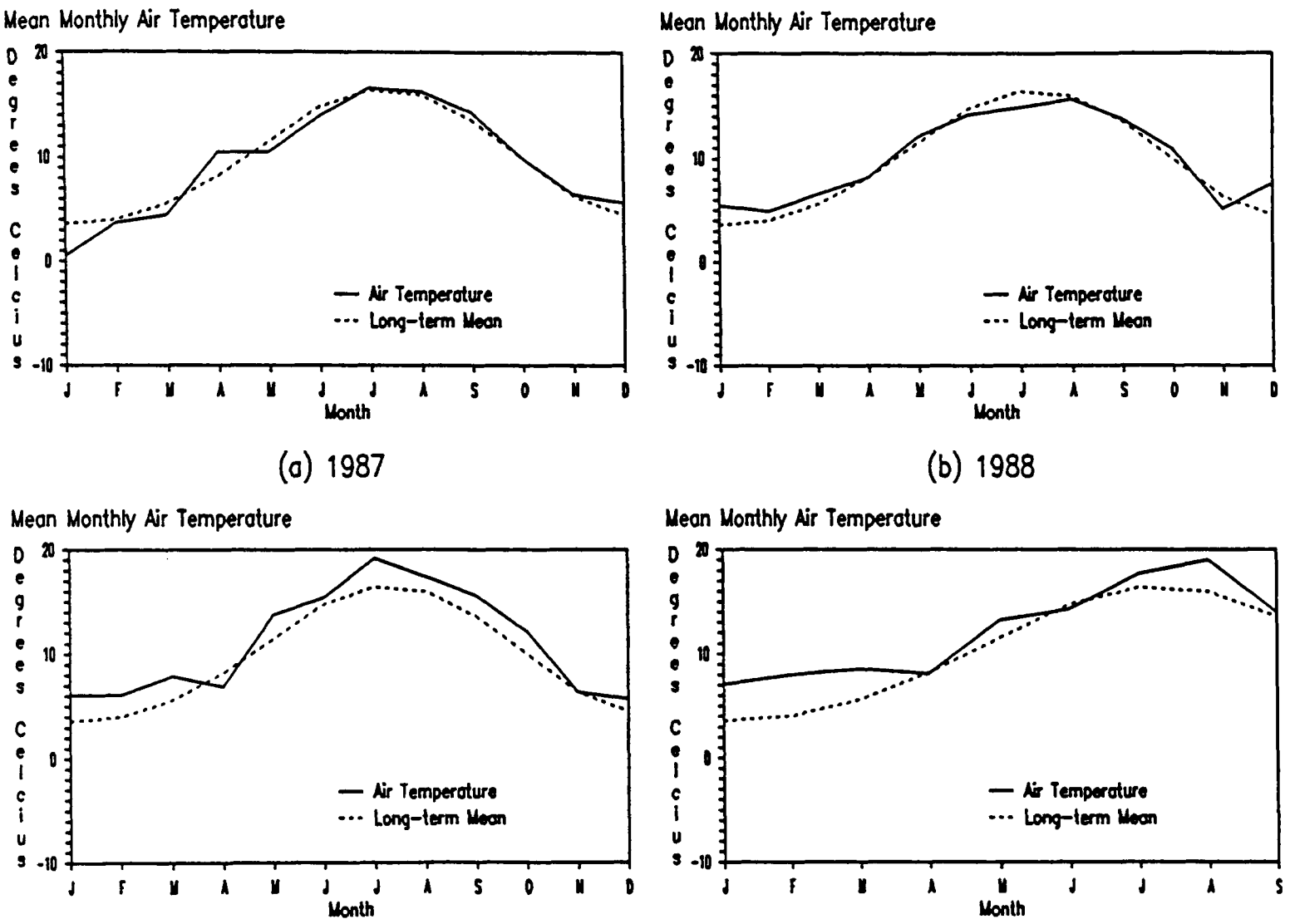


Figure A3.1. Monthly mean of daily air temperatures for the years 1987 to 1990. Long-term means calculated from 1815 to 1989.

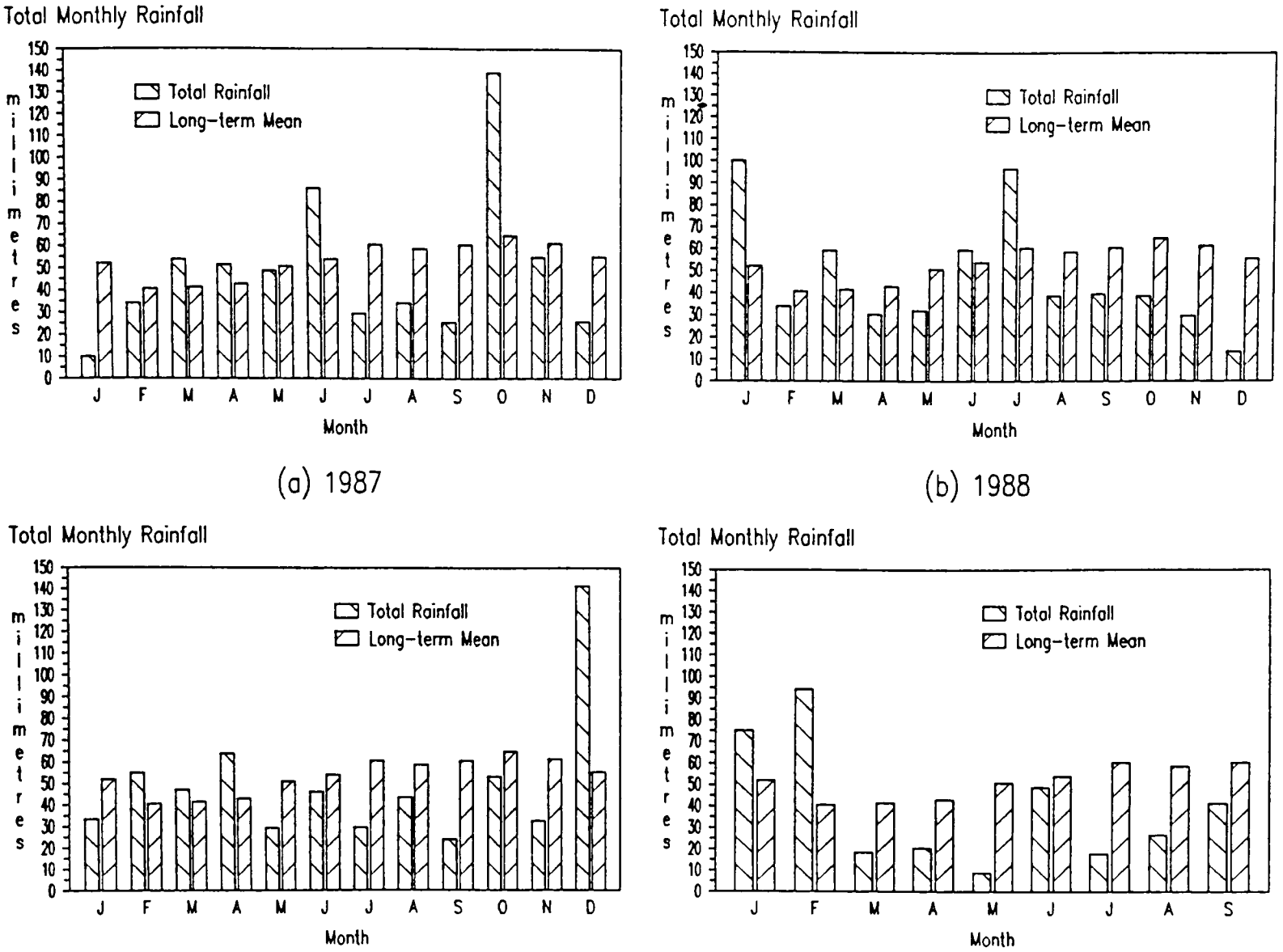


Figure A3.2. Total rainfall each month for the years 1987 to 1990. Long-term means calculated from 1767 to 1989.

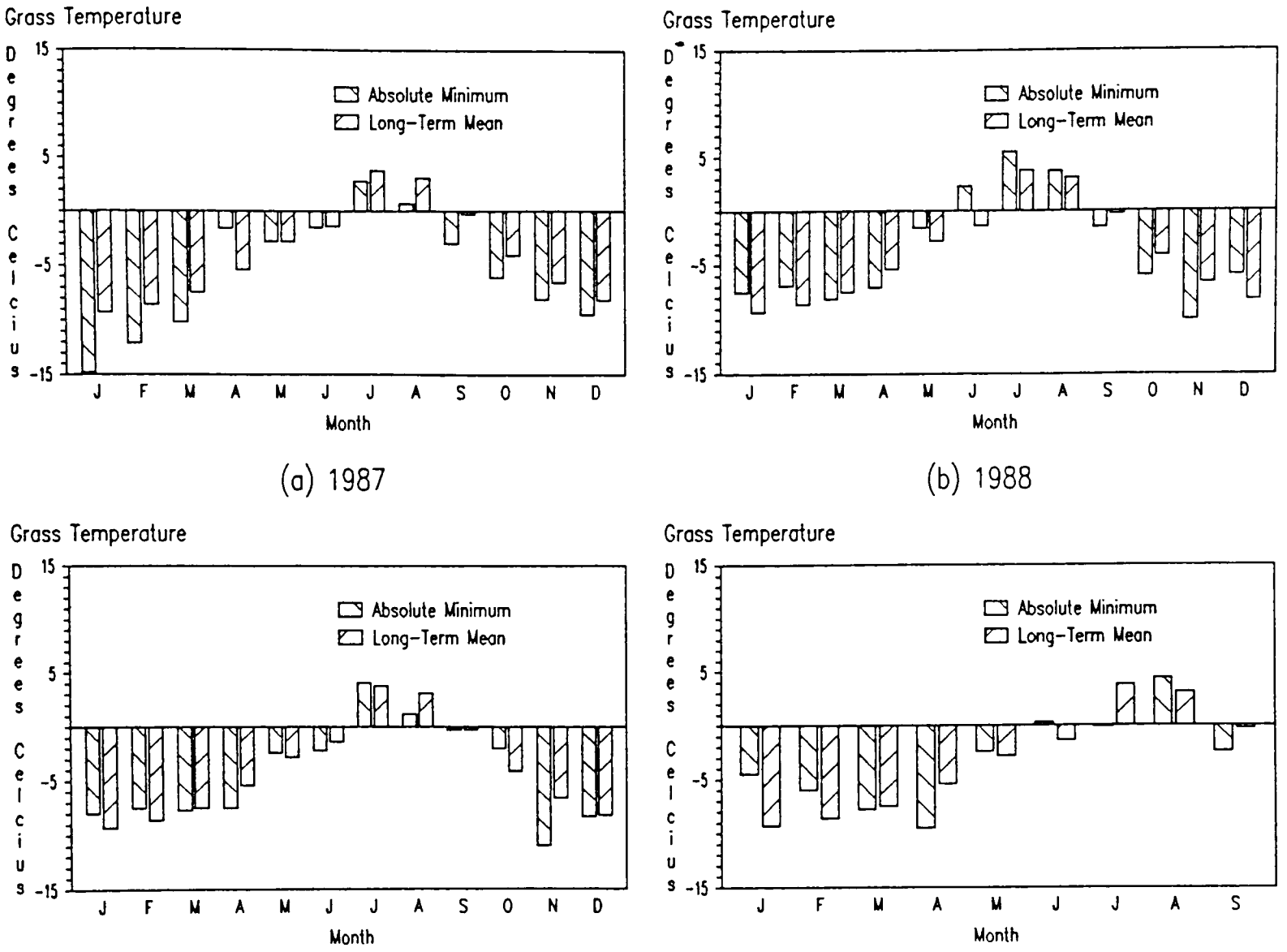
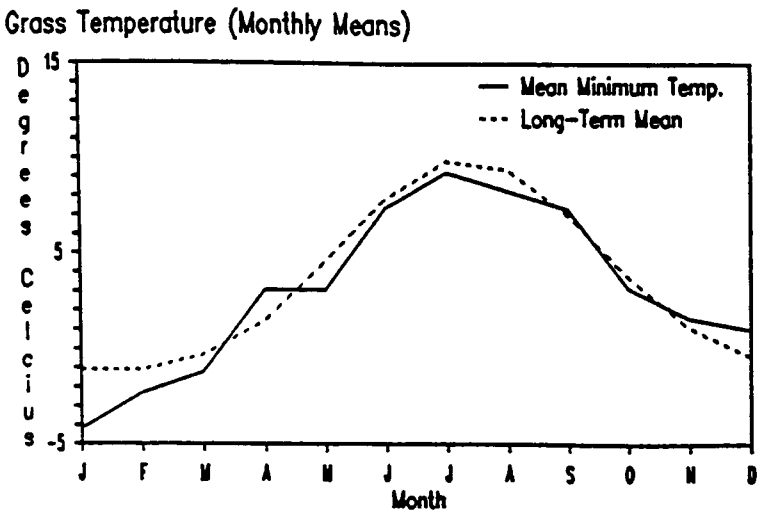
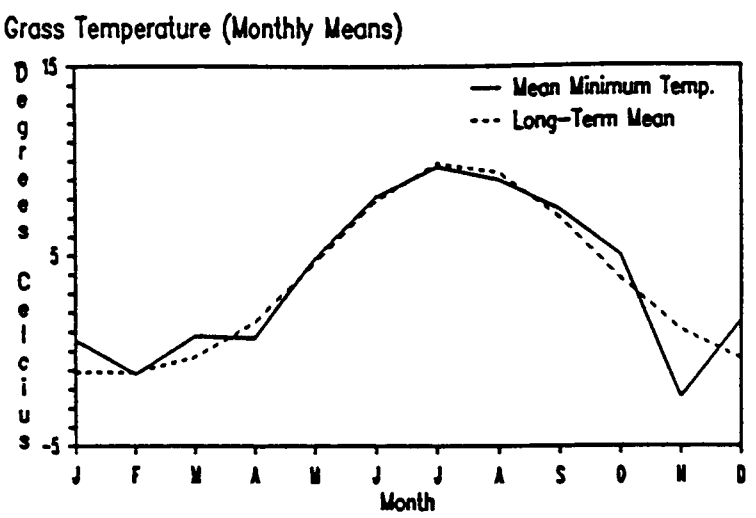


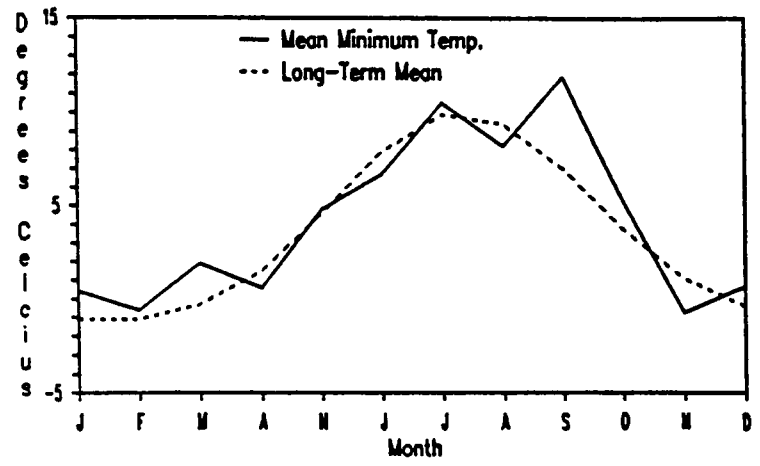
Figure A3.3. Absolute minimum grass temperature each month for the years 1987 to 1990. Long-term means calculated to 1989.



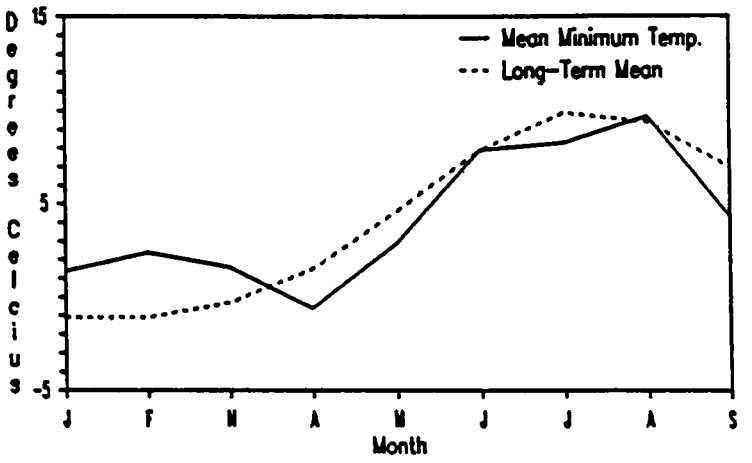
(a) 1987



(b) 1988



(c) 1989



(d) 1990

Figure A3.4. Monthly means of the daily grass minimum temperature for the years 1987 to 1990. Long-term means calculated from 1881 to 1989.

APPENDIX FOUR

Results of studies on topological specificity of oviposition.

Notes.

1. All results processed using Minitab Release 7.2, Minitab, Inc., AOS/VS version.

2. Text used - Minitab Handbook Second Edition (Ryan et al., 1985).

3. Chi-square test used = SUM[(observed-expected)^2/expected] (Ryan et al., 1985:261).

4. Data retained on :DIR:MINITAB:GALLPOSITION and :DIR:MINITAB:LRATIO

5. Statistical tables used were from Statistical methods in biology (Bailey, 1981: 209).

Area of leaf	Number of galls per leaf.											
	1	2	3	4	5	6	7	8	9	10	11	12
1A	0	2	2	12	4	11	7	6	4	1	0	0
2A	1	6	8	5	29	13	16	6	14	4	5	2
3A	0	5	4	7	25	14	11	15	13	4	5	0
4A	0	1	1	4	9	11	7	2	2	1	1	0
2A	3	9	6	23	18	16	7	16	6	4	0	0
2B	14	33	55	60	84	50	31	35	29	10	5	3
2C	14	27	46	51	69	48	31	27	26	8	2	0
2D	2	3	7	8	15	10	14	4	6	5	3	0
3A	9	13	11	12	20	17	9	16	5	5	0	0
3B	15	31	48	87	82	45	34	26	17	8	3	2
3C	14	31	58	63	60	50	33	21	18	13	2	2
3D	5	5	8	9	8	12	5	3	1	3	0	0
4A	9	7	5	11	10	10	8	7	7	4	2	0
4B	5	11	18	19	26	19	11	9	11	4	3	2
4C	5	10	19	24	30	15	8	7	8	3	1	1
4D	4	6	4	5	11	13	6	8	4	3	1	0
Total number of leaves in each group												
	100	100	100	100	100	59	34	26	19	8	3	1
Total number of galls in each group												
	100	200	300	400	500	354	238	208	171	80	33	12

Table A4.1. Results of counts of galls in each area of the leaf, for each of one to 12 galls per leaf.

Area of leaf	Number of galls per leaf.			
	13	14	Total	10-14 Total
1A	1	0	50	2
2A	2	0	111	13
3A	4	2	109	15
4A	0	0	39	2
1B	1	0	109	5
2B	2	1	412	21
3B	6	4	359	20
4B	1	0	78	9
1C	5	1	123	11
2C	4	2	404	19
3C	4	1	370	22
4C	3	1	63	7
1D	2	0	82	8
2D	0	1	139	10
3D	2	1	134	8
4D	2	0	67	6
Total number of leaves in each group				
	3	1	654	16
Total number of galls in each group				
	39	14	2649	178

Table A4.2. Results of counts of galls in each area of the leaf, for 13 and 14 galls per leaf, the total of all galls and the total of 10 to 14 galls per leaf.

Area of leaf	Number of galls per leaf.						
	1	2	3	4	5	6	7
1A	0.00000	0.53980	0.53980	3.2388	1.0796	2.96890	1.88930
2A	0.29877	1.79260	2.39013	1.4938	8.6642	3.88396	4.78026
3A	0.00000	1.37117	1.09694	1.9196	6.8559	3.83929	3.01658
4A	0.00000	0.27406	0.27406	1.0962	2.4665	3.01464	1.91841
1B	0.37004	1.11011	0.74008	2.8370	2.2202	1.97353	0.86342
2B	1.80076	4.24464	7.07440	7.7175	10.8045	6.43127	3.98739
3B	1.72714	3.33091	5.67489	6.2917	8.5123	5.92162	3.82438
4B	0.24434	0.36652	0.85521	0.9774	1.8326	1.22172	1.71041
1C	1.01921	1.47219	1.24570	1.3589	2.2649	1.92517	1.01921
2C	1.73601	3.58776	5.55525	10.0689	9.4902	5.20804	3.93497
3C	1.64135	3.63442	6.79987	7.3861	7.0344	5.86196	3.86889
4C	0.58049	0.58049	0.92879	1.0449	0.9288	1.39318	0.58049
1D	1.87776	1.46048	1.04320	2.2950	2.0864	2.08640	1.66912
2D	1.07657	2.36846	3.87566	4.0910	5.5982	4.09097	2.36846
3D	1.08340	2.16680	4.11692	5.2003	6.5004	3.25020	1.73344
4D	0.83102	1.24652	0.83102	1.0388	2.2853	2.70080	1.24652
Total	14.287	29.547	43.042	58.056	78.624	55.772	38.411

Table A4.3. Data from Table A4.1, leaves with one to seven galls, corrected to remove possible bias caused by the different sizes of areas 1A to 4D. (data from Table A4.1/mean size of area from Table A4.11).

Area of leaf	Number of galls per leaf.						
	8	9	10	11	12	13	14
1A	1.61940	1.07960	0.26990	0.00000	0.000000	0.26990	0.000000
2A	1.79260	4.18272	1.19506	1.49383	0.597532	0.59753	0.000000
3A	4.11352	3.56505	1.09694	1.37117	0.000000	1.09694	0.548470
4A	0.54812	0.54812	0.27406	0.27406	0.000000	0.00000	0.000000
1B	1.97353	0.74008	0.49338	0.00000	0.000000	0.12335	0.000000
2B	4.50189	3.73014	1.28625	0.64313	0.385876	0.25725	0.128625
3B	3.33091	3.20754	0.98694	0.24673	0.000000	0.74020	0.493468
4B	0.48869	0.73303	0.61086	0.36652	0.000000	0.12217	0.000000
1C	1.81192	0.56623	0.56623	0.00000	0.000000	0.56623	0.113245
2C	3.00909	1.96748	0.92587	0.34720	0.231469	0.46294	0.231469
3C	2.46202	2.11031	1.52411	0.23448	0.234478	0.46896	0.117239
4C	0.34830	0.11610	0.34830	0.00000	0.000000	0.34830	0.116099
1D	1.46048	1.46048	0.83456	0.41728	0.000000	0.41728	0.000000
2D	1.93783	2.36846	0.86126	0.64594	0.430629	0.00000	0.215314
3D	1.51676	1.73344	0.65004	0.21668	0.216680	0.43336	0.216680
4D	1.66203	0.83102	0.62326	0.20775	0.000000	0.41551	0.000000
Total	32.577	8.940	12.547	6.465	2.097	6.320	2.181

Table A4.4. Data from Table A4.1 and A4.2, leaves with eight to fourteen galls, corrected to remove possible bias caused by the different sizes of areas 1A to 4D. (data from Table A4.1/mean size of area from Table A4.11).

Area of leaf	Number of galls per leaf.	
	Total	10-14 Total
1A	13.4950	0.53980
2A	33.1630	3.88396
3A	29.8916	4.11352
4A	10.6883	0.54812
1B	13.4447	0.61673
2B	52.9937	2.70113
3B	44.2888	2.46734
4B	9.5295	1.09955
1C	13.9291	1.24570
2C	46.7567	2.19895
3C	43.3785	2.57926
4C	7.3142	0.81269
1D	17.1085	1.66912
2D	29.9287	2.15314
3D	29.0351	1.73344
4D	13.9195	1.24652
Total	408.865	29.609

Table A4.5. Data from Table A4.2, the total number of galls counted and the total number of galls on leaves with 10 to 14 galls per leaf. Data corrected to remove possible bias caused by the different sizes of areas 1A to 4D. (data from Table A4.1/mean size of area from Table A4.11).

Area of leaf	Number of galls per leaf.						
	1	2	3	4	5	6	7
1A	0.0000	1.8269	1.2541	5.5787	1.3731	5.3233	4.9186
2A	2.0912	6.0669	5.5530	2.5731	11.0198	6.9640	12.4449
3A	0.0000	4.6407	2.5485	3.3065	8.7198	6.8839	7.8534
4A	0.0000	0.9275	0.6367	1.8882	3.1371	5.4053	4.9944
1B	2.5901	3.7571	1.7194	4.8866	2.8238	3.5386	2.2478
2B	12.6043	14.3658	16.4361	13.2933	13.7420	11.5314	10.3808
3B	12.0890	11.2733	13.1846	10.8373	10.8266	10.6176	9.9564
4B	1.7103	1.2405	1.9869	1.6835	2.3308	2.1906	4.4529
1C	7.1339	4.9825	2.8941	2.3407	2.8807	3.4519	2.6534
2C	12.1511	12.1426	12.9066	17.3434	12.0703	9.3382	10.2443
3C	11.4885	12.3005	15.7983	12.7223	8.9468	10.5106	10.0723
4C	4.0631	1.9646	2.1579	1.7998	1.1813	2.4980	1.5113
1D	13.1433	4.9429	2.4237	3.9532	2.6536	3.7410	4.3454
2D	7.5354	8.0159	9.0044	7.0466	7.1202	7.3352	6.1661
3D	7.5832	7.3334	9.5649	8.9574	8.2677	5.8277	4.5128
4D	5.8166	4.2188	1.9307	1.7893	2.9066	4.8426	3.2452

Table A4.6. Number of galls in each area for one to seven galls per leaf. Data corrected to remove possible bias caused by the different sizes of areas 1A to 4D and expressed as a percentage frequency (data from Table A4.3/Totals from Table A4.3)x100.

Area of leaf	Number of galls per leaf.						
	8	9	10	11	12	13	14
1A	4.9710	3.7305	2.1511	0.0000	0.0000	4.2706	0.0000
2A	5.5026	14.4532	9.5247	23.1072	28.4992	9.4548	0.0000
3A	12.6270	12.3189	8.7426	21.2099	0.0000	17.3569	25.1521
4A	1.6825	1.8940	2.1842	4.2393	0.0000	0.0000	0.0000
1B	6.0580	2.5573	3.9323	0.0000	0.0000	1.9517	0.0000
2B	13.8192	12.8893	10.2515	9.9482	18.4043	4.0705	5.8986
3B	10.2247	11.0835	7.8659	3.8166	0.0000	11.7122	22.6298
4B	1.5001	2.5330	4.8686	5.6694	0.0000	1.9331	0.0000
1C	5.5619	1.9566	4.5128	0.0000	0.0000	8.9594	5.1933
2C	9.2368	6.7985	7.3792	5.3707	11.0399	7.3251	10.6149
3C	7.5575	7.2921	12.1472	3.6270	11.1834	7.4203	5.3764
4C	1.0691	0.4012	2.7759	0.0000	0.0000	5.5111	5.3241
1D	4.4832	5.0466	6.6515	6.4547	0.0000	6.6026	0.0000
2D	5.9484	8.1841	6.8642	9.9917	20.5388	0.0000	9.8740
3D	4.6559	5.9898	5.1808	3.3517	10.3345	6.8571	9.9367
4D	5.1018	2.8715	4.9674	3.2136	0.0000	6.5746	0.0000

Table A4.7. Number of galls in each area for eight to fourteen galls per leaf. Data corrected to remove possible bias caused by the different sizes of areas 1A to 4D and expressed as a percentage frequency. (data from Table A4.4/Totals from Table A4.4)x100.

Area of leaf	Number of galls per leaf.	
	Total	10-14 Total
1A	3.3006	1.8231
2A	8.1110	13.1175
3A	7.3109	13.8928
4A	2.6141	1.8512
1B	3.2883	2.0829
2B	12.9612	9.1227
3B	10.8321	8.3331
4B	2.3307	3.7136
1C	3.4068	4.2072
2C	11.4357	7.4266
3C	10.6095	8.7111
4C	1.7889	2.7447
1D	4.1844	5.6372
2D	7.3200	7.2719
3D	7.1014	5.8544
4D	3.4044	4.2099

Table A4.8. Total number of galls counted and the total number of galls on leaves with 10 to 14 galls per leaf. Data corrected to remove possible bias caused by the different sizes of areas 1A to 4D and expressed as a percentage frequency. (data from Table A4.5/Totals from Table A4.5)x100.

Leaf No.	Size of areas 1A to 4D (4 mm^2)																
	1A	2A	3A	4A	1B	2B	3B	4B	1C	2C	3C	4C	1D	2D	3D	4D	Sum
1	20	20	18	20	71	74	66	64	97	94	88	87	53	65	47	43	927
2	18	12	17	22	46	40	47	45	56	53	57	49	25	24	26	22	559
3	16	18	18	18	30	33	33	40	29	28	22	26	10	9	10	12	352
4	6	6	7	7	18	17	17	16	17	16	16	15	9	9	10	9	195
5	8	6	6	6	12	11	13	12	14	14	14	14	9	9	7	9	164
6	7	6	7	7	19	15	17	19	16	16	18	20	9	9	8	9	202
7	8	7	8	8	19	16	19	18	23	24	24	26	16	13	16	17	262
8	11	11	11	12	22	23	26	27	20	21	21	21	14	12	15	15	282
9	8	7	7	6	16	16	14	14	18	17	17	17	9	9	9	10	194
10	10	9	11	9	16	17	16	15	20	19	18	18	10	9	9	10	216

Table A4.9. The size of each of the areas 1A to 4D (x4 mm^2) and the total area (x4 mm^2) for ten randomly selected leaves.

Area of leaf	Running means expressed as a percentage of whole leaf						
	1st	2nd	3rd	4th	5th	6th	7th
1A	2.1575	2.6888	3.30766	3.24998	3.57559	3.55722	3.48525
2A	2.1575	2.1521	3.13927	3.12369	3.23066	3.18726	3.11362
3A	1.9417	2.4914	3.36551	3.42157	3.46896	3.46836	3.40908
4A	2.1575	3.0465	3.73558	3.69912	3.69100	3.65339	3.56769
1B	7.6591	7.9440	8.13694	8.41040	8.19173	8.39410	8.23093
2B	7.9827	7.5692	8.17113	8.30783	7.98773	7.89406	7.63875
3B	7.1197	7.7638	8.30087	8.40514	8.30948	8.32721	8.17359
4B	6.9040	7.4770	8.77257	8.63071	8.36798	8.54098	8.30230
1C	10.4639	10.2409	9.57346	9.35958	9.19498	8.98262	8.95348
2C	10.1402	9.8107	9.19200	8.94528	8.86354	8.70642	8.77126
3C	9.4930	9.8449	8.64659	8.53622	8.53630	8.59873	8.67896
4C	9.3851	9.0754	8.51238	8.30736	8.35320	8.61117	8.79867
1D	5.7174	5.0948	4.34352	4.41148	4.62675	4.59820	4.81372
2D	7.0119	5.6526	4.62069	4.61936	4.79305	4.73678	4.76893
3D	5.0701	4.8606	4.18740	4.42260	4.39174	4.31985	4.57514
4D	4.6386	4.2871	3.99444	4.14967	4.41730	4.42366	4.71864

Table A4.10. Areas from Table A4.9 expressed as running means, continued on Table A4.11. (Size of areas 1A to 4D from Table A4.9/Sum of areas From Table A4.9)x100. Means calculated from these data.

Area of leaf	Running means		
	8th	9th	10th
1A	3.53718	3.60235	**3.70508**
2A	3.21201	3.25603	**3.34710**
3A	3.47054	3.48584	**3.64651**
4A	3.65364	3.59132	**3.64886**
1B	8.17724	8.18504	**8.10728**
2B	7.70341	7.76385	**7.77451**
3B	8.30438	8.18350	**8.10589**
4B	8.46132	8.32300	**8.18515**
1C	8.72082	8.78276	**8.83041**
2C	8.60570	8.62317	**8.64048**
3C	8.52494	8.55137	**8.52957**
4C	8.62968	8.64448	**8.61337**
1D	4.83257	4.81109	**4.79294**
2D	4.70473	4.69745	**4.64437**
3D	4.66814	4.66492	**4.61510**
4D	4.79371	4.83381	**4.81339**

Table A4.11. Continuation of Table A4.10, areas from Table A4.9 expressed as running means. Tenth mean used in Tables A4.3 to A4.5.

Area of leaf	Number of galls per leaf						
	1	2	3	4	5	6	7
1A	3.70508	7.4102	11.1152	14.8203	18.5254	13.1160	8.8181
2A	3.34710	6.6942	10.0413	13.3888	16.7355	11.8487	7.9661
3A	3.64651	7.2930	10.9395	14.5860	18.2325	12.9086	8.6787
4A	3.64886	7.2977	10.9466	14.5954	18.2443	12.9170	8.6843
1B	8.10728	16.2146	24.3218	32.4291	40.5364	28.6998	19.2953
2B	7.77451	15.5490	23.3235	31.0980	38.8726	27.5218	18.5033
3B	8.10589	16.2118	24.3177	32.4236	40.5294	28.6949	19.2920
4B	8.18515	16.3703	24.5555	32.7406	40.9258	28.9754	19.4807
1C	8.83040	17.6608	26.4912	35.3216	44.1520	31.2596	21.0164
2C	8.64050	17.2810	25.9215	34.5620	43.2025	30.5874	20.5644
3C	8.52957	17.0591	25.5887	34.1183	42.6479	30.1947	20.3004
4C	8.61337	17.2267	25.8401	34.4535	43.0668	30.4913	20.4998
1D	4.79294	9.5859	14.3788	19.1718	23.9647	16.9670	11.4072
2D	4.64437	9.2887	13.9331	18.5775	23.2218	16.4411	11.0536
3D	4.61510	9.2302	13.8453	18.4604	23.0755	16.3374	10.9839
4D	4.81339	9.6268	14.4402	19.2536	24.0670	17.0394	11.4559

Table A4.12. Expected numbers of galls per leaf, for one to seven galls per leaf, calculated from the total numbers of galls on each leaf (Table A4.1) and the mean size of each area (Table A4.11, 10th mean). (Table A4.11x(Table A4.1/100)).

Area of leaf	Number of galls per leaf						
	8	9	10	11	12	13	14
1A	7.7066	6.3357	2.96406	1.22268	0.44461	1.44498	0.51871
2A	6.9620	5.7235	2.67768	1.10454	0.40165	1.30537	0.46859
3A	7.5847	6.2355	2.91721	1.20335	0.43758	1.42214	0.51051
4A	7.5896	6.2396	2.91909	1.20412	0.43786	1.42306	0.51084
1B	16.8631	13.8634	6.48582	2.67540	0.97287	3.16184	1.13502
2B	16.1710	13.2944	6.21961	2.56559	0.93294	3.03206	1.08843
3B	16.8602	13.8611	6.48471	2.67494	0.97271	3.16130	1.13482
4B	17.0251	13.9966	6.54812	2.70110	0.98222	3.19221	1.14592
1C	18.3672	15.1000	7.06432	2.91403	1.05965	3.44386	1.23626
2C	17.9722	14.7753	6.91240	2.85137	1.03686	3.36979	1.20967
3C	17.7415	14.5856	6.82366	2.81476	1.02355	3.32653	1.19414
4C	17.9158	14.7289	6.89070	2.84241	1.03360	3.35921	1.20587
1D	9.9693	8.1959	3.83435	1.58167	0.57515	1.86925	0.67101
2D	9.6603	7.9419	3.71550	1.53264	0.55732	1.81130	0.65021
3D	9.5994	7.8918	3.69208	1.52298	0.55381	1.79989	0.64611
4D	10.0119	8.2309	3.85071	1.58842	0.57761	1.87722	0.67387

Table A4.13. Expected numbers of galls per leaf, for eight to fourteen galls per leaf, calculated from the total numbers of galls on each leaf (Table A4.1 and A4.2) and the mean size of each area (Table A4.11, 10th mean). (Table A4.11x(Table A4.1 and A4.2/100)).

Area of leaf	Number of galls per leaf	
	Total	10-14 total
1A	98.148	6.5950
2A	88.665	5.9578
3A	96.596	6.4908
4A	96.658	6.4950
1B	214.762	14.4310
2B	205.947	13.8386
3B	214.725	14.4285
4B	216.825	14.5696
1C	233.917	15.7181
2C	228.887	15.3801
3C	225.948	15.1826
4C	228.168	15.3318
1D	126.965	8.5314
2D	123.029	8.2670
3D	122.254	8.2149
4D	127.507	8.5678

Table A4.14. Expected numbers of galls per leaf, for the total number of galls and the total number for 10-14 galls per leaf, calculated from the total numbers of galls on each leaf (Table A4.2) and the mean size of each area (Table A4.11, 10th mean). (Table A4.11x(Table A4.2/100))

Number of galls per leaf	Valve of Chi-squared	Probability d.f.=15
1	43.4063	< 0.001
2	85.7076	< 0.001
3	208.042	< 0.001
4	230.290	< 0.001
5	230.223	< 0.001
6	92.0502	< 0.001
7	74.0864	< 0.001
8	68.8534	< 0.001
9	85.4767	< 0.001
10	16.4969	> 0.05
11	40.0762	< 0.001
12	24.3544	> 0.05
13	15.3329	> 0.05
14	17.7211	> 0.05
TOTAL	955.109	< 0.001
10-14 TOTAL	50.9701	< 0.001

Table A4.15. Results of chi-squared test between the observed counts of the number of galls on leaves (Table A4.1 and A4.2) and the expected results based on the size of each area 1A to 4D (Table A4.12 to A4.14).

APPENDIX FIVE

Cocoon size.

	Length	Breadth		Length	Breadth
1	4.5	2.0	26	4.0	2.5
2	5.0	2.5	27	4.5	2.0
3	5.0	2.0	28	5.0	1.5
4	4.5	2.0	29	5.0	2.0
5	4.0	2.0	30	4.0	1.5
6	4.0	2.5	31	4.5	2.0
7	4.5	2.0	32	4.0	2.0
8	4.5	1.5	33	4.0	2.0
9	5.5	1.5	34	4.5	2.0
10	4.0	1.5	35	4.5	1.5
11	5.0	2.0	36	4.5	2.0
12	3.5	2.0	37	5.0	2.0
13	4.0	1.5	38	4.0	2.0
14	4.5	1.0	39	4.0	1.5
15	5.0	2.0	40	5.0	1.5
16	4.0	2.0	41	3.5	2.0
17	4.5	2.0	42	4.5	2.0
18	4.5	2.0	43	4.5	2.0
19	4.5	1.5	44	4.5	1.5
20	4.5	1.5	45	5.0	2.0
21	5.0	2.0	46	4.5	2.0
22	4.5	1.5	47	4.5	2.0
23	4.5	2.0	48	3.5	1.5
24	4.0	1.5	49	4.0	1.5
25	4.5	1.5	50	5.0	1.5

	N	MEAN	STDEV	MIN	MAX
Length	50	4.4400	0.4477	3.5000	5.5000
Breadth	50	1.8300	0.3131	1.0000	2.5000

Table A5.1. Results of the measurement of the length and breadth of 50 cocoons.

APPENDIX SIX

Insect Ringer.

NaCl	7.54 g/l
KCl	0.64 g/l
$MgCl_2.6H_2O$	1.73 g/l
$CaCl_2.6H_2O$	0.44 g/l
$NaHCO_3$	0.86 g/l
$NaH_2PO_4.2H_2O$	0.64 g/l
Glucose	6.13 g/l

Table A6.1. Chemical composition of insect Ringer solution used throughout this thesis.

APPENDIX SEVEN

Measurements of leaf and gall growth.

Day	Mean length of ten leaves (mm)	Mean length of ten galls (mm)
2	67.75	3.8
3	77.75	4.5
5	94.00	5.0
8	108.50	6.1
10	117.00	6.6
13	126.50	6.9
15	131.75	7.0
17	131.75	7.0

Table A7.1. The mean lengths of ten leaves and ten galls, measured over a period of 17 days.

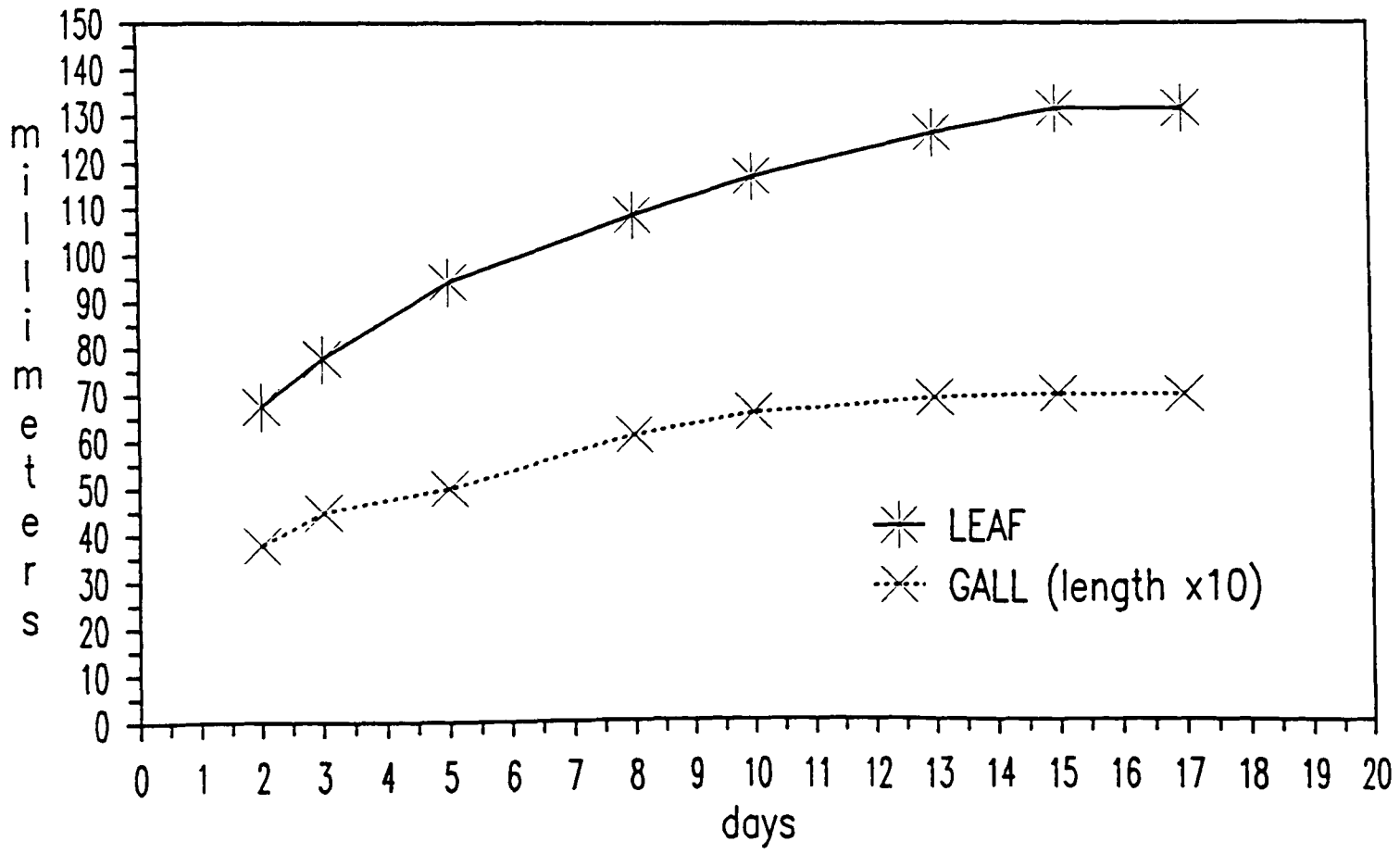


Figure A7.1. The mean length of ten leaves and ten galls, measured over a period of 17 days.

APPENDIX EIGHT

Measurements

e.p.u = eye piece unit as recorded using an eye piece graticule.

A8.1 Colleterial sac.

Diameter in e.p.u's	Total number
15	2
16	5
17	4
18	3
19	10
20	19
21	16
22	12
23	9
24	9
25	4
26	1
27	0
28	1

n = 93

mean = 20.85

1 e.p.u. = 0.047 mm therefore mean diameter of sac = 0.98 mm

Volume of sphere = $^4/_3$ pi x r^3

Volume of sac = 4.19 x 0.12

= 0.50 mm^3

Table A8.1. Data collected to determine the size and volume of the colleterial sac of Pontania proxima.

A8.2 Diameter of accessory gland tubule.

Diameter in e.p.u's for ten randomly selected tubules.

23, 19, 21, 18, 20, 26, 20, 23, 15, 16,

23, 25, 15, 24, 18, 17, 20, 23, 23, 18.

mean = 20.35

1 e.p.u. = 0.0037 mm therefore mean diameter of tubule = 0.075 mm

Table A8.2. Data collected to determine the mean diameter of tubules of the accessory gland of Pontania proxima.

APPENDIX NINE

HIGTON. R. N., (1989). Historical perspectives in the development of cecidology. Cecidology. 4(3): 5-9.

HISTORICAL PERSPECTIVES IN THE DEVELOPMENT OF CECIDOLOGY

Roger Higton

Department of Plant Sciences, University of Oxford, Oxford.

Introduction

> *"There is no better method for scientists of one period to bring to light their own unconscious, or at least undiscussed, presuppositions (which may insidiously undermine all their work) than to study their own subject in a different period".*
> (Cain 1957)

In discussing the evolution of Cecidology as a science, three stages of cognitive development can be identified. Each gives a clearer understanding of the study of galls from an historical perspective. These stages are:-

a) Recognition

b) Application

c) Understanding

Within this framework a brief study is given of the development of Cecidology up until the end of the 18th Century, by which time the link between gall inducer (at least for insects) and host had been established. One reason for this article is the lack of recent reviews on the history of Cecidology. I have not seen a copy of Bohner (1933-1936) and although Plumb (1953) provides a useful review, there does not seem to be a more recent history of the subject. I would be most grateful if other members of the BPGS could add to this contribution. Material covering this period of time or later, including local myths and folklore, would be most welcome.

Recognition

This can be described as when an entity is recognised as such. Galls are abnormal growths, which take many diverse forms, each form being constant in its characteristics, constant enough to be recognised. Morphologically, galls can be distinguished from the "normal" tissues of the plant, be it by modification of leaves, stems, roots, floral parts, etc. Mani (1964:16) estimates that 98% of plant galls occur on Angiosperms, while Meyer (1987:253) suggests that a very rough estimate for the total number of galls known to date, including those found on the cryptogams and gymnosperms, must not be far from 15,000. Even with these figures it would be most unwise to consider that the recognition phase is complete.

Senn (1941) records that in Historia Plantarum, Theophrastus (372-286 B.C.) mentions galls in five different passages. In one, oak galls and vine tendrils are recognised as being unlike normal structures found on plants, but no further conclusions are reached. Theophrastus even records the presence of an insect (which Senn identifies as *Neuroterus quercus-baccarum* L., but this conflicts with the translation by Hort 1916:201 who seems to be describing *Andricus curvator* HTG.) in a gall, but he did not make the connection between host and parasite. Indeed, part of Senn's translation reads "oak bears more things beside its fruits than any other tree" and goes on to include fungi and mistletoe under the same banner. So, there was no precept of understanding and this was to continue for approximately the next 1900 years.

Application

Within this model, application can be defined as a use of a recognised entity, which involves abstraction. This would include a specific function dependent on some particular property. For example, the use of Aleppo galls as missiles or counters would not in this model be an application; stones, sticks, teeth, etc could all function in the same way. However, the use of Aleppo galls (induced by *Cynips gallae-tinctoriae* Olivier) in tanning or Bassorah galls (induced by *Cynips insana* Westwood) in wool dyeing are applications *sensu stricto*.

The first known record of the application of galls occurs with Hippocrates (406-377 B.C.) and Theophrastus. The former recognised the Aleppo galls as having medicinal properties (Fagin 1918:159). Cosens (1916:89) gives their properties as those of an astringent, indeed Fagin (1918:159-160) reports the use of these galls as a tonic, an astringent in haemorrhages and a chemical antidote as late as 1849. She also cites it as the principal ingredient of Unguentum gallae, a preparation for external use listed in the British Pharmacopoeia and in the U.S. Pharmacopoeia, 1916.

Of the 10 oak galls described by Theophrastus in Historia Plantarum, the tanning and dyeing properties of the Aleppo and Bassorah galls are mentioned; added to this is the use of the gall induced by *Andricus theophrasteus* as a lamp wick. Other authors (Swanton 1912, Plumb 1953) attribute Theophrastus with alluding to the commercial and economic value of galls, through his mention of the superior quality of the Aleppo galls from Syria.

The medicinal qualities given to galls were also mentioned by the early writers, Dioscorides (1st Century A.D.) and Pliny the Elder (23-79 A.D.). Pliny (Cosens 1916:89) contributed very little by the way of any new knowledge, relying more on the work of his predecessors. He recommended the use of galls for 23 different ailments, ranging from burns to ulcerations of the mouth. Galls were to be either chewed or burnt and quenched in wine or a mixture of water and vinegar. Writers have inferred that Pliny had observed cynipids on oak leaves as he mentions a type of "gnat" on the leaves. He also describes galls with holes in of being of an inferior quality, but he did not link the galls, insects and their escape holes. Instead he assumed that gall-nuts were a fruit of the oak, produced in alternate years to the acorn.

Greene (1983:818) suggests that by the end of the 16th Century little more was known about galls than 2000 years before, indeed a translation from De Plantis (Cesalpino 1519-1603) reads "galls that grow on Robur ... which are to be judged excrementitious, since they have no other manifest use to the plants, not withstanding that some of them subserve useful purposes to men". The supposed medicinal quality of galls was still referred to in the various herbals of the intervening years. They were also treated as objects of superstition and as portends of the future. In 1616 Gervais Markham edited a revision of Richard Surflet's translation of "Maison Rustique" called "The Country Farme". "Maison Rustique" had originally been written by the Frenchman Charles Estienne in 1569. Under a heading "Signs foretokening fruitfulness", Markham wrote "He shall know a fruitfull and fertile year, if he shall see in the Oke apples, commonly called Gals, a Flie engendered."; elsewhere in the same work is written "... the greater sort of gals or apples have this propertie in them, namely to presage and foretell three things, that is to say, war, death, pestilence; for if you open them which are whole, you shall find therein a little flie or a spider, or a little worm ..." (Plumb 1953 also Folkard 1892:101). Cosens (1916) continues this theme by attributing Magnus (1193-1280) with the first proposal that galls have the power of prognostication, a view which was still accepted in the 17th Century by Lonicer and Mattioli. The supernatural qualities attributed to galls can be recognised in such names as "witches' broom" and "thunder bushes"; the belief that galls were formed by Satanic forces was still prevalent in the late 18th Century. Indeed, the superstition engendered by galls is well illustrated by Swanton (1914:2) who cites the contemporary example of cramp-balls being carried at the turn of the century. These are woody tumours of the stems of Oak, Beech and Holly, carried by people in South West Sussex, as their name suggests, as a safeguard against cramp.

Understanding

The understanding of insects as being the cause of gall formation is attributed to either Dr. Martin Lister (1683-1712), the physician-in-ordinary to Queen Anne (Swanton 1912:2) or, by a greater consensus, to Marcello Malpighi (1628-1694) (Cosens 1916:92, Fagin 1918:155, Plumb 1953). In his Anatome Plantarum (Pt.II, De gallis), London, 1679, Malpighi was not only the first person to describe galls in a systematic manner, but he also attributed their formation to the stinging of the plant by an insect and to the injection into the plant tissues of a liquid produced by an insect which he called "ichor". Reaumur (1683-1759) held that gall formation was not only due to the physical damage caused during oviposition, but also to the presence of the egg and the developing larvae. As Plumb (1953) points out, these two views form the basis of two different schools of thought with regard to gall induction, the stimulus and its mode of action; demonstrating that even after 300 years our understanding is still far from complete.

Acknowledgements

I wish to thank Dr. D. J. Mabberley for kindly reading and commenting on this article. I would also thank SERC, whose funding made this work possible. This article forms part of a D.Phil. thesis.

REFERENCES

BOHNER, K. (1933-1936). Geschichte der Cecidologie: Ein Beitrag zur Entwicklungsgeschichte naturwissenschaftlicher Forschung and ein Fuhrer durch die Cecidologie der Alten. Mittenwald, Nemayer.

CAIN, A. J. (1957). Logic and memory in Linnaeus's system of Taxonomy. Proceedings of the Linnean Society of London. 169: 144-163.

COSENS, A. (1916). The Founding of the Science of Cecidology. 46th Annual Report of the Entomological Society of Ontario for 1915: 88-93.

FAGIN, M. M. (1918). The Uses of Insect Galls. The American Naturalist LII: 155-176.

FOLKARD, R. (1892). Plant Lore, Legends and Lyrics. Second Edition. Sampson Low, Marston & Co. London.

GREENE, E. L. (1983). Landmarks of Botanical History. Volumes 1 & 2. EGERTON, F. N. (ed.). Stanford Unversity Press. California.

MANI, M. S. (1964). The Ecology of Plant Galls. Dr. W. Junk, The Hague.

MEYER, J. (1987). Plant Galls and Gall Inducers. Gebruder Borntraeger, Berlin. Stuttgart.

PLUMB, G. H. (1953). The Formation and Development of the Norway Spruce Gall caused by Adelges abietis L. The Connecticut Agricultural Experiment Station New Haven. Bulletin 566.

SENN, E. W. (1941). Oak Galls in the Historia Plantarum of Theophrastus. Transactions of the Royal Society of Edinburgh 60: 343-354.

SWANTON, E. W. (1912). British Plant Galls. A Classified Textbook of Cecidology. Methuen & Co. Ltd. London.

THEOPHRASTUS (1916). Enquiry into Plants and minor works on odours and weather signs. HORT, A. (trans.). William Heinemann, London.