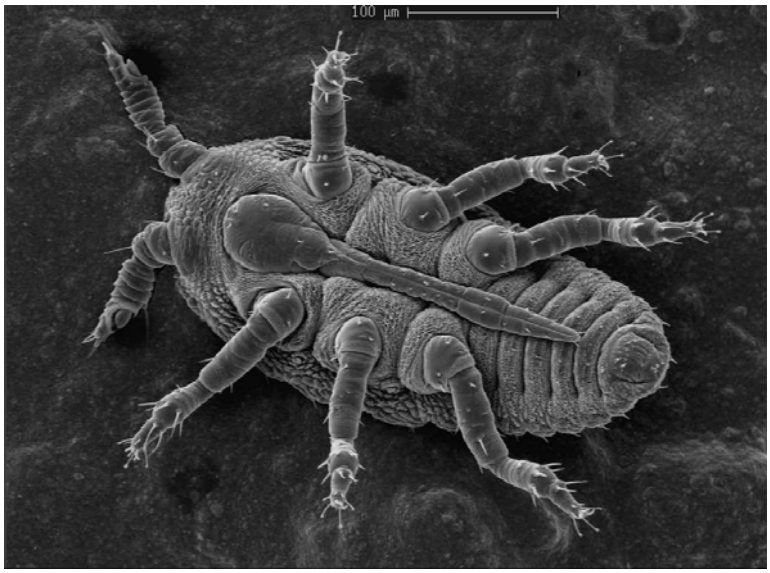
State Government Victoria Department of Primary Industries

Australian Government
Grape and Wine Research and Development Corporation

UNDERSTANDING THE FUNDAMENTAL INTERACTIONS BETWEEN GRAPEVINE PHYLLOXERA AND *VITIS* SPECIES



FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **DNR 01/3**

Principal Investigator: **KIM KINGSTON AND DR KEVIN POWELL**

RESEARCH ORGANISATION: DEPARTMENT OF PRIMARY INDUSTRIES – RUTHERGLEN CENTRE

Date: November 2006

UNDERSTANDING THE FUNDAMENTAL INTERACTIONS BETWEEN GRAPEVINE PHYLLOXERA AND VITIS SPECIES

A FINAL REPORT ON PROJECT DNR 01/3

AUTHORS

Kim Kingston[1,2], Kevin Powell[1] and Paul Cooper[2]

[1] DPI-Rutherglen, [2]Australian National University, Canberra

November 2006

DISCLAIMER

This publication may be of assistance to you but the State of Victoria and its employees do not guarantee that the publication is without flaw of any kind or is wholly appropriate for your particular purposes and therefore disclaims all liability for any error, loss or other consequence which may arise from you relying on any information in this publication.

© The State of Victoria Department of Primary Industries 2006.

This publication is copyright. No part may be reproduced by any process except in accordance with the provisions of the Copyright Act 1968.

This report has been compiled by Kim Kingston and Kevin Powell at DPI-Rutherglen, and Paul Cooper at ANU, Canberra. Photographs taken by DPI and ANU Staff.

DPI-Rutherglen
RMB 1145
Chiltern Valley Road
Rutherglen
Victoria 3685
AUSTRALIA
Telephone: +61 2 6030 4500
Facsimile: +61 2 6030 4600
Email: kevin.powell@dpi.vic.gov.au

TABLE OF CONTENTS

GLOSSARY **7**

ABSTRACT **11**

EXECUTIVE SUMMARY **13**

KEY RECOMMENDATIONS **14**

BACKGROUND **15**

PROJECT AIMS AND PERFORMANCE TARGETS **17**

SUB-PROJECT 1: CHARACTERISATION OF NUTRITIONAL PHYSIOLOGY OF GRAPE PHYLLOXERA THROUGH *IN VITRO* AND *IN PLANTA* STUDIES **19**

SUB-PROJECT 1.1

THE DEVELOPMENT OF AN ARTIFICIAL FEEDING SYSTEM FOR GRAPE PHYLLOXERA **21**

SUB-PROJECT 1.2

THE APPLICATION OF ELECTRICAL PENETRATION GRAPH TO GRAPE PHYLLOXERA FEEDING BEHAVIOUR STUDIES **47**

SUB-PROJECT 2: CHARACTERISATION OF DIGESTIVE AND SALIVARY SYSTEM OF GRAPE PHYLLOXERA USING MORPHOLOGICAL, BIOCHEMICAL AND GENETIC APPROACHES **81**

SUB-PROJECT 2.1

A REVIEW OF THE DIGESTIVE SYSTEM OF GRAPE PHYLLOXERA **83**

SUB-PROJECT 2.2

INVESTIGATING BACTERIAL SYMBIOTIC RELATIONSHIPS IN GRAPE PHYLLOXERA **109**

OVERSEAS TRAVEL REPORT **119**

APPENDIX 1: COMMUNICATION **123**

APPENDIX 2: INTELLECTUAL PROPERTY **127**

APPENDIX 3: REFERENCES **129**

APPENDIX 4: STAFF **137**

APPENDIX 5: RAW DATA **139**

APPENDIX 6: BUDGET RECONCILIATION **141**

GLOSSARY

Definition of terms used in the GWRDC Final Report – Project DNR 01/3.

Most definitions are adapted from Gullan, P. J. and Cranston, P. S. (2005). The Insects: an outline of entomology, 3rd edition. Blackwell Publishing. 505.

Definitions marked "*" are adapted from Triplehorn, C. A. and Johnson, N. F. (2005). Borror and DeLong's Introduction to the Study of Insects, 7th edition. Belmont, Thomson Brooks/Cole. 864.

Definitions marked "#" are adapted from Moore, R., Clark, W. D. and Vodopich, D. S. (1998). Botany, 2nd edition. WCB/McGraw-Hill. 886.

Adelgidae a taxonomic family of Aphidoidea; feed only on conifers, may form galls

Anterior towards the front (insect orientation)

Aphididae* a taxonomic family of Aphidoidea; soft-bodied insects, frequently found in large numbers on the host plant, suck sap from the stems or leaves of plants

Aphidoidea* a taxonomic superfamily of Sternorrhyncha; contains the families Phylloxeridae, Aphididae and Adelgidae

Apterous wingless form of an insect

Cortex# plant tissue located between the epidermis and the vascular bundles of stems and roots

Daktulosphaira vitifoliae the taxonomic species name for grape phylloxera (common name)

Dorsal upper surface, back (insect orientation)

Endosymbiont a specialised symbiont relationship where the micro-organism lives within the wall or cells of the insect, intracellular symbiosis

Gallicolae a gall-dwelling insect (ie grape phylloxera), particular stage that induces aerial galls on the host plant (ie grapevine)

Ganglion nerve centre

Gonopore opening of the genital duct, opening of the oviduct

Hemiptera a taxonomic order of insects; stylet mouthparts, wing reduction or absence common, immature stages (nymphs) usually resemble adults

Hemocoel main body cavity of insects, formed from an expanded 'blood' system

Hemolymph fluid (blood) filling the hemocoel

Homoptera a historical taxonomic order or suborder of Hemiptera containing cicadas, leafhoppers, spittle bugs, planthoppers, aphids, jumping plant lice, scale insects and whiteflies

Honeydew water-fluid, containing sugar, excreted from the anus of some Hemiptera

Instar insect growth stage between two successive moults

Intracellular inside the cell, in relation to the penetration track of the insect stylet

Labium part of the mouth structure of insects

Lateral side (insect orientation)

Longitudinal direction of the long axis of the body (lengthwise section)

Monophagous eat only one kind of food, typically specialised

Medial towards the middle (insect orientation)

Mycetocyte a cell containing symbiotic micro-organisms, scattered through the body and particularly in the fat body or aggregated organs

Mycetome an organ containing aggregations of mycetocytes

Nodosity galls (swellings) on grapevine roots induced by grape phylloxera feeding, especially on young, soft root material

Nymph immature insect after emerging from the egg, usually restricted to insects with incomplete metamorphosis

Oviduct depositor channel for mature eggs, connecting the ovaries and the gonopore

Oviparity reproduction in which eggs are laid

Oviposition process of egg passing from the external genital opening to the outside of the female insect

Ovipositor organ used for laying eggs

Parenchyma[#] plant tissue type characterised by relatively simple, living cells having only primary walls

Parthenogenesis development from an unfertilised egg

Phylloxeridae* a taxonomic family of Aphidoidea; typically monophagous plant feeders, induce gall development, complex life cycles: includes grape phylloxera

Posterior towards the rear (insect orientation)

Radicicolae gall-dwelling insect (ie grape phylloxera), particular stage that induces root tuberosities on the host plant (ie grapevine)

Sessile* attached or fastened, incapable of moving from place to place

Sternorrhyncha* a taxonomic suborder of Hemiptera; includes the superfamily Aphidoidea

Stylet needle-like structure, one of the elongate parts of piercing-sucking insect mouthparts

Symbiont an organism that lives in symbiosis with another, the organism may be extra- or intracellular

Symbiosis long-lasting, close and dependent relationship between organisms of two different species

Transovarial transmission transfer of symbiotic micro-organisms, between insect generations, via the eggs

Transverse right angle orientation to the longitudinal axis (crosswise section)

Tuberosity galls (swellings) on grapevine roots induced by grape phylloxera feeding, especially on mature, lignified root material

Ventral lower surface, underside (insect orientation)

ABSTRACT

Although resistant rootstocks are the main grape phylloxera management option worldwide, little is known of the interactions between grape phylloxera and *Vitis* species. This project significantly improved our understanding of the underlying physical and chemical basis of grape phylloxera-grapevine interactions. Several techniques, used for other sap-sucking insects, were modified to understand grape phylloxera feeding physiology, including: molecular to identify bacterial endosymbionts, electrophysiological to characterise feeding behaviour, microscopy for internal and external morphological features, and artificial diets for essential nutritional requirements. The advanced fundamental knowledge of grape phylloxera interactions have consolidated existing knowledge, value-added to current research programs and may improve future management strategies.

EXECUTIVE SUMMARY

This report describes the research and extension activities of a GWRDC-funded PhD scholarship project on grape phylloxera research and management conducted during the period April 2003 - July 2006. The research was undertaken by DPI-Rutherglen in collaboration with Dr Paul Cooper (ANU). The outputs of the project have resulted in an increased scientific knowledge of grape phylloxera and its fundamental nutritional requirements, feeding physiology, external and internal anatomy and physiology, and interactions with susceptible and resistant *Vitis* species. Knowledge of the biology and feeding behaviour of grape phylloxera is essential to ensure sustainable management of grapevines, including resistant rootstocks.

The four main research components of the project were (i) characterisation of nutritional requirements using an artificial diet feeding system, (ii) electrophysiological studies on grape phylloxera feeding behaviour, (iii) clarifying the status of endosymbionts using molecular techniques and (iv) characterising external and internal morphological features involved in digestion and feeding.

The research activities have allowed the development of improved techniques to study grape phylloxera's nutritional requirements through the development of the first artificial diet feeding system for radicicolae grape phylloxera. Both chemical and physical components of the artificial diet system were extensively studied, including a comparison of two feeding chambers and twenty diet formulations. This system can be further optimised to study the influence of nutritional modifications on the development, survival and fecundity of the insect. By using this system as a model, we could determine how changes in management practices may influence the nutritional status of the host plant, and how this change may impact on grape phylloxera management. The system could also be used to screen novel antimetabolites as alternative management options for grape phylloxera, and in endosymbiont studies

Electrophysiological techniques were used for the first time on several life stages of grape phylloxera, and fourteen waveforms were characterised on susceptible *Vitis vinifera*. The potential for using this technique to understand grape phylloxera interactions with resistant rootstocks was highlighted through a 'proof-of-concept' approach using immune and resistant American *Vitis* species.

Bacterial endosymbiont relationships with radicicolae grape phylloxera were examined using DNA techniques, and although grape phylloxera did not appear to share the same symbiotic relationship as aphids, it may have a transient endosymbiont relationship.

The internal and external morphological features of grape phylloxera involved in feeding and digestion have been characterised in detail using light and electron microscopy techniques. The insect was shown to have a modified gut structure, which compensates for a high egg production capacity by minimising potential waste excretion. Sensory pits were observed on the insect antennae, and the length of stylet (feeding appendage) measured. These features are important in determining location of the insects feeding site.

Research results have been passed onto industry and researchers at all levels including international and national conference presentations. Significant collaborative networks were developed with international groups in Germany, The Netherlands and Austria during the course of the project.

KEY RECOMMENDATIONS

USE THE NOVEL MODIFIED 'HUMIDITY' ARTIFICIAL DIET CHAMBER TO OPTIMISE THE ARTIFICIAL DIET SCREENING SYSTEM TO:

i. compare nutritional requirements of selected grape phylloxera genotypic classes to improve our knowledge of grapevine resistance mechanisms, and the potential for resistance breakdown

ii. screen a range of novel protection and anti-metabolic control agents

iii. analyse the chemical constituents of *Vitis vinifera* and resistant *Vitis* species root extracts to determine what chemical compounds could influence grape phylloxera feeding (by either inhibition or stimulation) and screen optimal concentrations using the rapid artificial diet system

USE THE EPG SYSTEM TO OPTIMISE ROOTSTOCK SCREENING PROTOCOLS AND UNDERSTAND RESISTANCE MECHANISMS BY:

i. correlating EPG waveforms with grape phylloxera biological activity on susceptible and resistant host-plants

ii. comparing the feeding behaviour of selected grape phylloxera genotypic classes on selected resistant rootstocks

iii. utilising the EPG system for studies on other sap-sucking pests, including disease vectors, of grapevines

USE THE DATA OBTAINED FROM EXTERNAL AND INTERNAL MORPHOLOGICAL STUDIES TO:

i. further examine the relationship between stylet length and cellular feeding sites of different grape phylloxera life stages

ii. examine the influence of sensory appendages on host-plant location

iii. confirm the presence or absence of a waste excretory mechanism

iv. extend the molecular approaches developed to characterise the identity and role of bacterial endosymbionts in radicicolae grape phylloxera, and compare endosymbiont relationships with those identified in European gallicolae grape phylloxera

v. identify the digestive enzymes in the midgut region of grape phylloxera, and investigate their significance

BACKGROUND

To date the only effective grape phylloxera management practice is the use of American resistant rootstocks, yet little is known of the phenotypic basis of this resistance. This project aims to significantly improve our understanding of the underlying physical and chemical basis of grape phylloxera-grapevine interactions. A variety of techniques, already in existence for other sap-sucking insects, will be used to understand grape phylloxera feeding physiology. The advancement of our fundamental knowledge of factors which are important for grape phylloxera nutrition and which influence feeding behaviour, and consequently damage levels, will consolidate existing knowledge, value-add to current research programs and may identify future novel management strategies for grape phylloxera.

The International Symposium on Grapevine Phylloxera Management held in Melbourne in 2000 highlighted the need to focus research on the fundamental interactions between grape phylloxera, its host-plant and the environment (Powell and Whiting, 2000). Only by understanding these complex interactions can we ensure that any future methods developed for the detection or management of grape phylloxera are sustainable. Studies conducted on the feeding physiology of aphid pests (closely related to phylloxerids) have contributed to a more detailed understanding of their interactions with crop plants.

An understanding of the fundamental interactions between grape phylloxera and its host-plant, in conjunction with existing grape phylloxera-related research activities, will lay the foundations for future sustainable grape phylloxera management.

PROJECT AIMS

The project aims were to:

i. develop an understanding of nutritional factors which influence feeding behaviour of grape phylloxera on ungrafted and grafted vines through the development of an *in vitro* feeding bioassay.

ii. utilise the *in vitro* system for rapid screening of natural and novel antimetabolic control agents.

iii. characterise and describe the structure and biochemistry of the grape phylloxera digestive and salivary system.

iv. identify and localise microbial endosymbionts which could be targeted as a novel approach to grape phylloxera management.

v. examine the interaction of grape phylloxera with susceptible and resistant grapevines using electrophysiological techniques.

PROJECT AIMS AND PERFORMANCE TARGETS

OUTCOMES

An improved understanding of the interaction between grape phylloxera and grapevine (susceptible and resistant) including nutritional factors that influence feeding behaviour and the structure and biochemistry of the grape phylloxera digestive and salivary system.

Potential discovery of novel control methods for grape phylloxera.

OUTPUTS AND PERFORMANCE TARGETS

Outputs	**Performance Targets**	**Report Section**
Sub-project 1: Characterisation of nutritional physiology of grape phylloxera through *in vitro* and *in planta* studies		
1. Artificial diet bioassay system developed to allow rapid screening for the effects of various compounds on grape phylloxera	Artificial diet components defined Artificial diet system tested with grape phylloxera Artificial diet system tested with model control agents against grape phylloxera	Results in Sub-project 1.1
2. Grape phylloxera feeding behaviour at a cellular level characterised on resistant and susceptible grapevines	Pilot electrophysiological system (EPG) developed for grape phylloxera EPG waveforms characterised for grape phylloxera EPG feeding patterns on susceptible and resistant vines characterised	Results in Sub-project 1.2
3. Role of endosymbionts defined	Aposymbiotic grape phylloxera reared *in vitro* and nutritional components supplied by endosymbionts defined	Discussed in Sub-project 1.1 and 2.2
4. Nutritional factors influencing grape phylloxera feeding behaviour on resistant and susceptible grapevines identified	Range of nutritional factors screened for feeding stimulant or feeding deterrent activity screened *in vitro*	Discussed in Sub-project 1.1 and 1.2
5. Antimetabolic compounds, for use as grape phylloxera control options, identified	Range of plant derived proteins and enzyme inhibitors screened *in vitro*	Discussed in Sub-project 1.1

Sub-project 2: Characterisation of digestive and salivary system of grape phylloxera using morphological, biochemical and genetic approaches		
1. Characterisation and localisation of grape phylloxera digestive system and endosymbionts	Light microscopy study completed Electron microscopy study completed Gut and salivary gland morphology characterised Endosymbionts localised and characterised	Microscopy results in Sub-project 2.1 and 2.2 (endosymbionts)
2. Digestive enzymes of grape phylloxera identified	Enzyme bioassays on grape phylloxera completed	Discussed in Sub-project 2.1

ALTERED OUTPUTS AND PERFORMANCE TARGETS

The following outputs and performance targets were removed from the project as achievement of these outputs was reliant upon the progress, and the relative success of other experimental approaches. This was outlined in the original project proposal. For referencing to the project proposal, the original numbering of outputs has been maintained in this section.

Outputs	**Performance Targets**
Sub-project 1: Characterisation of nutritional physiology of grape phylloxera through *in vitro* and *in planta* studies	
6. Grapevine-grape phylloxera interactions characterised using 'hairy root' system	*'Hairy root' culture transformation system established* *Co-cultivation of grape phylloxera with 'hairy roots' established* *Gene constructs affecting root morphology and/or biochemistry introduced and an assessment of effects on grape phylloxera feeding and development completed*
Sub-project 2: Characterisation of digestive and salivary system of grape phylloxera using morphological, biochemical and genetic approaches	
3. Genes involved in grape phylloxera growth and reproduction identified	*CDNA libraries screened with grape phylloxera DNA and RNA* *Clones isolated some DNA sequence defined* *Sequence compared with known sequences by database analysis* *RT-PCR gene expression study completed*

SUB-PROJECT 1: CHARACTERISATION OF NUTRITIONAL PHYSIOLOGY OF GRAPE PHYLLOXERA THROUGH *IN VITRO* AND *IN PLANTA* STUDIES

SUB-PROJECT 1.1: THE DEVELOPMENT OF AN ARTIFICIAL FEEDING SYSTEM FOR GRAPE PHYLLOXERA

SUB-PROJECT 1.2: THE APPLICATION OF ELECTRICAL PENETRATION GRAPH TO GRAPE PHYLLOXERA FEEDING BEHAVIOUR STUDIES

SUB-PROJECT 1.1

THE DEVELOPMENT OF AN ARTIFICIAL FEEDING SYSTEM FOR GRAPE PHYLLOXERA

1 INTRODUCTION

An artificial diet is mix of synthesised ingredients that provide food and nutrition to a living organism. An artificial diet may contain a fully defined list of chemicals (holidic diet), a mix of defined and non-defined compounds (meridic diet) or be a mix of non-defined, non-purified compounds (oligidic diet) (Cohen, 2003).

The development of an insect artificial diet system provides fundamental information on the organisms optimal nutritional requirements. An optimised artificial diet also provides a model system for the investigation of chemical differences associated with host-plant resistance and susceptibility, and for rapid *in vitro* screening of possible anti-metabolic control compounds and novel protection agents. In addition, artificial diets can also be used to understand what degree of nutritional advantage is provided to the insect by bacterial endosymbionts. By establishing diet systems for insects with and without bacterial symbiotic associations, the level of nutritional dependence the insect places on the bacterial endosymbiont association may be determined.

Other Insect Models

Artificial diets have been formulated for a number of plant-sucking insect species within the orders of Thysanoptera and Hemiptera. The success of these artificial diets has been varied, with experiments reporting insect development through the nymphal life stages, but failing to support adult development (Jancovich *et al.*, 1997); other diets have maintained an insect population for several weeks and through several generations (Auclair, 1965); and limited diet formulations are able to support the same insect colony for extended periods of time, up to 26 years (van Emden, 2003). Insects grown on artificial diet systems typically experience extended developmental rates, and reduced weight gains in comparison with plant fed insects. However there are exceptions where insect developmental time on artificial diets has been similar to the developmental time on a natural plant food source. Adult insects in this system were observed to live longer and lay more eggs when feeding on the artificial diet (Debolt, 1982).

Many variations in diet formulation and chamber design exist, with specific modifications often required to reflect the natural food source of the insect. Since the first use of a double layer of Parafilm® to encase the liquid artificial diet (Mittler and Dadd, 1962), the use of Parafilm® has become a common part of the artificial diet chamber for plant-sucking insects. The transparent nature of Parafilm®, and the self-sealing characteristics allow of insect observation in the diet chamber, and prevents the leakage of the chamber with insect stylet penetration (van Emden, 2003).

Potential Applications of Artificial Diets

Artificial diets provide a method for maintaining insect colonies in a laboratory environment, in the absence of a natural (plant) food source, and also a model system to undertake biological studies. Artificial diets have been used to determine the impact of specific compounds, eg sugars and amino acids, on insect survival and food uptake (Srivastava and Auclair, 1971; Febvay *et al.*, 1988), to examine the nutritional contribution of bacterial symbiotic relationships (Sasaki *et al.*, 1991), and to investigate the impacts of possible control agents (Powell *et al.*, 1993).

Research Aims

The only published attempt to develop an artificial diet for grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) resulted in fourth instar gallicolae insects surviving a maximum of period of 15 days on a 2.5% glucose plus 2.5% fructose, or a 5% sucrose plus amino acids diet formulation (Wohrle, 1999; Forneck and Wohrle, 2003). In Australian vineyards, radicicolae grape phylloxera pose the greatest threat to grapevine health and production, and prior to this project, no artificial diet studies had been conducted on radicicolae grape phylloxera. For the purpose of examining the essential nutritional requirements of grape phylloxera, with the long-term aim of developing a model system for the testing of novel control agents, the development of an artificial diet for radicicolae grape phylloxera was investigated.

2 MATERIAL AND METHODS

2.1 Insect Collection

Radicicolae grape phylloxera were originally collected from infested vineyards in Victoria, Australia. Populations were maintained at the Department of Primary Industries – Rutherglen Centre on excised *Vitis vinifera* L. cv. Sultana root pieces (approximately 1 cm width x 10 cm length), prepared using a protocol slightly modified from Granett, *et al*. (1985). The modifications were: (1) roots washed clean of attached soil with a soft brush under running water; (2) roots soaked for 5-minutes in Ridomil® Gold Plus systemic fungicide solution (2.3 g/L); (3) roots triple rinsed with sterile distilled water prior to air drying; and (4) the size of the petri dish was increased to 15 cm, with the increase in area reducing the impact of water condensation. Grape phylloxera populations were incubated in the dark at a constant temperature of 25 ±3°C.

Egg hatcheries were established to enable the simultaneous collection of a large number of first instar insects. Eggs from excised root pieces were placed onto moist filter paper in a 35 mm petri dish. The petri dish was sealed with Parafilm® M membrane and incubated in the dark at a constant temperature of 25±3°C. The hatcheries were monitored daily until sufficient first instars were available for the artificial diet experiment (2-3 days post establishment). Insects were then transferred directly from the hatchery into the artificial diet chamber without prior feeding on grapevine root material.

Only apterous radicicolae grape phylloxera were used for this study. For collection from excised root pieces, developmental life stages were determined by comparative increases in size. If present, neighbouring moulted cuticles were counted to confirm the insect life stage. Insects were handled using a moist sable-haired paintbrush to avoid damage. First instars were collected prior to feeding while actively moving on the excised root piece, or from egg hatcheries. Intermediate life stages (second-fourth instars) were gently removed from the excised root surface with a moist paintbrush.

2.2 Chemicals and Materials

All chemical reagents (Table 1) were obtained from Sigma-Aldrich. Grape phylloxera artificial diet solutions were prepared using acid-washed glassware, ultra-pure water and high purity, non-contaminated chemicals. Diet solutions were pH adjusted with 0.5M KOH and 0.5M HCl to a range of 4.5-7.5pH (Table 1), to investigate the impact of artificial diet pH on grape phylloxera survival rates. Diet solutions were filter sterilised with a Millipore™ 25 mm Millex® filter unit (0.22 μm) and stored in sterile plastic vials at -20°C. During diet experiments, stock diet solutions were stored at 4°C.

2.3 Artificial Diet Chamber Design

Two artificial diet chamber designs were trialled for grape phylloxera. Experiments involving both designs were set up in replicated blocks, although the application (and renewal) of the diet solution was performed in treatment groups to avoid cross-contamination of solutions. Replicated blocks represented one chamber per artificial diet solution managed as one group; treatment groups represented all chambers exposed to the same artificial diet solution. Artificial diet chambers were incubated in the dark at a constant temperature of 25±3°C. Artificial diet solutions were renewed every two days to avoid deterioration of the chemical components and reduce microbial contamination.

Grape phylloxera survival, location and feeding status was monitored daily (either once or twice) in replicated blocks. For monitoring, artificial diet chambers were viewed with a stereo microscope to count the number of insects alive and dead. The filter paper chamber required inversion of the chamber to view insects below the filter paper; inversion was not necessary to view all insects in the water humidity chamber. Insects in a stationary position on the diet membrane, with the stylet in a vertical position, were recorded as 'probing' the diet solution. Monitoring of artificial diets continued until all insects in all chambers were dead.

Images of grape phylloxera on the membrane surface of the artificial diets were captured with a SPOT RT™ (Diagnostic Instruments) camera mounted onto an Olympus AX70 Provis microscope. External cold source lighting was provided to increase the contrast of the images.

2.3.1 Filter Paper Chamber Design

Based on Powell, *et al*. (1993), the filter paper chamber consisted of a sterile 35 mm diameter petri dish with a sterile 32 mm diameter filter paper in the base moistened with 50 µl sterile ultra-pure water (Figure 1a). Insects were placed into the chamber with a moist sable-haired paintbrush and the chamber covered immediately with a layer of stretched Parafilm® M membrane. Insects were 'starved' inside the chambers, in the dark, for a period of 1-2 hours to reduce the impacts of prior grapevine feeding. First instar insects collected from the egg hatcheries were not starved for an additional period of time prior to the addition of the artificial diet solution. The hatchery first instar insects did not have food in their digestive system due to a lack of exposure to grapevine material.

Following the starvation period, the artificial diet solution (250 µl) was placed in the middle of the membrane surface, and covered with a second layer of stretched Parafilm® M. A water weight of 30-50 ml was placed above the Parafilm® M membrane surface to increase the pressurisation of the diet solution (except for diet experiments 1-3 in Table 2a). Diet experiment 3 provided a comparison between the survival rate of grape phylloxera under non-pressurised and pressurised chamber conditions.

The filter paper chamber artificial diet solution was renewed by removing the double Parafilm® membrane from the diet chamber. Insects in contact with the diet membrane at the time of renewal were carefully repositioned onto the moist filter paper with a moist sable-haired paintbrush. The diet chamber was recovered with a new layer of stretched Parafilm®, and fresh diet solution enclosed with a second layer of new stretched Parafilm®.

2.3.2 Humidity Chamber Design

The humidity chamber was simplified from the model previously used to develop an artificial diet for gallicolae grape phylloxera (Wohrle, 1999; Forneck and Wohrle, 2003). The humidity chamber consisted of a sterile 35 mm diameter petri dish with the sterile lid of a 0.5 ml tube in the base, filled with 50-70 µl of sterile water and covered with a pre-moistened circle of fine mesh (Figure 1b). The mesh provided a cover over the water filled tube lid to allow insects to move across the water surface and reduce the impact of accidental drowning. The original chamber design (Wohrle, 1999; Forneck and Wohrle, 2003) used a saturated solution of K_2SO4 to fill the tube lid in the base of the chamber. Water was used for these experiments following initial trials (data not shown) which provided evidence that contact with the K_2SO_4 solution had a negative impact on grape phylloxera survival rates.

Insects were placed into the chamber with a moist sable-haired paintbrush and the chamber covered immediately with a layer of stretched Parafilm® M membrane. The lid of the petri dish was also covered with a layer of stretched Parafilm® and placed directly above the diet chamber membrane layer. The two membrane surfaces were kept aligned by placing a 30 ml water weight on the petri dish lid (Figure 2). Insects were 'starved' inside the chambers, in the dark, for a period of 1-4 hours. First instar insects collected from the egg hatcheries were not starved for an additional period of time prior to the addition of the artificial diet solution. The hatchery first instar insects did not have food in their digestive system due to a lack of exposure to grapevine material.

Following the starvation period, the two membrane surfaces were separated and the artificial diet solution (50-70 µl) was placed in the middle of the diet chamber membrane surface. The petri dish lid membrane surface was replaced back on top of the diet chamber to seal the diet solution, and the water weight placed back onto the petri dish lid to increase the pressurisation of the diet solution. The volume of diet solution used for the humidity chamber was reduced from the filter paper chamber due to the alterations in the method of diet enclosure. Higher volumes of diet solution were unable to be contained in the diet enclosure during the application of the petri dish lid membrane.

The artificial diet solution in the humidity chamber design could be renewed without disturbing insect activity. The water weight and petri dish lid membrane surface was removed, and the diet solution gently poured off the membrane surface of the diet chamber. Both Parafilm® membranes were rinsed with 1 ml of sterile water and blotted dry with a clean tissue. Fresh diet solution was placed onto the diet chamber membrane and recovered with the petri dish lid membrane and water weight.

Table 1. Formulations for 20 artificial diet solutions trialled to increase the survival time of grape phylloxera within an artificial diet system. Dietary components consisted of a range of sugars and amino acids (AA); values represent g/100ml ultra pure water. Solution pH range is also indicated.

	5% sucrose	10% sucrose	20% sucrose	5% glucose	5% fructose	5% sucrose + 5 AA	5% sucrose + AA	5% glucose + AA	5% fructose + AA
sugar									
D (+) sucrose	5	10	20			5	5		
D (+) glucose				5				5	
D (-) fructose					5				5
amino acid									
L-arginine-HCl						0.4	0.4	0.4	0.4
L-glutamine						0.6			
L-histidine-HCl						0.2	0.2	0.2	0.2
L-isoleucine							0.2	0.2	0.2
L-leucine							0.2	0.2	0.2
L-lysine-HCl							0.2	0.2	0.2
L-methionine						0.1	0.1	0.1	0.1
L-phenylalanine						0.1	0.1	0.1	0.1
L-threonine							0.2	0.2	0.2
L-tryptophan							0.1	0.1	0.1
L-valine							0.2	0.2	0.2
pH values									
	4.5	7.0	7.0	7.5	7.5	7.0	4.5	4.5	6.5
	6.0						6.5	6.5	
	7.0								
	7.5								

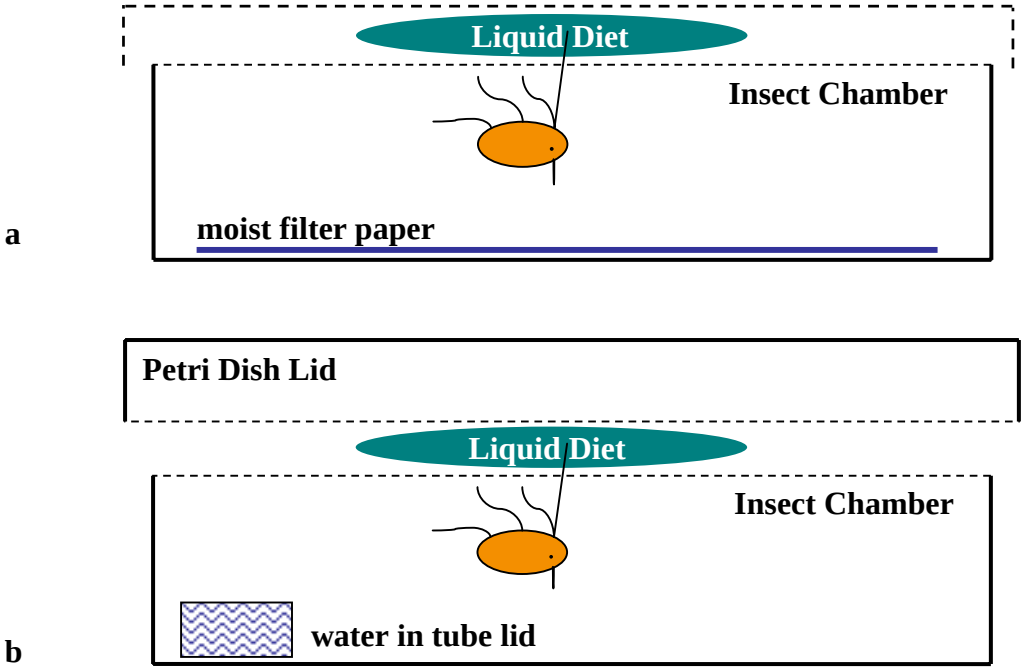


Figure 1. Diagrammatic representation of the two chamber designs trialled for grape phylloxera artificial diet experiments. The solid line represents the 35 mm diameter petri dish used for the diet chamber, the dashed line represents a layer of Parafilm® M. The insect is depicted on the diet membrane, inserting its stylet into the liquid diet.

a) filter paper chamber with moist filter paper in the base of the petri dish and the diet enclosed within a double layer of Parafilm® M;

b) humidity chamber with the lid of a 0.5 ml tube (containing water) providing chamber humidity, the chamber is enclosed with a layer of Parafilm® M and the diet enclosed with a second layer of Parafilm® M attached to the lid of the petri dish.



Figure 2. Photograph of the humidity chamber with a 30 ml water weight positioned above to keep the diet and petri dish lid membrane surfaces aligned, and to increase the pressurisation of the diet solution.

2.4 Artificial Diet Formulation

A range of artificial diet formulations were trialled for grape phylloxera (Table 1). Diet formulations varied in sugar type, sugar concentration, amino acid profile and pH. These formulations were based on current knowledge of the feeding site of grape phylloxera (see below). Controls for all artificial diet experiments were ultra-pure water adjusted to the same pH as the experimental diet solution. An additional control treatment of 'no diet' was occasionally used to observe insect survival time in the absence of any fluid diet solution.

2.4.1 Sugar Type and Concentration

The majority of artificial diet formulations contained sugar in the form of sucrose. Variation in the sucrose concentration was based on successful aphid diets with a range of 15-35% (Mittler, 1988). Generally, 5% sugar was incorporated into the diet formulation. This was based on chemical analysis of grape phylloxera feeding sites that identified sucrose to have a concentration range 13-55 mg/g dry weight (Ryan *et al.*, 2000). Sucrose, glucose and fructose were identified as the main sugars at the feeding sites. The variable sugar types were trialled in diet experiments 6, 8-10 and 12 (Tables 2a and 2b). Glucose was re-examined in diet experiments 10 and 12 due to evidence of starch (a glucose complex) accumulation at grape phylloxera feeding sites (Forneck *et al.*, 2002).

2.4.2 Amino Acids

Amino acids were added to grape phylloxera artificial diet formulations due to evidence of increased amino acid concentrations at the feeding site of radicicolae grape phylloxera (Kellow, 2000).

The amino acid profile for diet experiment 5 (Table 2a) containing 5 amino acids were based on changes in the grapevine root amino acid profile associated with grape phylloxera induced gall formation (Kellow, 2000). Histidine, arginine and phenylalanine recorded the largest increase in concentration with gall formation, and glutamine occurred at the highest concentration of all amino acids detected. Methionine recorded a mid-range increase in concentration, but was included in the 5 amino acid profile (instead of other amino acids with a higher increase in concentration) due to evidence that the addition of methionine, in association with sucrose, enhanced the uptake of artificial diet solutions by aphids (Mittler, 1988).

The amino acid profile for diet experiments 7, 9-15 (Table 2b) containing 10 amino acids were defined by the amino acids considered 'essential' for insect nutrition (Cohen, 2003). In the absence of previous artificial diet formulations for grape phylloxera, concentrations for both amino acid diets were based on a holidic diet developed for the pea aphid, *Acrythosiphon pisum* (Harris) (Homoptera: Aphididae) (Akey and Beck, 1971).

2.4.3 pH Levels

Artificial diet experiments 1-8 (Tables 2a and 2b) were adjusted to a neutral or slightly alkaline pH, based on the aphid model. Aphids obtain optimal growth and reproduction in artificial diet systems with slightly alkaline pH (7.4-7.8), reflecting the pH of the phloem sap of their preferred host-plants (Mittler, 1988). Cohen (2003) reported that aphid diets are generally formulated to be slightly acidic to improve palatability and microbial control. Slightly acidic grape phylloxera artificial diets were trialled in diet experiments 5, 9 and 10 (Tables 2a and 2b). The pH of the xylem sap in canes of *V. vinifera* are reported to be in the acidic range, pH 4.2-4.8 (Stoll *et al.*, 2000). To reflect the pH of the food source of grape phylloxera, acidic artificial diets were trialled in diet experiments 5, 11-15 (Tables 2a and 2b).

Although the specific pH of parenchyma cell contents was not directly measured, the pH of the grapevine root food source of grape phylloxera was confirmed by measurements from micro-propagated *V. vinifera* cv. Shiraz (Kellow *et al.*, 2002). Leaf, stem and root materials from the micro-propagated grapevine were ground with a small amount of water in a mortar and pestle. The ground mix was pH tested with indicator tape, pH range 2-9 (Merck). Each sample was measured in triplicate.

2.4.4 Grapevine Root Extract

Grapevine root extracts were added to diet formulations to provide a 'natural' feeding stimulus. The root extracts contained ground root material from the natural grapevine diet of grape phylloxera, and provided a more complex diet formulation in comparison with the chemically defined, holidic, artificial diets.

Micro-propagated *V. vinifera* cv. Shiraz roots used as a diet formulation in diet experiment 6 (Table 2a) were prepared similar to root material used for pH measurement. Modifications were the grinding of three complete root systems, with the addition of 2.5 ml sterile water pH 7.5. The root solution was left to diffuse for 10 minutes, and then filter sterilised with a Millipore™ 25 mm Millex® filter unit (0.22 µm) and stored in sterile plastic vials at 4°C. The root extract was pH tested with indicator tape, pH range 2-9 (Merck). The root extract diet solution was prepared immediately prior to the start of the artificial diet experiment, and stored at 4°C during the experiment.

Micro-propagated *V. vinifera* cv. Shiraz roots were prepared for addition to the artificial diet formulation in diet experiment 15 (Table 2b) using an modified method. The root material from a micro-propagated grapevine was ground in liquid nitrogen with a mortar and pestle, and added in liquid form (200 µl) to the 5% sucrose pH 4.5 plus amino acids diet solution (600 µl). The final pH of the diet solution was not tested. The root extract was stored at 4°C during the diet experiment to preserve the contents for diet renewal.

Table 2a. Grape phylloxera artificial diet experiments conducted using the filter paper chamber, indicating life stage and diet formulation (plus five amino acids denoted as +5AA). Control diets for each experiment are highlighted in **bold**, diet formulation characteristics in *italic* indicate non-artificial diet solution variables. Life stage (n) value represents number of insects per diet formulation.

trial number	life stage (n)	chamber design	artificial diet formulation				
			a	b	c	d	e
1	hatchery first instar (25)	filter paper	**no diet**	**water pH 7.0**	5% sucrose pH 7.0	10% sucrose pH 7.0	20% sucrose pH 7.0
2	first instar + intermediate (15)	filter paper	*crawler* **water pH 7.0**	*crawler* 10% sucrose pH 7.0	*intermediate* **water pH 7.0**	*intermediate* 10% sucrose pH 7.0	
3	first instar (15)	filter paper	***no pressure*** 10% sucrose pH 7.0	*water pressure* 10% sucrose pH 7.0			
4	first instar (15)	filter paper	**no diet**	5% sucrose pH 7.0			
5	hatchery first instar (15)	filter paper	**water pH 7.0**	5% sucrose pH 7.0	5% sucrose + 5AA pH 7.0	5% sucrose pH 6.0	5% sucrose pH 4.5
6	hatchery first instar (20)	filter paper	**water pH 7.5**	root extract pH 5.0	5% sucrose pH 7.5	5% glucose pH 7.5	5% fructose pH 7.5

Table 2b. Grape phylloxera artificial diet experiments conducted using the humidity chamber, indicating life stage and diet formulation (plus amino acids denoted as +AA, 10 amino acid mix). Control diets for each experiment are highlighted in **bold**. Life stage (n) value represents number of insects per diet formulation.

trial number	life stage (n)	chamber design	artificial diet formulation			
			a	b	c	d
7	intermediate (15)	humidity chamber	**water pH 7**	5% sucrose + 5AA pH 7.0		
8	intermediate (10)	humidity chamber	**water pH 7.5**	5% sucrose pH 7.5	5% fructose pH 7.5	5% glucose pH 7.5
9	intermediate (20)	humidity chamber	**water pH 7**	5% fructose + AA pH 6.5	5% sucrose + AA pH 6.5	
10	intermediate (30)	humidity chamber	**water pH 7**	5% glucose + AA pH 6.5	10 AA pH 6.5	
11	intermediate (30)	humidity chamber	**water pH 4.5**	5% sucrose + AA pH 4.5		
12	intermediate (40)	humidity chamber	**no diet**	**water pH 4.5**	5% sucrose + AA pH 4.5	5% glucose + AA pH 4.5
13	intermediate (40)	humidity chamber	**water pH 4.5**	5% sucrose + AA pH 4.5		
14	hatchery first instar (30)	humidity chamber	**water pH 4.5**	5% sucrose + AA pH 4.5		
15	first instar (40)	humidity chamber	**water pH 4.5**	5% sucrose + AA pH 4.5	5% sucrose + AA pH 4.5 + root extract	

2.5 Statistical Analysis

Statistical analysis was performed with R statistical software (R.DevelopmentCoreTeam, 2006). Survival analysis was used to determine the fraction of grape phylloxera within each diet experiment that would survive for each monitoring period, and the rate of death for that time interval. For each artificial diet experiment, data was formatted to represent individual insect time until death for all diet formulations. Survival analysis was performed using the Kaplan-Meier estimate to determine the survival distribution, and plotted. The log-rank test was performed to verify statistical difference (95%) between the survival distribution of the artificial diet formulations. Diet formulations were only compared within a single artificial diet experiment.

Insects within artificial diet experiments that died due to causes other than the diet formulation, or were removed from the experiment due to external damage to the diet chamber, were excluded (censored) from the survival analysis. Missing insects were identified when the total insect number per chamber did not equal 'n'. The live/dead status of missing insects was calculated by prior and post insect counts. Non-recovered missing insects were censored, all other missing insect data points were adjusted to represent the most probable live/dead status of the insect. Insects were observed to move from the humidity chamber tube lid surface and were therefore not censored from the analysis. Artificial diets involving censored data were diet experiments 1, 2, 4, 5, 6 and 11 (Tables 3a and 3b).

Artificial diet experiments with a significant log-rank test p-value were further analysed as a two-treatment survival analysis comparison with the control. This was to determine which individual diet formulation was significantly different from the control. Experiments containing multiple treatments were also ran as two-treatment comparisons with the control to investigate individual relationships.

3 RESULTS

3.1 Artificial Diet Chamber Design

3.1.1 Filter Paper Chamber

The filter paper chamber design for grape phylloxera artificial diets produced variable humidity and condensation levels within the chamber, confounding grape phylloxera survival on an artificial diet due to restricted insect mobility and access to the diet solution. As individual insects moved across the diet membrane surface they became immobilised in (relatively) large condensed droplets of water. Insects would also become trapped in water droplets below the filter paper surface. In both cases, the insects were inhibited from interacting with the artificial diet solution, resulting in a negative impact on the aim of the experiment to compare insect survival on variable diet formulations. The continual disturbance of the sessile feeding grape phylloxera necessary during diet renewal appeared to have a negative impact on insect fitness and therefore their ability to fed.

3.1.2 Humidity Chamber Design

In comparison with the filter paper chamber, the humidity chamber design produced more even condensation levels within the chamber, and smaller droplets that had less impact on the movement of the insect across the membrane surface. Droplets of water did still occur (Figure 3), which would adhere to the insect as they crossed the membrane surface, but they did not immobilise the insects to the same level as in the filter paper chamber, and the insects continued to interact with the diet solution. The absence of the filter paper prevented insects from becoming trapped below the surface, and also improved the visibility of the diet chamber for monitoring. The design modification allowed for renewal of the artificial diet solution with reduced disruption to the insect. Insects were observed to remain in a stationary feeding position on the membrane surface during and post diet renewal.

Although a small proportion of insects (generally 1-2, independent of total insect number) in every humidity chamber would interact with the water chamber and potentially drown, the addition of the fine mesh to cover the top of the water chamber did allow some insects to successfully move over the surface of the water chamber and re-interact with the artificial diet membrane. The number of insects recorded on the water chamber surface varied with observation time, although determination of the number of insects 'saved' from drowning was difficult to assess as there was limited opportunity to track the activity of individual insects.

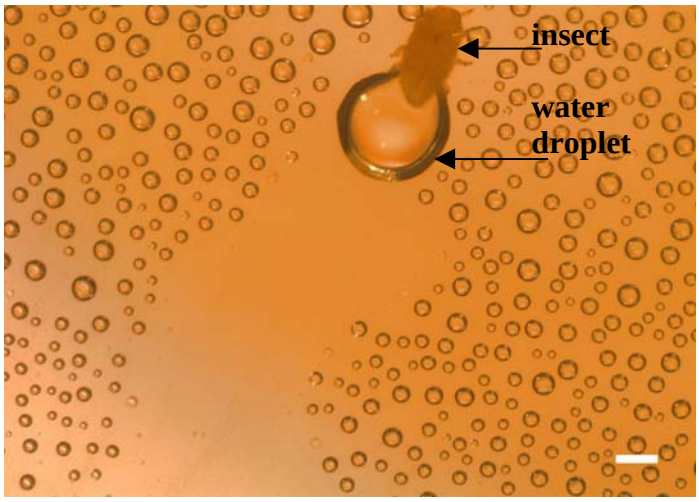


Figure 3. An intermediate grape phylloxera on the underside of the humidity chamber diet membrane, larger droplets of condensed water were observed in the filter paper chamber. Condensed droplets of water adhered to the insect walking across the membrane surface, leaving a dry membrane surface in its trail. As the water droplet near the posterior end increased in size, the mobility of the insect decreased. Scale bar approximately 200 µm.

Table 3a. Summary of survival analysis results for grape phylloxera artificial diet experiments conducted using the filter paper chamber. Diet formulations as described in Table 2a, control diets for each experiment are highlighted in **bold**. Mean, standard error of mean (s.e.), median values are presented for each diet formulation. Significant log-ranked test p-values, and the diet formulations significantly different from the control diet are highlighted in ***bold italic***.

trial number	life stage (n)	value	artificial diet formulation					log-rank test p-value
			a	b	c	d	e	
1	hatchery first instar (25)	mean	**2.96**	**3.00**	3.00	3.28	3.22	
		s.e.	**0.127**	**0.160**	0.294	0.155	0.264	0.72
		median	**3**	**3**	3	3	3	
2	first instar + intermediate (15)	mean	**3.27**	3.18	**3.38**	3.17		
		s.e.	**0.148**	0.251	**0.329**	0.363		0.727
		median	**3**	3	**3.5**	3		
3	first instar (15)	mean	**4.20**	4.07				
		s.e.	**0.253**	0.236				0.628
		median	**4**	4				
4	first instar (15)	mean	**2.35**	***3.13***				
		s.e.	**0.252**	***0.185***				< ***0.05***
		median	**2**	***3***				
5	hatchery first instar (15)	mean	**3.86**	3.07	3.69	3.44	3.28	
		s.e.	**0.540**	0.476	0.578	0.476	0.383	0.755
		median	**4**	3	3	3	3	
6	hatchery first instar (20)	mean	**2.75**	3.05	2.75	2.93	2.95	
		s.e.	**0.233**	0.111	0.171	0.220	0.165	0.903
		median	**3**	3	3	3	3	

Table 3b. Summary of survival analysis results for grape phylloxera artificial diet experiments conducted using the humidity chamber. Diet formulations as described in Table 2b, control diets for each experiment are highlighted in **bold**. Mean, standard error of mean (s.e.), median values are presented for each diet formulation. Significant log-ranked test p-values, and the diet formulations significantly different from the control diet are highlighted in ***bold italic***.

trial number	life stage (n)	value	artificial diet formulation				log-rank test p-value
			a	b	c	d	
7	intermediate	mean	**4.37**	5.03			
	(15)	s.e.	**0.428**	0.567			0.215
		median	**4.5**	4.5			
8	intermediate	mean	**5.20**	3.95	5.25	4.55	
	(10)	s.e.	**0.549**	0.390	0.486	0.559	0.154
		median	**4.5**	4.5	4.5	4.5	
9	intermediate	mean	**4.75**	4.83	5.33		
	(20)	s.e.	**0.498**	0.475	0.402		0.267
		median	**4.5**	4.5	5.5		
10	intermediate	mean	**3.80**	3.73	3.28		
	(30)	s.e.	**0.224**	0.245	0.171		0.214
		median	**3.5**	3.5	3.5		
11	intermediate	mean	**4.79**	4.18			
	(30)	s.e.	**0.454**	0.250			0.184
		median	**3.5**	4.25			
12	intermediate	mean	**4.80**	**4.80**	***6.54***	5.05	
	(40)	s.e.	**0.272**	**0.347**	***0.376***	0.355	***< 0.001***
		median	**5**	**5**	***6.5***	5.5	
13	intermediate	mean	**4.00**	4.11			
	(40)	s.e.	**0.200**	0.250			0.525
		median	**4**	4			
14	hatchery first	mean	**2.30**	2.67			
	instar	s.e.	**0.192**	0.211			0.315
	(30)	median	**2**	3			
15	first instar	mean	**2.77**	3.36	3.15		
	(40)	s.e.	**0.249**	0.269	0.268		0.33
		median	**2.25**	3	2.5		

3.2 Artificial Diet Survival Time

There was a 1-day increase in grape phylloxera survival rates with changing from the filter paper chamber to the humidity chamber (Tables 3a and 3b). This increase in survival rate was also associated with a change in the predominant grape phylloxera life stage used for the artificial diet experiments from first instars to intermediate insects.

In total, 20 diet formulations were trialled on grape phylloxera (Table 1). Using both artificial diet chamber designs, grape phylloxera exposed to controls of either no diet or water survived several days without nutritional intake. The median survival time of first instar insects on controls ranged from 2-4 days across all artificial diet experiments, while the median for intermediate life stages was 3-6.5 days (Tables 3a and 3b). Intermediate insects had previously fed on grapevine root material, which may have enhanced survival rates or fitness levels in comparison with first instars.

3.2.1 Survival Time – Filter Paper Chamber

The filter paper chamber was used to investigate grape phylloxera survival rates for six artificial diet experiments (Table 2a). All of these experiments, except for artificial diet experiment 2, used the first instar life stage. Diet experiment 2 compared the survival of the first instar and intermediate life stages. Across the six filter paper chamber experiments, diet formulation compared sucrose at a range of concentration levels (5, 10 and 20%), sucrose at a range of pH levels (4.6, 6.0, 7.0, 7.5), sucrose with other sugars (glucose and fructose) and a sucrose diet with the addition of a five amino acid mix.

The only filter paper chamber artificial diet to have a significant impact on grape phylloxera survival time was diet experiment 4 (Table 3a). The first instar median survival time was extended by 1-day, in comparison with the control, when insects were exposed to the diet solution of 5% sucrose pH 7.0. All insects were recorded dead under both the control and the diet solution at day four (Table 4), however the rate of death was reduced during the first two days of the artificial diet experiment when grape phylloxera were exposed to the 5% sucrose pH 7.0 diet (Figure 4).

Using intermediate insects in comparison with the first instar life stage (Table 3a, diet experiment 2) did not extend the survival time of grape phylloxera insects. The application of pressure from the water weight on the diet membrane (diet experiment 3) did not increase insect survival time, however insects in the pressurised chamber were observed to be more active on the diet membrane surface with higher levels of probing activity. Altering the diet formulation of a sucrose artificial diet from 5-20% (diet experiment 1), or changing the pH from 7.0-4.5 (diet experiment 5), did no extend the median survival time of first instars beyond 3-days (Table 3a). Changing the type of sugar or the use of grapevine root extract (diet experiment 6), also did not have an impact of insect survival time. The pH of the root extract was 5.0. This aligned with the pH of the root and the stem of the micro-propagated grapevine (Table 5). The leaf of the grapevine was more acidic, pH 4.0.

3.2.2 Survival Time – Humidity Chamber

The humidity chamber was used to investigate grape phylloxera survival for nine artificial diet experiments (Table 2b). These experiments were mainly performed on the intermediate life stages, although diet experiments 14 and 15 were performed on the first instar life stage. Across the nine experiments, diet formulation compared sugars (sucrose, glucose and fructose) with the addition of a ten amino acid mix at a range of pH levels (4.5, 6.5, 7.0 and 7.5).

The only humidity chamber artificial diet formulation to have a significant impact on grape phylloxera survival time was tested in diet experiment 12 (Table 3b). Intermediate grape phylloxera exposed to a diet of 5% sucrose pH 4.5 plus amino acids survived an additional 2.5 days (median) over both the no diet ($p < 0.001$) and water pH 4.5 ($p < 0.05$) controls. The artificial diet formulation of 5% glucose pH 4.5 plus amino acids did not significantly extend the survival time of the insects (no diet $p = 0.173$, water pH 4.5 $p = 0.872$). The proportion of surviving insects was higher for the 5% sucrose pH 4.5 plus amino acid diet solution, in comparison with all other formulations, throughout the 11 days of the experiment (Figure 5). The maximum survival time for insects feeding on control diets was seven days, while insects exposed to the 5% sucrose pH 4.5 plus amino acid diet survived for a maximum period of 11 days (Table 6).

The significant increase in survival time due to grape phylloxera exposure to the artificial diet formulation 5% sucrose pH 4.5 plus amino acids was not observed in diet experiments 11, 13 (intermediate life stages), 14 or 15 (first instars). However in each of the diet experiments there was in increase in the median survival time of insects exposed to the 5% sucrose pH 4.5 plus amino acids diet (Table 3b). Artificial diet formulations involving 5% sucrose plus five amino acids (diet experiment 7), a change in sugar type (diet experiment 8) and the addition of amino acids to varying sugar types (diet experiments 9 and 10) also did not have a significant impact on insect survival time.

3.3 Stylet Interactions

Grape phylloxera insects examined with a stereo microscope were observed in a stationary position on the diet membrane with the stylet extended from the labium and penetrating the diet membrane surface. A crystallisation of the diet solution was observed at the tip of the stylet of intermediate insects ($n = 7$) in diet experiments 11 and 12 (Figure 6). The crystallisation was only observed when the insects were exposed to the diet formulation 5% sucrose pH 4.5 plus amino acids. A component of the amino acid mix (suspected to be L-isoleucine), less readily dissolved during the preparation of this diet formulation. Insects on the 5% sucrose pH 4.5 plus amino acids diet formulation, with their stylet in a vertical position through the diet membrane, were also observed to move the stylet in a ‘backwards and forwards’ movement.

Table 4. Survival analysis for grape phylloxera artificial diet experiment 4, first instar life stage in the filter paper chamber feeding on diet formulations 'no diet' or '5% sucrose pH 7.0'.

formulation	time (days)	insects at risk	insects dead	survival	standard error
no diet	0	15	0	1.000	0
	1	15	2	0.867	0.0878
	2	12	8	0.289	0.1215
	3	3	1	0.193	0.1129
	4	2	2	0.000	
5% sucrose pH 7.0	0	15	0	1.000	0
	1	15	0	1.000	0
	2	15	3	0.800	0.103
	3	12	7	0.333	0.122
	4	5	5	0.000	

'Insects at risk' is the number of insects alive within the diet chamber prior to observation, 'insects dead' is the number of insects observed dead for each time interval. Gaps in 'insects at risk' and 'insects dead' values are due to censored insects.

Table 5. Micro-propagated grapevine pH values obtained from ground leaf, stem and root material.

sample	pH value
grapevine leaf	4.0
grapevine stem	5.0
grapevine root	5.0

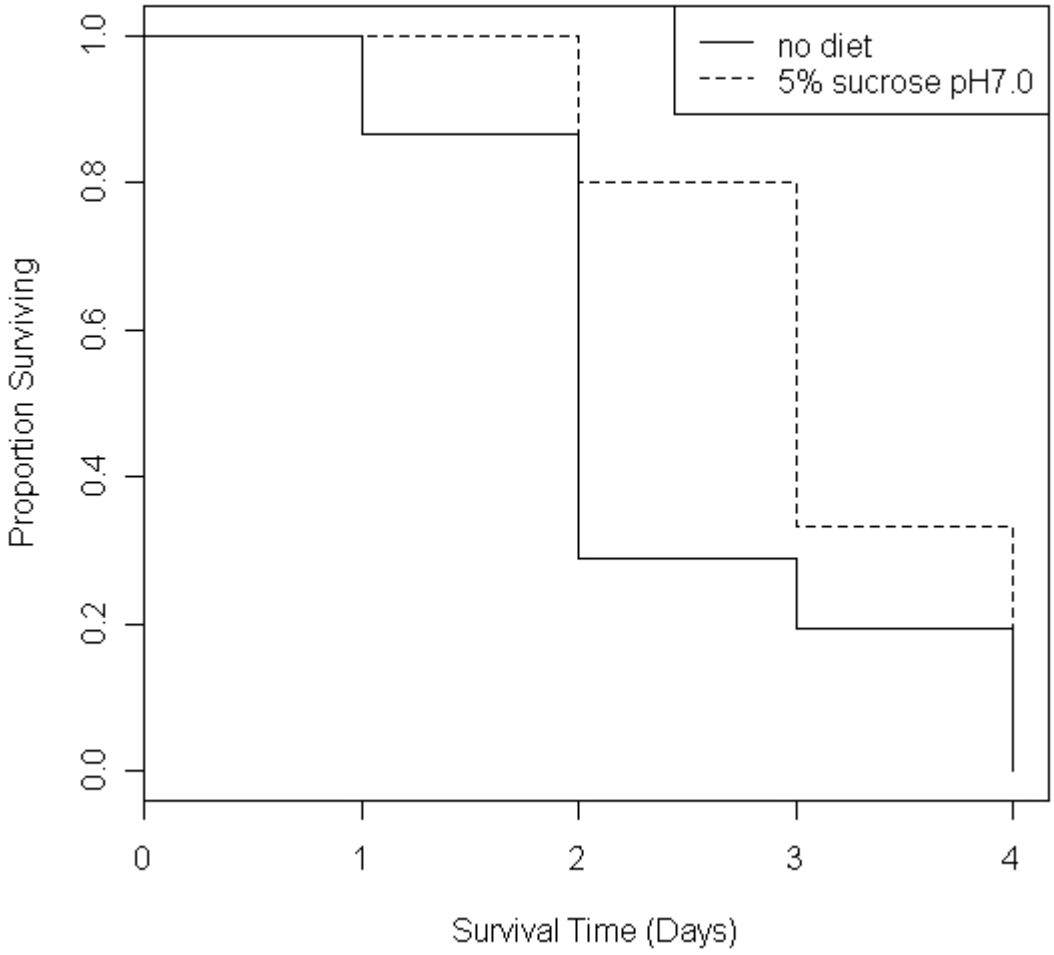


Figure 4. Grape phylloxera artificial diet experiment 4 survival analysis plot, proportion of population surviving over time (days). First instar life stage in the filter paper chamber feeding on 'no diet' or '5% sucrose pH 7.0'.

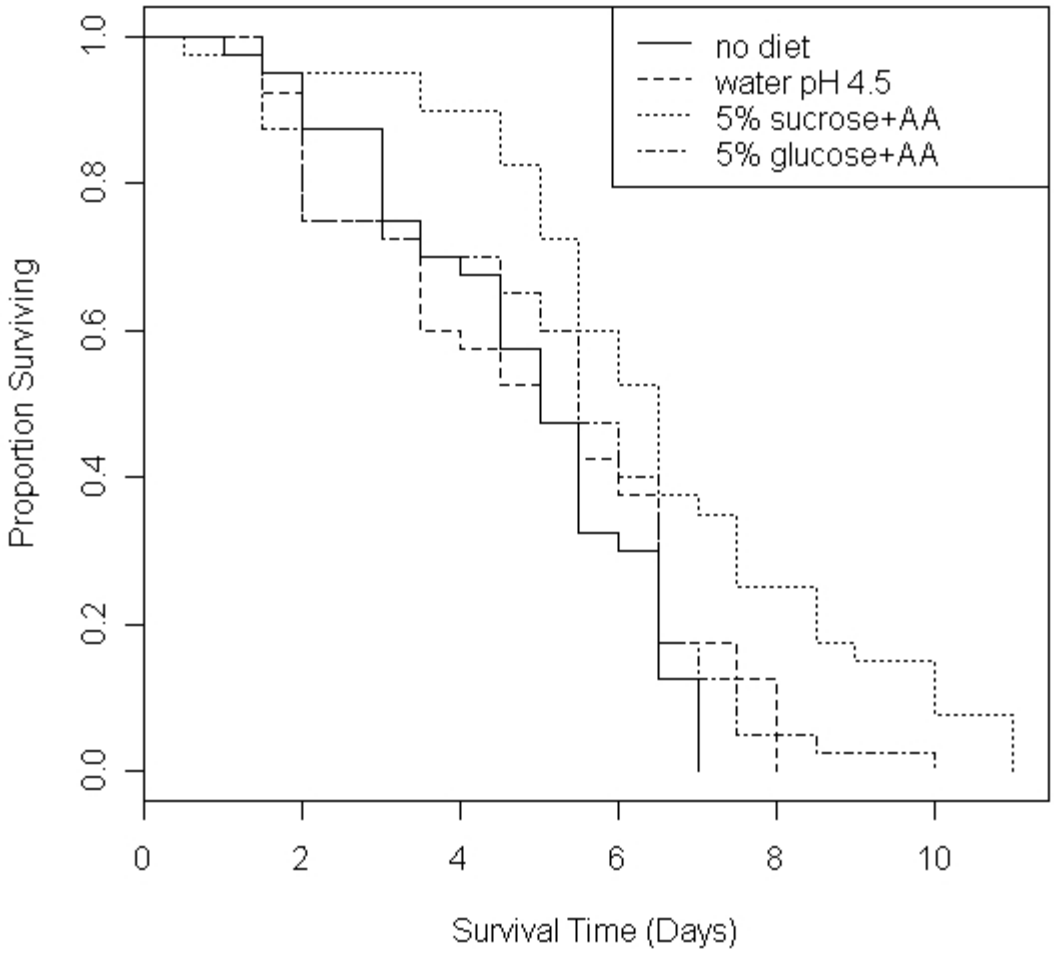


Figure 5. Grape phylloxera artificial diet experiment 12 survival analysis plot, proportion of population surviving over time (days). Intermediate life stage in the humidity chamber feeding on 'no diet', 'water pH 4.5', '5% sucrose pH 4.5 plus amino acids (AA)' or '5% glucose pH 4.5 plus amino acids (AA)'.

Table 6. Survival analysis for grape phylloxera artificial diet experiment 12, intermediate life stage in the humidity chamber feeding on diet formulation 'no diet', 'water pH 4.5', '5% sucrose pH 4.5 plus amino acids' or '5% glucose pH 4.5 plus amino acids'.

formulation	time (days)	insects at risk	insects dead	survival	standard error
no diet	0	40	0	1.000	0
	1.0	40	1	0.975	0.0247
	1.5	39	1	0.950	0.0345
	2.0	38	3	0.875	0.0523
	3.0	35	5	0.750	0.0685
	3.5	30	2	0.700	0.0725
	4.0	28	1	0.675	0.0741
	4.5	27	4	0.575	0.0782
	5.0	23	4	0.475	0.0790
	5.5	19	6	0.325	0.0741
	6.0	13	1	0.300	0.0725
	6.5	12	7	0.125	0.0523
	7.0	5	5	0.000	
water pH 4.5	0	40	0	1.000	0
	1.5	40	3	0.925	0.0416
	2.0	37	7	0.750	0.0685
	3.0	30	1	0.725	0.0706
	3.5	29	5	0.600	0.0775
	4.0	24	1	0.575	0.0782
	4.5	23	2	0.525	0.0790
	5.0	21	2	0.475	0.0790
	5.5	19	2	0.425	0.0782
	6.0	17	2	0.375	0.0765
	6.5	15	8	0.175	0.0601
	7.5	7	2	0.125	0.0523
	8.0	5	5	0.000	
5% sucrose 4.5 plus amino acids	0	40	0	1.000	0
	0.5	40	1	0.975	0.0247
	1.5	39	1	0.950	0.0345
	3.5	38	2	0.900	0.0474
	4.5	36	3	0.825	0.0601
	5.0	33	4	0.725	0.0706
	5.5	29	5	0.600	0.0775
	6.0	24	3	0.525	0.0790
	6.5	21	6	0.375	0.0765
	7.0	15	1	0.350	0.0754
	7.5	14	4	0.250	0.0685
	8.5	10	3	0.175	0.0601
	9.0	7	1	0.150	0.0565
	10.0	6	3	0.075	0.0416
	11.0	3	3	0.000	

Table 6 continued.

formulation	time (days)	insects at risk	insects dead	survival	standard error
5% glucose	0	40	0	1.000	0
pH 4.5 plus	1.5	40	5	0.875	0.0523
amino acids	2.0	35	5	0.750	0.0685
	3.0	30	1	0.725	0.0706
	3.5	29	1	0.700	0.0725
	4.5	28	2	0.650	0.0754
	5.0	26	2	0.600	0.0775
	5.5	24	5	0.475	0.0790
	6.0	19	3	0.400	0.0775
	6.5	16	9	0.175	0.0601
	7.0	7	2	0.125	0.0523
	7.5	5	3	0.050	0.0345
	8.5	2	1	0.025	0.0247
	10.0	1	1	0.000	

'Insects at risk' is the number of insects alive within the diet chamber prior to observation, 'insects dead' is the number of insects observed dead for each time interval. Gaps in 'insects at risk' and 'insects dead' values are due to censored insects. Time intervals without an observed death of an insect are not presented as the survival distribution value remains the same as the previous time interval.

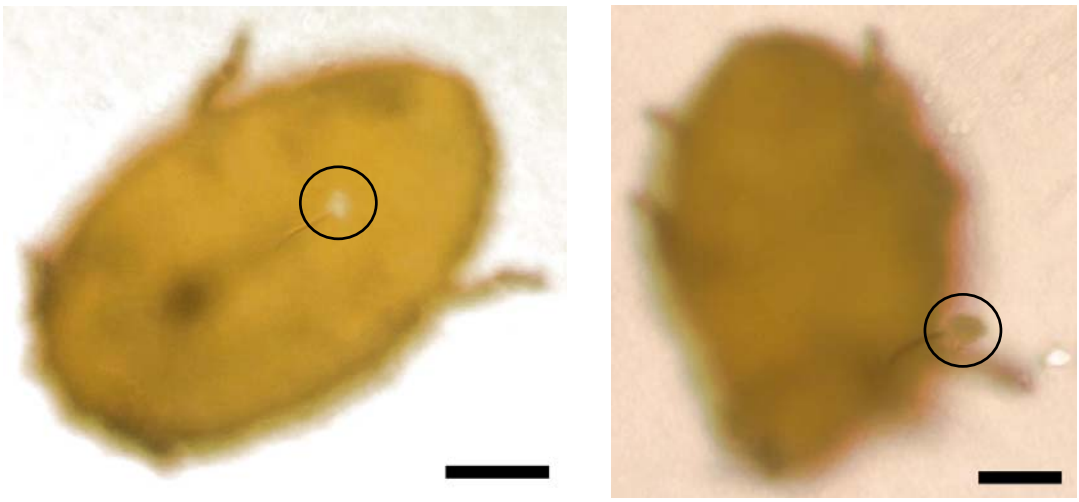


Figure 6. Intermediate grape phylloxera observed in artificial diet experiment 11, feeding on diet 5% sucrose pH 4.5 plus amino acids. The insect is positioned on the underside of the diet membrane with its stylet extended from the labium into the diet solution, a crystallisation of the diet solution has formed at the tip of the stylet. Scale bar approximately 100 μm.

4 DISCUSSION

Grape phylloxera survival on artificial diets was obtained for a maximum period of 10 days. The humidity chamber design was best suited to the sessile feeding grape phylloxera, as the insects were able to maintain a constant feeding location following stylet piercing of the diet membrane due to less disturbance during diet renewal. The application of the water weight did not result in an increase in the survival time of the insects, but was required in the humidity chamber design to keep the two layers of Parafilm® M aligned. Evidence of insect interaction with the diet solution was provided by the formation of a crystallisation in the diet solution near the tip of the stylet for insects feeding on the 5% sucrose pH 4.5 plus amino acids diet formulation. The identity of this crystallisation was unknown, but may represent the precipitation of the chemical reagent observed to delay dissolving during the preparation of the diet solution. The formation of the crystallisation provided evidence that grape phylloxera were piercing the diet membrane and interacting with the diet solution.

Diet Chamber Design

Water droplets condensed on the diet membrane of all chambers, but appeared to impede the insects in the filter paper chamber more than in the humidity chamber. Water droplets were larger in the filter paper chamber, and typically first instar insects were used in this chamber, increasing the ratio in the size of the water droplet to the insect. Intermediate insects in the humidity chamber were observed to still collect the water droplets, although movement across the diet membrane surface was still possible.

Diet Formulation

Variation in diet formulation had limited impact on grape phylloxera survival in comparison with the control diet. Two diet experiments (4 and 12) provided significantly increased insect survival times, however these results were not reproducible and require further investigation. Both diet formulations contained 5% sucrose, suggesting that sucrose is a more suitable form of sugar for grape phylloxera feeding in comparison with fructose and glucose. Filtered and non-filtered root extracts were trialled as a precaution against the accidental removal of feeding stimulant chemicals by the filtration process. However the addition of either root extract solutions did not have a significant impact on grape phylloxera survival rates.

The acidic pH of the grapevine root (pH 5.0) was similar to the diet formulation in diet experiment 12 (5% sucrose pH 4.5 plus amino acids) that significantly enhanced grape phylloxera survival. The pH of the significant diet formulation in diet experiment 4 (5% sucrose pH 7) was however neutral, making interpretation of the optimal pH for grape phylloxera artificial diets difficult. A pH similar to the natural food source would be expected to be the preferred diet formulation, however the non-reproducibility of this result makes this problematic to conclude.

The addition of amino acids to the diet formulation was to reflect the grape phylloxera natural food source of grapevine parenchyma cell contents. With the addition of amino acids to the artificial diet there was a general increase in the survival time of grape phylloxera, however this increase also coincided with the use of the intermediate life stage

(instead of first instar), and the use of the humidity chamber (instead of the filter paper chamber). These confounding factors make the interpretation of cause-and-effect difficult.

5 CONCLUSION

This project experimented with the development of the first artificial feeding system for radicicolae grape phylloxera. The survival time of grape phylloxera in response to artificial diet formulations was variable. Out of 20 diet formulations trialled, 2 had a significant increase on insect survival time, however these results were not reproducible in additional experiments with the same diet formulations. Variable survival results are anticipated with the development of an artificial feeding system for a novel insect species, and are expected to improve as knowledge of the insects essential dietary requirements increase. Grape phylloxera are closely related to aphids, but due to differences in diet nutrition, cell contents compared with phloem sap, the direct application of an aphid diet to grape phylloxera was not successful. The confirmed interaction of grape phylloxera with the artificial diet, by the development of a crystallisation at the tip of the stylet, is encouraging for the future development of this procedure.

6 RECOMMENDATIONS

Future Applications

In future grape phylloxera artificial diet studies, the two diet formulations displaying significant increases in insect survival rates, and in particular the 5% sucrose pH 4.5 plus amino acid diet, require further modification before the diet can be regarded as optimal. However, once an optimal artificial diet feeding system for grape phylloxera is defined, then comparison of the nutritional requirements of several grape phylloxera genotypic classes can be made. This would improve our understanding of the variable survival rates of grape phylloxera in resistant rootstock trials, and lead to knowledge of grapevine resistance mechanisms and the potential for breakdown. The artificial diet system could also be used for the screening of novel anti-metabolic control agents. Control agents that have been used for other plant-sucking insect pests include lectins, protease inhibitors and amylase inhibitors (Gatehouse *et al.*, 1980; Gatehouse *et al.*, 1986; Gatehouse *et al.*, 1995).

Optimising Artificial Diet Feeding System

This proof-of-concept study has highlighted that an *in vitro* feeding bioassay can be developed to study the nutritional requirements of radicicolae grape phylloxera. The physical environment of the feeding chamber is a major component of an artificial diet bioassay system. Two feeding chambers were compared in this project, and the 'humidity chamber' design had significant advantages over the more conventional 'filter paper chamber' design. By modifying the humidity within the feeding chamber, grape phylloxera movement was not impeded by excessive build-up of condensed droplets of water in the chamber. In addition, the use of a pressurised system, although not increasing insect survival, did reduce the level of insect handling required during operation of the artificial diet experiment. The ability to change the artificial diet without opening the

chamber improved the efficiency of the process, reduced the risk of bacterial contamination, and decreased insect disturbance.

Optimising Artificial Diet Formulation

Before being able to optimise an artificial diet formulation for grape phylloxera, a defined chemical profile needs to be obtained from the natural food source, *Vitis vinifera* parenchyma cells. Obtaining a chemical and pH profile of the contents of parenchyma cells is difficult, therefore the sub-optimal artificial diet presented was based on previous diet research and published sugar, amino acid and pH values from *V. vinifera* crude extracts. Twenty diet formulations were tested, highlighting the complexity of developing an optimal artificial diet for insects, and two artificial diets significantly improved the survival of grape phylloxera. This result highlights the potential for the future development of a grape phylloxera artificial diet system, and the application of the system for the screening of novel control agents.

Interestingly, the addition of a grapevine root extract did not enhance grape phylloxera survival rates. This could be due to a concentration effect, as the extract would have been diluted in the artificial diet, potentially masking any effect. In future studies it would be worthwhile analysing the chemical constituents of the *V. vinifera* root extract to determine what chemical compounds could influence grape phylloxera feeding (by either inhibition or stimulation) and once identified, optimise their concentration in the artificial diet system. In addition, extracts of resistance rootstocks could be added to diets to determine their impact on grape phylloxera feeding, survival and development.

Optimising Insect Life-stage Selection for Diet studies

Two life stage categories (first instar and intermediate) were tested in the artificial diet system, and data suggested that the survival rate of insects was related to life-stage. Intermediate instars generally survived longer on artificial diets than first instars. This could be an indicator of several factors: (i) dietary requirements may change for different grape phylloxera instars, which may ultimately relate to changes in vine physiology, (ii) a higher natural mortality in first instars, (iii) potential handling issues with first instars which may affect survival, and/or (iv) the impact of stored food products in the digestive system of intermediate instars. Choice of life stage for further artificial diet studies is clearly an important factor to consider.

SUB-PROJECT 1.2

THE APPLICATION OF ELECTRICAL PENETRATION GRAPH TO GRAPE PHYLLOXERA FEEDING BEHAVIOUR STUDIES

1 INTRODUCTION

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae) feed only on *Vitis* species, causing economic loss to vineyards planted with the susceptible *V. vinifera*. The grafting of American *Vitis* species as resistant rootstocks to *V. vinifera* is the main management option for grape phylloxera infested vineyards; however there are reports in America and Europe of adaptation by grape phylloxera leading to the breakdown of this resistance (Granett *et al.*, 1987; Boubals, 1994). Most studies on the interaction between grape phylloxera and *Vitis* species have focused on population dynamics (Powell *et al.*, 2003) and the genetic variability of the insect (Corrie, 2003). There is limited understanding of how grape phylloxera feeds on susceptible and resistant varieties of the *Vitis* host plant. Understanding grape phylloxera feeding behaviour on the host plant is fundamentally important for ensuring the long-term success of resistant rootstocks as a management tool, and for the identification of factors (such as nutrition) that influence insect-host plant relationships that could potentially be manipulated for vineyard management systems.

Electrical Penetration Graph

To assist with interpreting grape phylloxera host plant interactions, this project has utilised the Electrical Penetration Graph (EPG) technique. EPG amplifies an electrical signal that is created when the piercing mouthpart (stylet) of an insect penetrates into a food substrate. The EPG amplifier detects changes in electrical conductivity within the stylet canal of the insect and the plant tissue of the food source (Tjallingii, 2000). A detailed description of the standard EPG system was presented by Walker (2000), and is briefly outlined here (Figure 1). The EPG amplifier consists of two electrical components, the voltage source and the input resistor. An electrical current travels from the voltage source, through the output wire, to the plant electrode, which is inserted into the moist soil of a potted plant. The insect is connected to an insect electrode, consisting of a short length of 8-20 μm gold wire, with conductive adhesive (silver paint). The insect electrode is then connected via the input wire to the input resistor. When the insect inserts the stylet into the plant, an electrical circuit is completed and the electrical current flows from the voltage source, through the plant-insect biological connection, through the input resistor and back to the voltage source. The biological connection introduces both variable resistance and variable voltage into the circuit. These signals are recorded by the EPG amplifier and are used to interpret insect feeding behaviour.

Since the development of the EPG concept (McLean and Kinsey, 1964), and further development by Tjallingii (Van Helden and Tjallingii, 2000), the EPG technique has become established as a methodology suitable for studying the feeding behaviour for a range of piercing insects. EPG analysis can differentiate between the feeding activities of

stylet penetration, stylet pathway to the food source, salivation and ingestion, and assist with determining host plant resistance mechanisms and virus transmission. The majority of published research in the field involves Hemipteran taxa, including members of the families: Aphididae, or aphids (Sandanayaka and Hale, 2003), Pemphigidae, or root feeding aphids (Cole *et al.*, 1993), Miridae, or mirids (Cline and Backus, 2002), Cicadellidae, or leafhoppers (Backus *et al.*, 2005), Aleyrodidae, or whiteflies (Jiang and Walker, 2003) and Phylloxeridae, or phylloxerids (Harrewijn *et al.*, 1998). Studies have also been undertaken on insects from other orders, including Thysanoptera: Terebrantia, or thrips (Kindt *et al.*, 2003), and Acarina: Tetranychidae, or mites (Guo and Zhao, 2000).

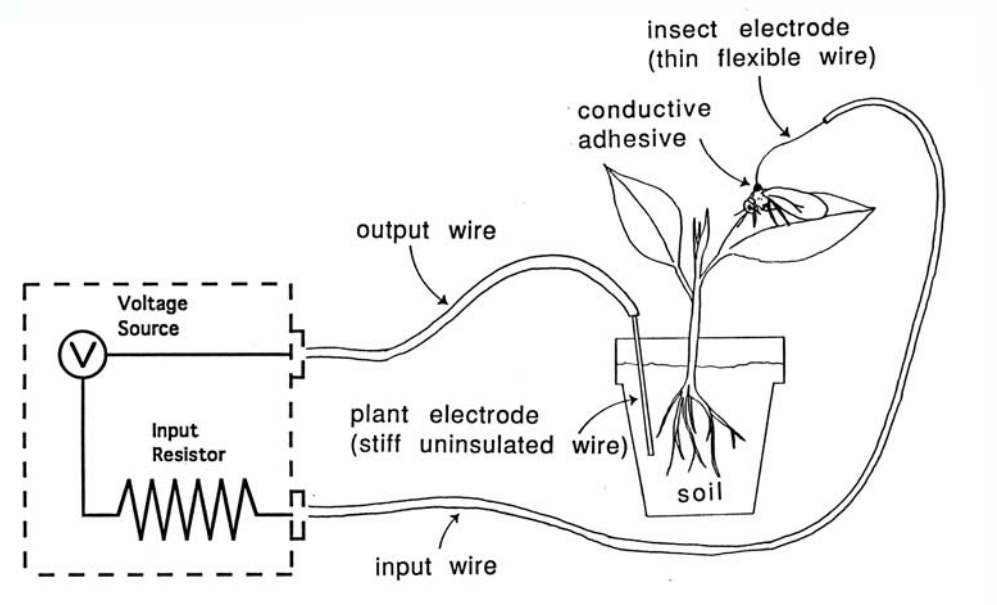


Figure 1. Diagram of the EPG components, where the amplifier (represented by the dashed box) is connected to the insect-plant system (from Walker, 2000). The main components of the system are labelled with arrows.

This project used the EPG technique to primarily investigate the feeding behaviour of a single grape phylloxera genotypic class on susceptible *V. vinifera*, with the intention of expanding the study in the future to include resistant rootstock varieties, and other grape phylloxera genotypic classes, to understand host-plant resistance mechanisms. The aim of the research was to determine the feeding activity required by grape phylloxera for the establishment of the grapevine root feeding site, root gall initiation and insect population development. EPG technology has previously been applied to only one member of the Phylloxeridae family, *Phylloxera coccinea* Heyden (Harrewijn *et al.*, 1998). Before commencement of this research, anticipated difficulties with the application of the EPG technique to grape phylloxera were based on the relatively small size of the insect, its root feeding habit and the anticipated food source of parenchyma cell contents. A major concern was how these factors would impact on (and potentially reduce) the strength of the

EPG signal. However the literature provided examples where each of these factors were previously (singly) overcome, including the application of EPG to: similar-sized whitefly insects (Hemiptera: Aleyrodidae) (Lei *et al.*, 1996); aphids feeding on lettuce roots (Homoptera: Pemphigidae) (Cole *et al.*, 1993); and western flower thrips feeding on non-vascular plant tissue (Thysanoptera: Terebrantia) (Harrewijn *et al.*, 1996).

Research Aims

This report highlights the first time application of the EPG technique to radicicolae grape phylloxera. Modifications to the EPG system described in Figure 1 are detailed and current methodologies are discussed. Data analysis focuses on comparing continual feeding EPG patterns on *V. vinifera* root material from intermediate life stages with the active, gall initiating first instars, and with the reproductive adult. No previous Aphididae studies have involved comparing adult EPG feeding patterns with earlier life stages (Van Helden and Tjallingii, 2000). Limited 'proof-of-concept' analysis was also conducted to characterise grape phylloxera initial probing activity on *V. vinifera*, and to determine feeding patterns on resistant and immune rootstock material.

2 MATERIAL AND METHODS

2.1 Insect and Plant Material

Radicicolae grape phylloxera were originally collected from infested vineyards in the King Valley, Victoria, Australia. Grape phylloxera from this region have been identified as a single genotypic class, G4 (Corrie *et al.*, 2002). Populations were maintained at the Department of Primary Industries – Rutherglen Centre on excised *V. vinifera* root pieces (approximately 1 cm width x 10 cm length), prepared using a protocol slightly modified from Granett, et al. (1985). The modifications were: (1) roots washed clean of attached soil with a soft brush under running water; (2) roots soaked for 5 minutes in Ridomil® Gold Plus systemic fungicide solution (2.3 g/L); (3) roots triple rinsed with sterile distilled water prior to air drying; and (4) the size of the petri dish was increased to 15 cm, with the increase in area reducing the level of water condensation within the chamber. Grape phylloxera populations were incubated in the dark at a constant temperature of 25 ±3°C prior to EPG recording.

Only apterous, radicicolae grape phylloxera were used for this study. Grape phylloxera were selected for EPG recording based on life stage and location on excised root pieces. Using a stereo microscope, insects were measured with a graduated eyepiece with life stage being determined by comparative increases in size. The presence of eggs located externally near the posterior end of the insect confirmed the adult reproductive stage. If present, neighbouring moulted cuticles were counted to confirm the insect life stage. Life stages used for EPG analysis were first instars, third and fourth instars (grouped as 'intermediates') and adults. Insects feeding in an isolated location were preferentially connected to the EPG insect electrode in order to record individual grape phylloxera-grapevine root interactions. However isolated insects were not exclusively used due to the group distribution of grape phylloxera on gall sites limiting their availability.

Lignified grapevine root material for excised root pieces (Granett *et al.*, 1985) were sourced from non-phylloxera infested vineyards to prevent the potential cross contamination of G4 with other grape phylloxera genotypic classes. Susceptible (*V. vinifera* cv. Sultana and Shiraz), resistant (*V. riparia* x *V. rupestris* cv. Schwarzmann and *V. champini* cv. Ramsey) and immune (*V. cinerea* x *V. riparia* cv. Börner) *Vitis* species were utilised for grape phylloxera feeding behaviour trials. Sterile tissue culture grapevines (Kellow *et al.*, 2002) of these species were also established.

2.2 Insect Electrode and EPG Recording Positions

The EPG insect electrode consisted of 3-5 cm of 12.5 µm (0.0005 in) diameter gold wire (Sigmund Cohn, Mount Vernon, NY) connected with conductive silver paint (water-based) to the input wire. Using a stereo microscope, the end of the gold wire was repeatedly dipped into the silver paint until a droplet formed. The gold wire was held with self-closing forceps to prevent the wire from slipping. The silver paint droplet was then carefully placed onto the abdomen of the insect and held for a few seconds until dry and the gold wire was fixed in place (Figure 2). Due to the survival of grape phylloxera for up to eight days without feeding (Kingston, et al., 2005, see final report sub-project 1.1**)**, individuals used for EPG recording were not starved prior to set up.

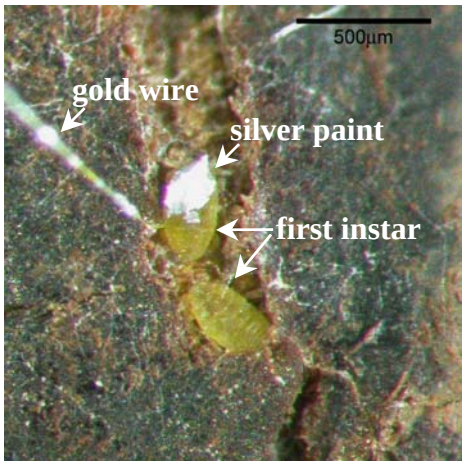


Figure 2. First instar grape phylloxera connected to the insect electrode, consisting of a short section of 12.5 µm gold wire attached with conductive silver paint, scale bar 500 µm. The main features of the insect electrode are labelled with arrows.

Two insect recording positions were developed for grape phylloxera EPG recordings. The 'active feeding' insect position was performed to gain an initial understanding of grape phylloxera feeding waveforms as no published EPG data was available for comparison. The 'probe initiation' insect position was recorded to understand the active feeding waveforms in relation to the complete feeding behaviour profile (stylet penetration, stylet pathway and ingestion) required by grape phylloxera to establish feeding and survive on *Vitis* species.

2.2.1 Active Feeding

Grape phylloxera were individually wired to the insect electrode without disturbance from the established excised root food source. The insect was then connected to the EPG amplifier for recording. Due to the sessile feeding nature of grape phylloxera, it was assumed that these insects were actively feeding at the time of data acquisition. If the insect was accidentally disturbed during set up, and the stylet was removed from the excised root food source, then the insect was reclassified as being in a probe initiation EPG recording position.

2.2.2 Probe Initiation

Grape phylloxera individuals were wired to the insect electrode and then gently disturbed from the established excised root food source with a sable-haired paintbrush. The insect was left hanging from the insect electrode for a short period of time until attached to the EPG amplifier and positioned onto a new food source. A stereo microscope was used to assist in this positioning. Probe initiation EPG recordings were performed using both excised root and tissue culture recording systems (described below).

2.3 Plant Electrode and EPG Recording Systems

The plant electrode was modified from the stiff, uninsulated copper wire connected to the output wire and inserted into the potted plant (Figure 1). The plant electrode consisted of a 1 cm brass pin which could be used with both the excised root and tissue culture recording systems. Excised roots were used to maintain the grape phylloxera populations in the laboratory, and provided the simplest plant set up for data acquisition. The tissue culture method allowed recording of grape phylloxera feeding on a whole plant, in the absence of a soil environment.

2.3.1 Excised Root

Excised root pieces, sourced from CSIRO Merbein, were typically 10 cm in length x 6-10 mm in diameter. Standardisation of the root piece diameter was dependent upon variability of the lignified root material available. Excised root pieces were prepared as per Granett, et al. (1985), with modifications as outlined earlier. Grape phylloxera populations were established on the excised root pieces prior to use for EPG recording. The ends of the excised root pieces were wrapped in damp cotton wool, with the modified plant electrode placed between the root exterior surface and the cotton wool at one end (Figure 3). Wetting the cotton wool provided a suitable level of conductivity for the movement of an electrical current for EPG recording.

2.3.2 Tissue Culture

Micro propagated grapevines were prepared as outlined by Kellow, et al. (2002). Slight modifications included: (1) agar medium was not supplemented with benzyl aminopurine or napthaleneacetic acid; (2) plantlets were not transferred to a second agar medium prior to the perlite-based medium; and (3) after transfer to the perlite-based medium, tissue culture vines were not inoculated with grape phylloxera eggs. Once the tissue culture vines had established, they were removed from the perlite-based medium and positioned on a 15 cm petri dish lined with filter paper. The modified plant electrode was placed in contact with the filter paper, which was kept wet throughout the recording (Figure 3). Wetting the filter paper assisted in maintaining the structure of the tissue culture vine and provided a suitable level of conductivity for the movement of an electrical current.

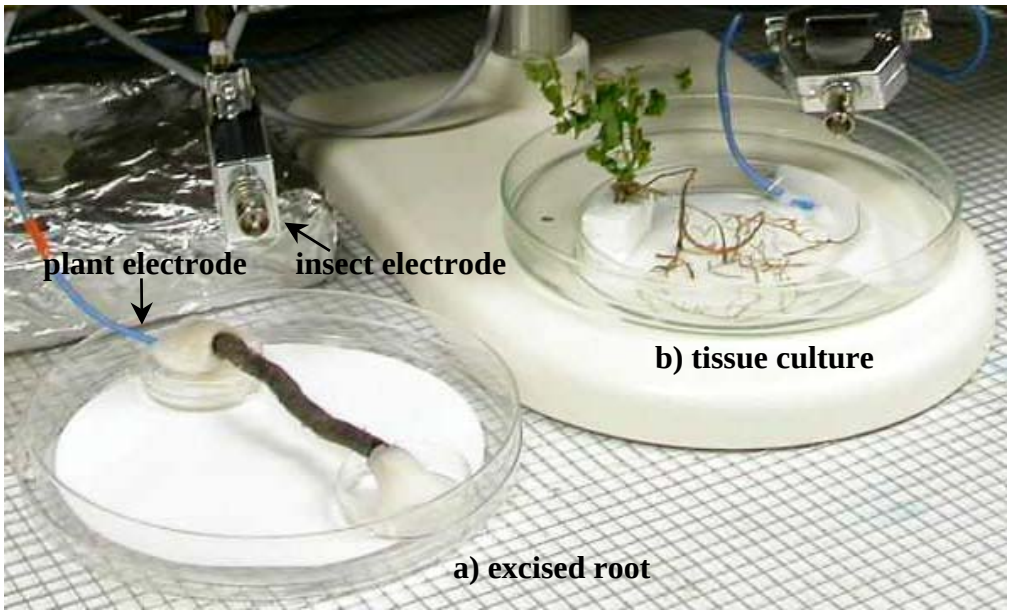


Figure 3. The plant EPG recording system for the a) excised root and b) tissue culture protocols. The plant electrode (EPG output wire) and insect electrode (EPG input wire) are labelled on the excised root system.

2.4 EPG Equipment

A DC (direct current) voltage EPG amplifier was used for all recordings. The Giga-8 series EPG amplifier (Wageningen University, Laboratory of Entomology), with the potential to simultaneously record the feeding activity of eight insects, operated under an input resistance of 1 giga Ohm ($10^9\Omega$), 50x amplification and an input bias current <1 pA. The number of channels utilised per recording varied from 1-6. All recordings were performed at room temperature (23±2°C) within a Faraday cage covered with black cardboard to provide a dark environment for the radicicolae grape phylloxera (Figure 4). There was no standardisation for the timing of the recordings over the 24 hour/day period, and recording length varied from 0.5-8 hours (the majority of recordings were five hours in length).



Figure 4. The faraday cage used for all EPG recordings; the access door was opened during the set up of the plant and insect electrodes, and closed during recordings to reduce the level of background electrical interference. The pictured set up contains the tissue culture plant system with the stereo microscope (right) and two-excised root plant systems (left). The wire exterior of the faraday cage was covered with black cardboard to provide a dark environment for the radicicolae grape phylloxera.

2.5 Waveform Characterisation

EPG data was captured and analysed using PROBE 3.0 software (Wageningen University, Laboratory of Entomology). Prior to waveform analysis, EPG recordings were reviewed to identify common waveforms. Waveforms were characterised by amplitude (height of wave presented in mV), wave duration (width of wave in seconds), repetition rate (number of waves per second), and a general description of the waveform shape (Figure 5). Waveform amplitude was corrected as a percentage of the highest waveform amplitude (waveform 9) for waveforms which occurred in probe initiation recordings, and presented as %V. For waveform measurements, 5 second views of the EPG recordings were examined. Waveform duration was measured for all complete waves within the 5 second view, and converted (1/width of wave in seconds) into Hertz (Hz). The number of complete waves for the same 5 second view was divided by five for the per second repetition rate (Hz), and the amplitude of the wave was recorded. Five 5 second views were measured per recording. When the waveform occurred for a sufficient period of time, there was a 5 second gap in between consecutive measurements of the same recording. This gap was decreased or increased (depending upon waveform occurrence) to allow for the measurement of five 5 second views. Where possible, the same waveform was reviewed at a range of voltage levels to provide information about the signal origin, resistance (R) or electromagnetic force (emf), of the wave.

The majority of waveforms were defined from insects in the active feeding position, and are categorised as continual feeding waveforms. Waveforms associated with probe initiation are categorised as probe waveforms. Waveforms that were observed relatively infrequently during waveform analysis were initially categorised as 'undefined' and reviewed post analysis to determine additional waveform groupings. Waveforms that occurred only once during all recordings remained classified as undefined.

Recordings were reviewed to determine regular waveform sequence transitions and the frequency of waveforms. No correlation studies have been performed to identify biological function of the waveforms; therefore a waveform numbering system was applied to prevent the implication of biological meaning.

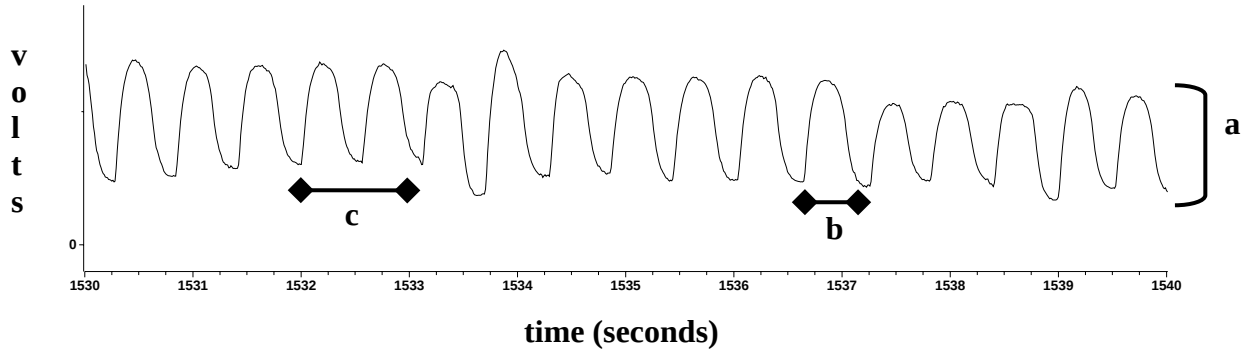


Figure 5. Key points for waveform characterisation during EPG analysis included: a) amplitude or height of wave presented in mV, b) wave duration or width of wave in seconds, c) repetition rate or wave number per second, and a general description of the wave shape.

2.6 Waveform Analysis

PROBE 3.0 was used to monitor the duration and occurrence of each of the characterised waveforms within each EPG recording. Minor waveform interruptions of 1-2 waves were ignored and the dominant waveform was recorded. Silent periods between waveforms were considered part of the previous waveform duration, unless there was a change in baseline voltage and evidence for waveform transition. A silent period was defined as a temporary return to the baseline voltage level between waves, but for EPG analysis did not infer a change in waveform or the removal of the insect stylet from the food source.

Waveform transition was generally defined as the continued occurrence of the new pattern for >5 seconds. Exceptions were made for short period, high amplitude waves. This rule reduced the complexity of the analysis, although some information may have been excluded. As this was the initial application of EPG to the feeding behaviour of grape phylloxera, and no correlation data identifying feeding location was available, there was a deliberate decision not to over analyse the data. Most recordings were five hours in length. Eight hour recordings were only analysed for five hours to increase the standardisation of recording length; no new waveforms were observed in the three hours not analysed.

Excised root pieces were pre-colonised by a population (G4 genotypic class) of grape phylloxera, while tissue culture plants had not previously been exposed to grape phylloxera feeding activity. Duplicate recordings of the same insect and root material combination were discarded so each recording analysed represented a new insect-plant combination. The same root material may have been used for more than one insect recording. Progressive life stage recordings were made on the same excised root piece, resulting in the (low) possibility of the same insect being recorded on the same root material at a different life stage. Individual insects on the root pieces were not identifiable.

The plant and insect EPG recording structure was used to determine the relative success of the trialled procedures, but did not define groupings for waveform analysis. Recordings were analysed dependent upon waveform occurrence. Distinction was made between recordings presenting continuous feeding waveforms only and recordings representing probe (and feeding) waveforms. If probe waveforms only occurred for a short period of time at one end of the recording, they were excluded to allow analysis as a continuous feeding recording.

2.7 Statistical Analysis

PROBE 3.0 collected data was transferred to Microsoft® Excel to calculate waveform duration values. GenStat-Eighth Edition© (Lawes Agricultural Trust) was used to calculate summary statistics for the data, including total time spent in each waveform, number of occurrence of waveforms and average time spent in each waveform. Data was grouped by life stage to calculate the average percent time spent in each waveform out of the total recording time. This calculation removed any time bias introduced by variable EPG recording length, and combined waveform totals were corrected to equal 100 percent. All waveform events were included in the analysis, including the final waveform occurrence that may have prematurely ended due to the completion of the EPG recording time. Because of the regular occurrence of the common waveforms, this was not expected to impact highly on analysis.

Due to low sample numbers, continual feeding analysis data within life stage groups were combined from susceptible *V. vinifera* cv. Sultana and Shiraz root material. Data for intermediate (third and fourth) instars were also combined to represent a 'base-level' feeding behaviour in comparison with first instar and adults. GenStat-Eighth Edition© summary of statistics values compared the mean time and variance of waveform duration to confirm that there was no grouping of the separate factors prior to further statistical analysis.

Seven insects were analysed for each life stage. Sample numbers were too low for statistical analysis, and descriptive data only is presented. In comparing all life stages, ANOVA was used to determine if there was a difference in waveform duration with life stage development.

Sample number (n = 3) for probe initiation and rootstock recordings were too low for statistical analysis. Characterisation of waveforms and waveform distribution data are presented, although no conclusive statements are able to be made regarding the feeding behaviour of grape phylloxera in these recordings.

3 RESULTS

EPG recordings were successfully acquired from both the excised root and tissue culture recording systems. Grape phylloxera in the active feeding position remained stationary and appeared to continue feeding once connected to the EPG amplifier, indicating that tethering the insect to the gold wire did not deter feeding. Grape phylloxera in the probe initiation position were observed to move along the root surface once connected to the gold wire, although movement was partially restricted by the rigidity and length of the wire. Once insects had initiated feeding, there was no indication of disturbance to normal feeding behaviour caused by connection to the EPG amplifier. EPG recordings were successfully performed on all feeding life stages of grape phylloxera.

3.1 EPG Recording Success Rate

EPG success was determined by the presence or absence of recognisable waveforms. Failed recordings were identified as receiving no EPG signal (a flat 0V line), a non-reactive test calibration pulse or a high voltage noise rhythm that masked feeding waveforms. The majority of recordings were performed with insects in the active feeding position on the excised root plant system (Table 1). On susceptible root material, 70% (28/40) of recordings were successful, and 25% (2/8) on resistant root material. No EPG recordings were attempted with insects in the active feeding position on the tissue culture plant system as the tissue culture plants were not previously colonised by a grape phylloxera population.

EPG recordings with insects in the probe initiation position were performed using both the excised root and the tissue culture plant recording system (Table 1). Probe initiation recordings on the excised root EPG plant system were successful for 25% (5/20) of recordings on susceptible root material; no successful probe initiation recordings were obtained on resistant excised root material. Combining both susceptible and resistant root material, probe initiation recordings on the tissue culture plant system were successful for 25% (3/12) of recordings.

Waveform analysis was performed on continual feeding recordings from insects in the active feeding position on susceptible excised root material. There was no difference in the success rate of these EPG recordings following separation by insect life stage (Table 2). To ensure equal units for statistical analysis, life stage sample number was limited by the least successful life stage, adults. Seven adult recordings were suitable for continual feeding waveform analysis; 21 recordings were analysed across all life stages. The total EPG recording time of the analysed recordings varied; first instar EPG recordings were 203 ± 97 (mean ± standard deviation) minutes in length, intermediate instars 248 ± 99 minutes, and adults 140 ± 112 minutes.

Table 1. Number of successful grape phylloxera EPG recordings comparing plant system (excised root and tissue culture) with insect position (active feeding and probe initiation).

	active feeding			**probe initiation**		
	susceptible		resistant	susceptible		resistant
	Shiraz	Sultana	all	Shiraz	Sultana	all
excised root	8 (10)	20 (30)	2 (8)	0 (1)	5 (19)	0 (2)
tissue culture	NA	NA	NA	2 (11)	NA	1 (1)

Susceptible and resistant root material indicated. Total number of attempted recordings is presented in parentheses, some plant system-insect position combinations were not attempted (NA).

Table 2. Number of successful grape phylloxera EPG recordings on the excised root plant recording system with insects in the active feeding position, recordings differentiated by life stage.

life stage	**susceptible**		**resistant**
	Shiraz	Sultana	all
first instar	0 (1)	10 (15)	1 (5)
intermediate	5 (5)	5 (8)	1 (3)
adult	3 (4)	5 (7)	0 (0)

Susceptible and resistant plant material indicated, recordings on susceptible roots used for continual feeding waveform analysis. Total number of attempted recordings is presented in parentheses.

3.2 Waveform Characterisation

Initial analysis of grape phylloxera continual feeding recordings identified 11 re-occurring waveforms (Table 3). An additional three waveforms were observed in probe initiation recordings (Table 4). These waveforms have been labelled numerically, with sub-numbering indicating a variation within the main waveform. A visual representation of these waveforms (Figures 6 and 7) highlights the variation in appearance of grape phylloxera EPG patterns. No correlation studies were performed on these waveforms to infer biological function.

Waveforms 2 and 3 occur in all recordings and regularly interchange between each other. Waveform 2 was a regular monophasic pattern, with an irregular biphasic interruption. Waveform 3 was a biphasic pattern with an irregular silent period. There was variation in the relative size of the positive and negative peaks. Waveform 4 was a similar biphasic pattern with no silent period, and occurred in 71% of recordings. Waveform 4 interchanged with waveform 3, and occasionally waveform 2. These three waveforms were the most dominant in grape phylloxera continual feeding EPG recordings.

Waveforms 2.2 and 2.4 occurred only in recordings from first and intermediate instars, and were short interruptions or variations to waveform 2. Waveform 3.4 occurred only in first instar recordings, and interchanged with waveform 3.

Waveform 5 was a more complex monophasic pattern in comparison with waveform 2. Waveform 5 interchanged with waveform 2 during recordings, and was only observed in intermediate (n = 1) and adult (n = 4) life stages. Waveform 6 was a complex mix of waveforms 2 and 3, and occurred only in recordings from intermediates. Waveform 6 interchanged between waveforms 2 and 3. Waveform 7 was a complex mix of undefined waveforms that required further observation in additional recordings in order to be classified.

Waveforms 8 and 10 indicated changes in amplitude rather than a unique pattern occurrence. Waveform 8 was a 3V change in the baseline of the recording without any interruption to other waveforms. Waveform 8 interchanged between waveforms 2, 3, 7 and 6, and was only observed in recordings from intermediates. Waveform 10 was a high voltage interruption to waveforms 2 and 3. Waveform 10 was observed in all life stages.

Table 3. Grape phylloxera continual feeding EPG waveform characteristics and measurements. Waveforms defined by type, amplitude (mV and %V), repetition rate (Hz), duration (Hz) and signal origin, for definitions see text. A description of the waveform and frequency of observation from each life stage grouping (n = 7) is provided. Amplitude (mV and %V) n value indicates the number of recordings viewed for measurements; repetition rate n value indicates the number of 5-second views measured, and wave duration n value indicates the number of waves measured.

waveform		**waveform measurements**					**waveform characteristics**	
label	type	amplitude (mV)	amplitude (% V)	repetition rate (Hz)	duration (Hz)	signal origin (R/emf)	description	frequency (n = 7)
2	Monophasic	295 ± 239 (n = 6)	14 ±17 (n = 3)	3.4 ± 1.3 (n = 90)	6.2 ± 2.9 (n = 1526)	R/emf	sharp positive peak with waves in baseline, irregular biphasic interruption	first instar: 7 intermediate: 7 adult: 7 **combined: 21**
2.2	Monophasic	600 ± 361 (n = 3)	14 ± 0 (n = 1)	2.1 ± 0.85 (n = 8)	4.0 ± 1.8 (n = 83)	unknown	increase in amplitude interruption to waveform 2, momentary change in baseline voltage	first instar: 1 intermediate: 3 adult: 0 **combined: 4**
2.4	Monophasic	138 ± 18 (n = 2)	5.4 ± 0 (n = 1)	2.2 ± 0.35 (n = 3)	6.8 ± 4.3 (n = 33)	unknown	continuation of waveform 2, momentary change in baseline voltage	first instar: 1 intermediate: 1 adult: 0 **combined: 2**
3	Biphasic	657 ± 334 (n = 6)	44 + 0 (n = 1)	1.9 ± 0.9 (n = 83)	5.7 ± 1.1 (n = 768)	emf	sharp positive and negative peak, irregular silent period between waves	first instar: 7 intermediate: 7 adult: 7 **combined: 21**
3.4	Biphasic	413 ± 301 (n = 2)	–	1.6 ± 0.95 (n = 5)	7.5 ± 3.8 (n = 41)	unknown	continuation of waveform 3, momentary change in baseline voltage	first instar: 2 intermediate: 0 adult: 0 **combined: 2**

Table 3 continued.

waveform		waveform measurements					waveform characteristics	
label	type	amplitude (mV)	amplitude (% V)	repetition rate (Hz)	duration (Hz)	signal origin (R/emf)	description	frequency (n = 7)
4	Biphasic	703 ± 237 (n = 4)	23 ± 15 (n = 2)	7.1 ± 0.88 (n = 40)	7.6 ± 1.0 (n = 1413)	emf	sharp positive and negative peak, no silent period between waves	first instar: 3 intermediate: 7 adult: 5 **combined: 15**
5	Monophasic	370 ± 98 (n = 5)	–	4.3 ± 0.67 (n = 14)	5.9 ± 3.0 (n = 301)	unknown	sharp positive peak with waves along peak	first instar: 0 intermediate: 1 adult: 4 **combined: 5**
6	Mono-Biphasic combination	1317 ± 633 (n = 3)	127 ± 0 (n = 1)	0.8 ± 0.43 (n = 15)	1.5 ± 0.65 (n = 60)	unknown	complex mix of waveforms 2 and 3 with deep negative peak, increased amplitude	first instar: 0 intermediate: 3 adult: 0 **combined: 3**
7	Undefined	variable	variable	variable	variable	unknown	mixed group of single occurrence patterns	first instar: 2 intermediate: 1 adult: 1 **combined: 4**
8	Altered baseline voltage	change 3396 ± 246 (n = 2)	–	–	–	unknown	approximate 3V amplitude change in baseline voltage, continuation of waveforms	first instar: 0 intermediate: 2 adult: 0 **combined: 2**
10	High amplitude interruption	% increase 337 ± 149 (n = 5)	18 ± 0 (n = 1)	variable	variable	R	high amplitude interruption to waveforms, no change in baseline voltage	first instar: 1 intermediate: 3 adult: 1 **combined: 5**

Table 4. Grape phylloxera probe initiation EPG waveform measurements and characteristics. Waveforms defined by type, amplitude (mV and %V), repetition rate (Hz), duration (Hz) and signal origin, for definitions see text. A description of the waveform and frequency of observation from each life stage grouping (intermediate and adult only) are provided. Amplitude (mV and %V) n value indicates the number of recordings viewed for measurements; repetition rate and wave duration measurements were not attempted.

waveform		**waveform measurements**					**waveform characteristics**	
label	type	amplitude (mV)	amplitude (% V)	repetition rate (Hz)	duration (Hz)	signal origin (R/emf)	description	frequency (n = 3)
1	Non-penetration	–	–	–	–	–	0V, flat line pattern	intermediate: 1 adult: 2 **combined: 3**
9	Altered baseline voltage and waveform	1800 ± 1700 (n = 3)	100 ± 0 (n = 3)	–	–	R	high amplitude pattern followed by a change in the waveform, at the beginning of continual feeding waveforms	intermediate: 1 adult: 2 **combined: 3**
9.1	Altered baseline voltage and waveform	2150 ± 1750 (n = 3)	120 ± 40 (n = 3)	–	–	R	high amplitude pattern similar to waveform 9 but not followed by a change in waveform	intermediate: 1 adult: 2 **combined: 3**

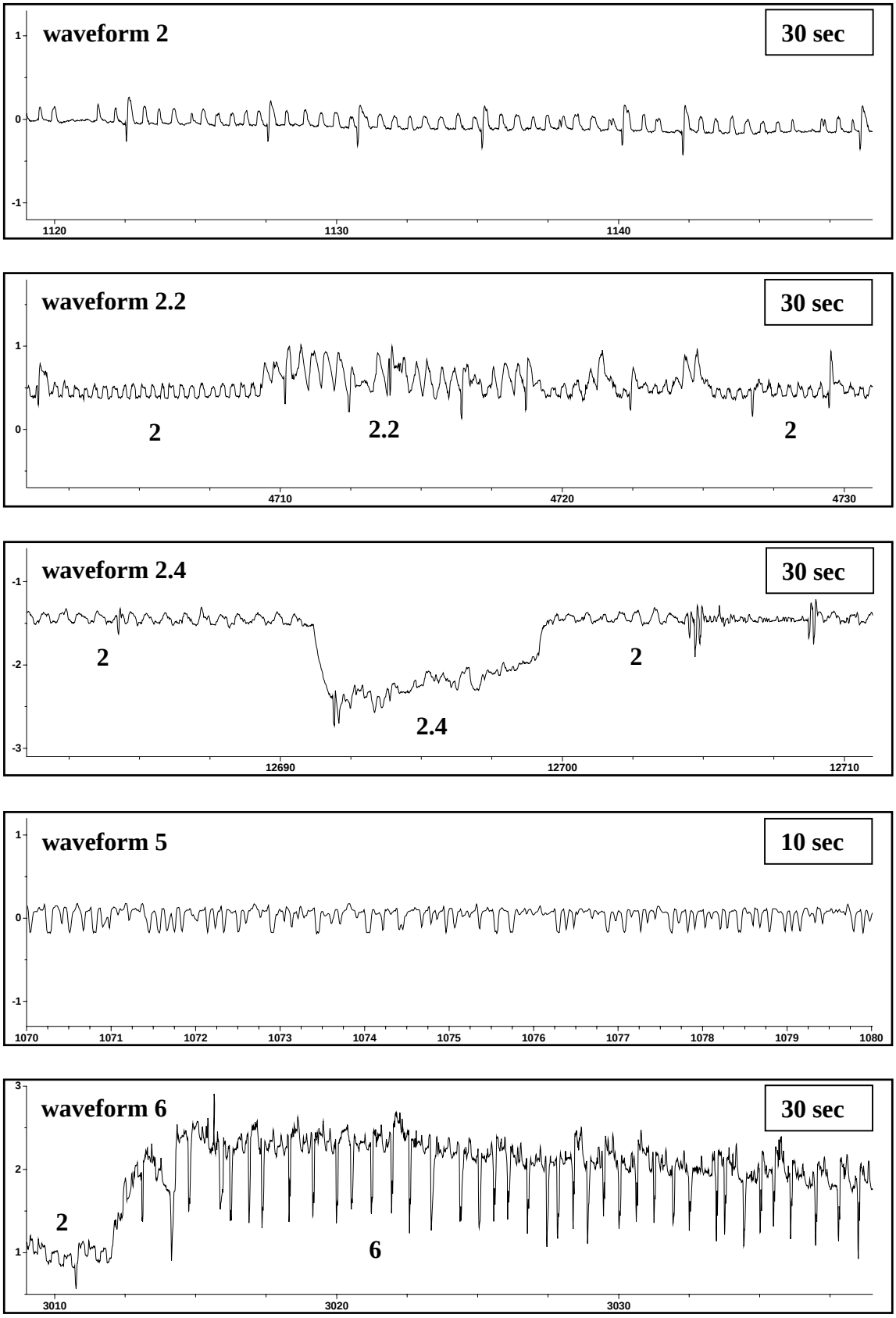


Figure 6. Visual representation of grape phylloxera monophasic EPG waveforms (including mono-biphasic waveform 6), timeframe and waveform transitions indicated.

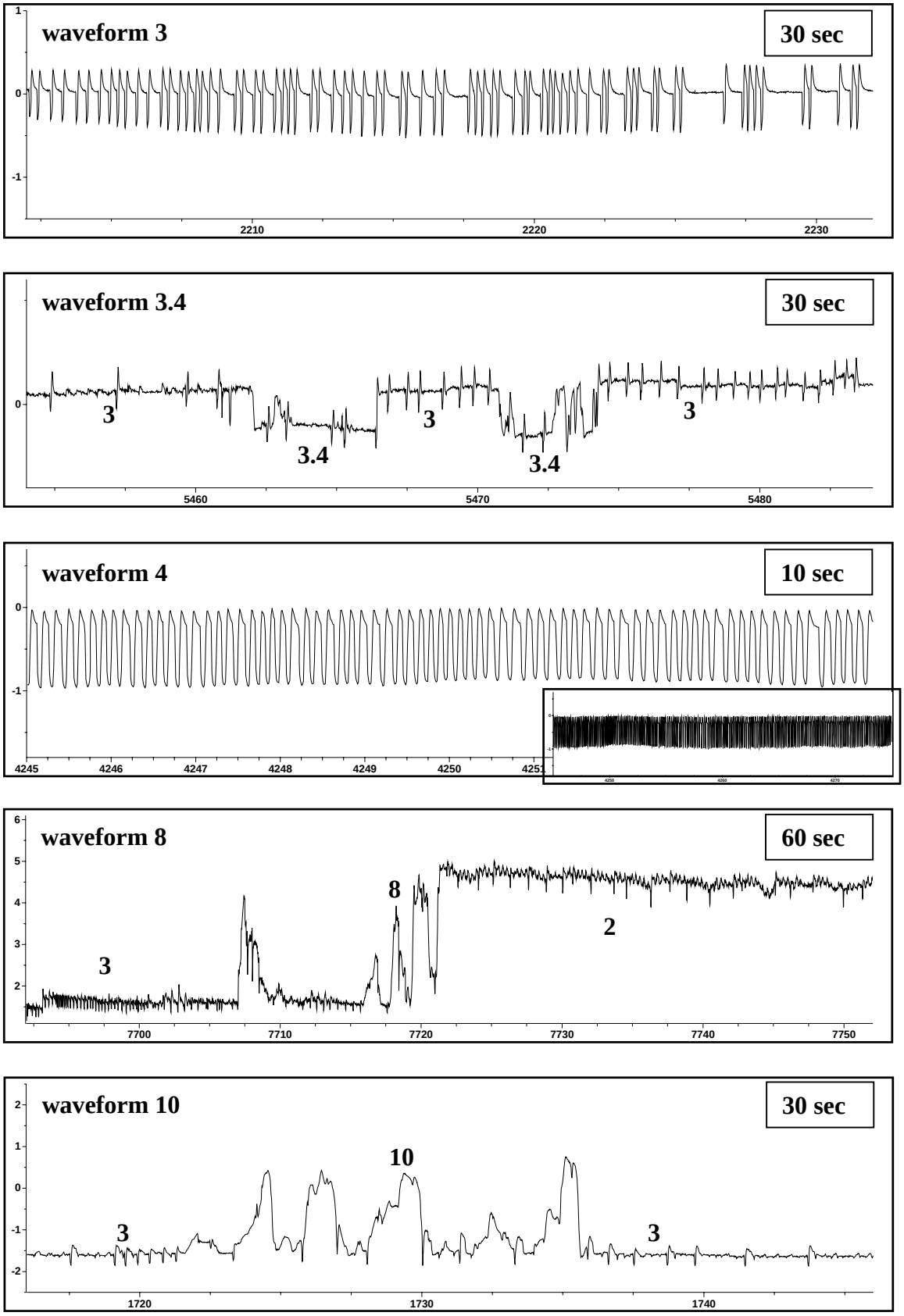


Figure 7. Visual representation of grape phylloxera biphasic and high voltage EPG waveforms, timeframe and waveform transitions indicated. Waveform 4 insert is the pattern at a 30 second timeframe.

3.3 Continual Feeding Analysis

EPG recordings were analysed for first instar, intermediate and adult life stages of grape phylloxera where continual feeding waveforms were observed (n = 7). To correct for variation in the total recording time between recordings and life stages (Table 5), waveforms between life stages were compared by the parameters of mean duration time and the percentage of total recording time spent within each waveform.

ANOVA was only possible on waveforms that occurred in the majority of recordings, being waveforms 2, 3 and 4. Waveform 2 indicated a 90% significant decrease in the percentage of total recording time spent in the waveform from first instars to the adult life stage (Table 5). There were no significant differences between life stages for waveform 3. Waveform 4 occurred for significantly less mean time in the first instar life stage in comparison with adults, and all life stages were significantly different when comparing percentage of total recording time. The average percentage of total recording time for each waveform is presented by life stage in Figures 8a-8c. The first instar life stage distribution was dominated by waveform 2 (72%) and waveform 3 (22%). Intermediates had the highest diversity of waveforms observed (10 waveforms), and the adult life stage had the most even distribution with 4/6 waveforms occurring for >15% of the total recording time.

Within life stage variation for waveform distribution as a percentage of total recording time was greatest for adult grape phylloxera (Figure 9c). The first instar life stage was dominated by waveforms 2 and 3 across all replicates (Figure 9a), intermediates by waveforms 2, 3 and 4 (Figure 9b), and adults by a complex mix of waveforms 2, 3, 4 and 5 (Figure 9c).

Table 5. Grape phylloxera life stage comparison of waveform parameters for continual feeding EPG recordings on susceptible excised root material. Parameters presented (total duration time, frequency, mean duration time and percentage of total recording time), are the mean ± standard deviation. ANOVA presented in **bold** for 'waveform x life stage' comparisons of mean duration time and percentage of time available.

wave form	life stage	total time (sec)	frequency	mean t (sec)	% recording time
2	first instar	9012 ± 4549	77 ± 46	133 ± 68	75 ± 13[a]
	intermediate	9186 ± 4041	68 ± 31	155 ± 53	59 ± 10[ab]
	adult	4055 ± 3923	38 ± 27	109 ± 71	48 ± 26[b]
	ANOVA p			**ns**	**0.063**
2.2	first instar	27 ± 0	2 ± 0	14 ± 0	0.36 ± 0
	intermediate	59 ± 34	4 ± 3.5	19 ± 13	0.33 ± 0.2
	adult	not observed	not observed	not observed	not observed
2.4	first instar	13 ± 0	2 ± 0	6.5 ± 0	0.084 ± 0
	intermediate	6 ± 0	3 ± 0	2.1 ± 0	0.034 ± 0
	adult	not observed	not observed	not observed	not observed
3	first instar	2925 ± 2338	82 ± 56	34 ± 11	23 ± 11
	intermediate	3971 ± 1886	82 ±36	53 ± 19	28 ± 6.7
	adult	2060 ± 2076	44 ± 38	45 ± 48	21 ± 17
	ANOVA p			**ns**	**ns**
3.4	first instar	13 ± 13	2 ± 1.4	5.3 ± 3.0	0.16 ± 0.20
	intermediate	not observed	not observed	not observed	not observed
	adult	not observed	not observed	not observed	not observed
4	first instar	154 ± 230	13 ± 20	11 ± 2.6[a]	0.93 ± 1.3[a]
	intermediate	1177 ± 972	22 ± 18	55 ± 29[ab]	10 ± 8.7[b]
	adult	2005 ± 1889	25 ± 25	109 ± 84[b]	21 ± 12[c]
	ANOVA p			**0.036**	**0.001**
5	first instar	not observed	not observed	not observed	not observed
	intermediate	2012 ± 0	22 ± 0	91 ± 0	11 ± 0
	adult	1399 ± 1598	13 ± 6.9	84 ± 82	29 ± 33
6	first instar	not observed	not observed	not observed	not observed
	intermediate	207 ± 199	1 ± 0	207 ± 199	1.2 ± 1.1
	adult	not observed	not observed	not observed	not observed
7	first instar	629 ± 578	2 ± 1.4	556 ± 682	4.5 ±2.1
	intermediate	737 ± 0	2 ± 0	369 ± 0	4.1 ± 0
	adult	26 ± 0	1 ± 0	26 ± 0	0.36 ± 0
8	first instar	not observed	not observed	not observed	not observed
	intermediate	59 ± 70	3 ± 1.4	16 ± 16	0.33 ± 0.39
	adult	not observed	not observed	not observed	not observed
10	first instar	14 ± 0	2 ± 0	7.2 ± 0	0.19 ± 0
	intermediate	37 ± 35	3.3 ± 2.1	13 ± 8.9	0.26 ± 0.27
	adult	169 ± 0	14 ± 0	12 ± 0	2.4 ± 0

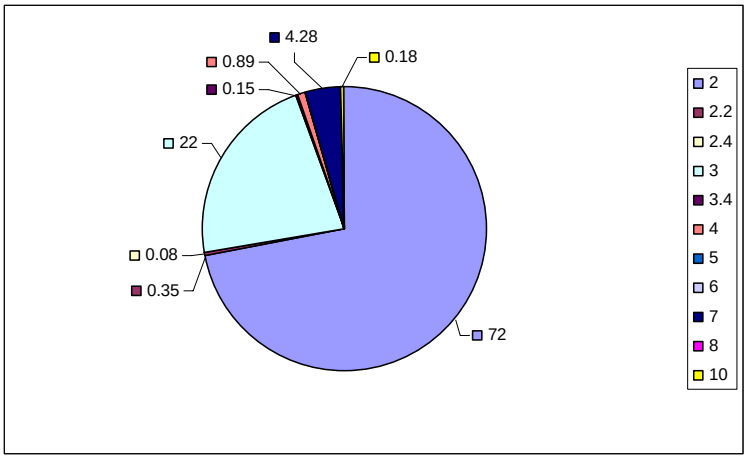


Figure 8a. First instar grape phylloxera average waveform distribution as a percentage of total recording time, n=7. The colour-coded key indicates individual waveforms. Combined replicate averages have been corrected to equal 100%.

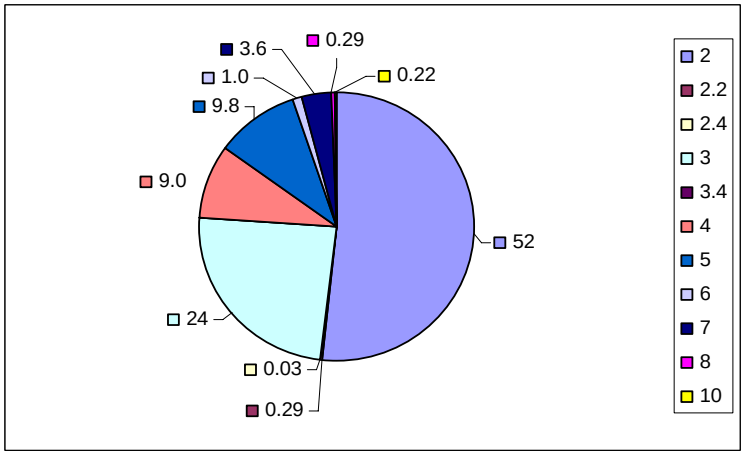


Figure 8b. Intermediate grape phylloxera average waveform distribution as a percentage of total recording time, n=7. The colour-coded key indicates individual waveforms. Combined replicate averages have been corrected to equal 100%.

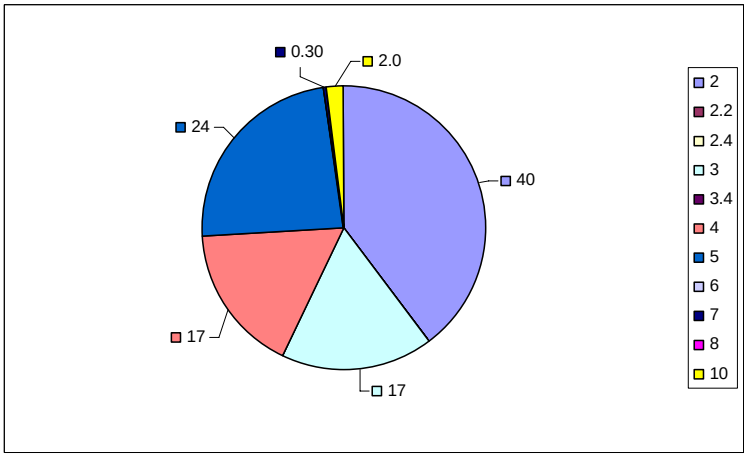


Figure 8c. Adult grape phylloxera average waveform distribution as a percentage of total recording time, n=7. The colour-coded key indicates individual waveforms. Combined replicate averages have been corrected to equal 100%.

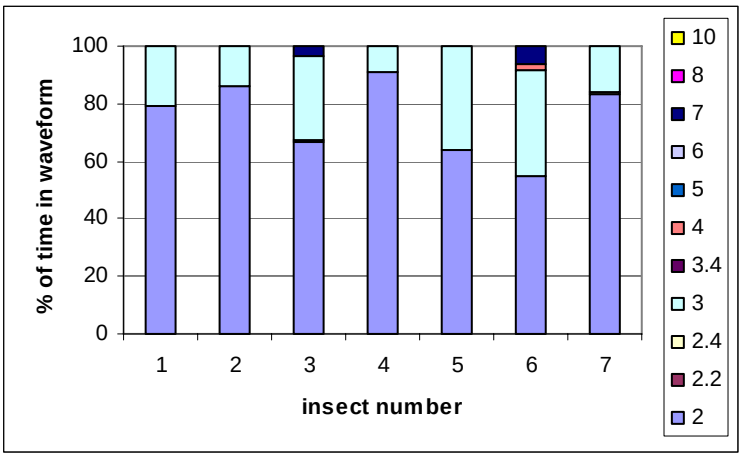


Figure 9a. First instar grape phylloxera waveform distribution as a percentage of total recording time for the seven individual insects used for continual feeding analysis. Individual waveforms indicated by the colour-coded key.

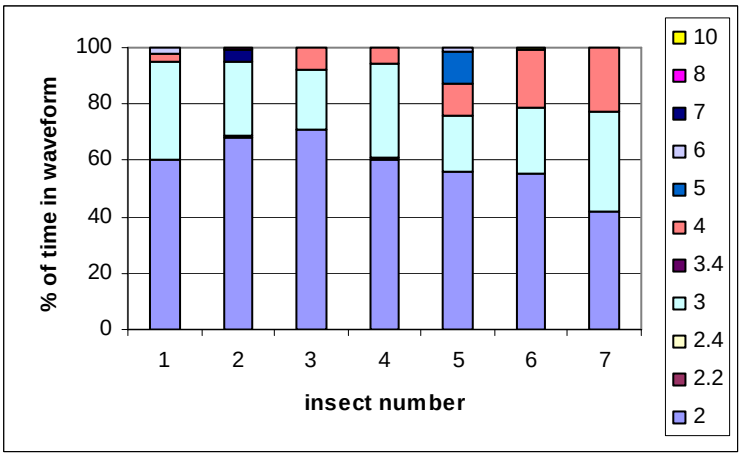


Figure 9b. Intermediate grape phylloxera waveform distribution as a percentage of total recording time for the seven individual insects used for continual feeding analysis. Individual waveforms indicated by the colour-coded key.

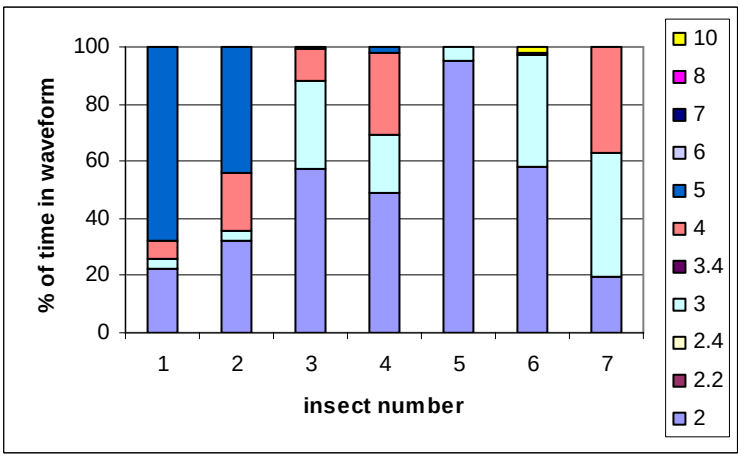


Figure 9c. Adult grape phylloxera waveform distribution as a percentage of total recording time for the seven individual insects used for continual feeding analysis. Individual waveforms indicated by the colour-coded key.

3.4 Probe Recordings

Grape phylloxera probe initiation, or plant penetration, EPG recordings were observed to have three re-occurring waveforms that were not observed in the continual feeding recordings (Table 4). Waveform 1 represented non-penetration, and the EPG pattern displayed a non-responsive flat line indicating that there was no electrical contact in the insect and plant biological system. Waveform 9 was a high voltage pattern observed as a transition between waveform 1 and the continual feeding waveforms shown in Table 3. Waveform 9 represented electrical contact with the plant and penetration of the stylet into a food source. At the end of the continual feeding patterns the EPG pattern would return to waveform 1, indicating that there was no longer electrical contact between the insect and the plant, and that the insect had removed the stylet from the food source. Waveform 9.1 was similar in structure to waveform 9, and occurred prior to the continual feeding patterns. Waveform 9.1 also occurred during continual feeding patterns without the EPG pattern returning to waveform 1, suggesting that waveform 9.1 represented pre-probing and stylet re-positioning activity.

Three grape phylloxera EPG recordings involving probing patterns have been analysed (Table 6). Although 22 recordings displayed potential probing patterns, further analysis was not possible due to electronically induced background noise in the system and the complexity of the patterns. The three analysed recordings were all performed on susceptible (*V. vinifera* cv. Sultana) excised root material; two insects were adults and one an intermediate life stage. In each of the recordings, there were several probe waveform – continual feeding waveform transition cycles. Waveforms 2 and 3 were the only continual feeding patterns observed in all of these recordings. Waveforms 2.4 and 4 were observed in two of the three recordings, and waveforms 2.2, 6, 7 and 10 were only observed in a single recording. Waveforms 2.2, 7 and 10 were only observed in the intermediate life stage recording. Continual feeding waveforms 3.4, 5 and 8 were not observed in any of the probe recordings, and the percent amplitude values for these waveforms (Table 3) could not be calculated.

Multiple probing attempts, involving waveforms 9.1 and 9, were made before insects settled into a regular feeding pattern (Figure 10). Waveforms 2 and 4 were both observed immediately following waveform 9, although extended periods of feeding activity were only observed when waveform 2 was the first continual feeding pattern. The waveform distribution varied for each of the probe initiation recordings (Figure 11), although all insects were observed to settle into feeding activity for between 20-80% of the total recording time.

Table 6. Comparison of waveform parameters of grape phylloxera probe initiation EPG recordings from the susceptible excised root plant system. Sample number n = 1, absolute parameters (total duration time, frequency, and percentage of total recording time) are presented as totals; mean parameters (mean duration time) are the mean ± standard deviation.

wave form	resistant rootstock	total time (sec)	frequency	mean t (sec)	% recording time
1	adult	8427	15	562 ± 1286	78
	adult	456	9	51 ± 60	13
	intermediate	3092	35	88 ± 387	43
2	adult	1165	19	61 ± 80	11
	adult	1696	11	154 ± 177	50
	intermediate	3505	92	38 ± 68	49
2.2	adult	not observed	not observed	not observed	not observed
	adult	not observed	not observed	not observed	not observed
	intermediate	286	35	8.2 ± 7.2	4.0
2.4	adult	12	5	2.4 ± 0.96	0.11
	adult	not observed	not observed	not observed	not observed
	intermediate	18	5	3.6 ± 2.4	0.25
3	adult	47	8	5.8 ± 2.9	0.44
	adult	390	14	28 ± 38	11
	intermediate	32	3	11 ± 2.3	0.45
4	adult	1114	14	80 ± 155	10
	adult	704	9	78 ± 130	21
	intermediate	not observed	not observed	not observed	not observed
6	adult	not observed	not observed	not observed	not observed
	adult	47	1	47 ± 0	1.4
	intermediate	not observed	not observed	not observed	not observed
7	adult	not observed	not observed	not observed	not observed
	adult	not observed	not observed	not observed	not observed
	intermediate	54	1	54 ± 0	0.76
9	adult	15	5	3.0 ± 2.9	0.14
	adult	8.9	3	3.0 ± 2.8	0.26
	intermediate	46	26	1.8 ± 1.5	0.64
9.1	adult	20	13	1.5 ± 1.1	0.19
	adult	113	7	16 ± 34	3.3
	intermediate	93	22	4.2 ± 4.0	1.29
10	adult	not observed	not observed	not observed	not observed
	adult	not observed	not observed	not observed	not observed
	intermediate	73	16	4.5 ± 3.4	1.0

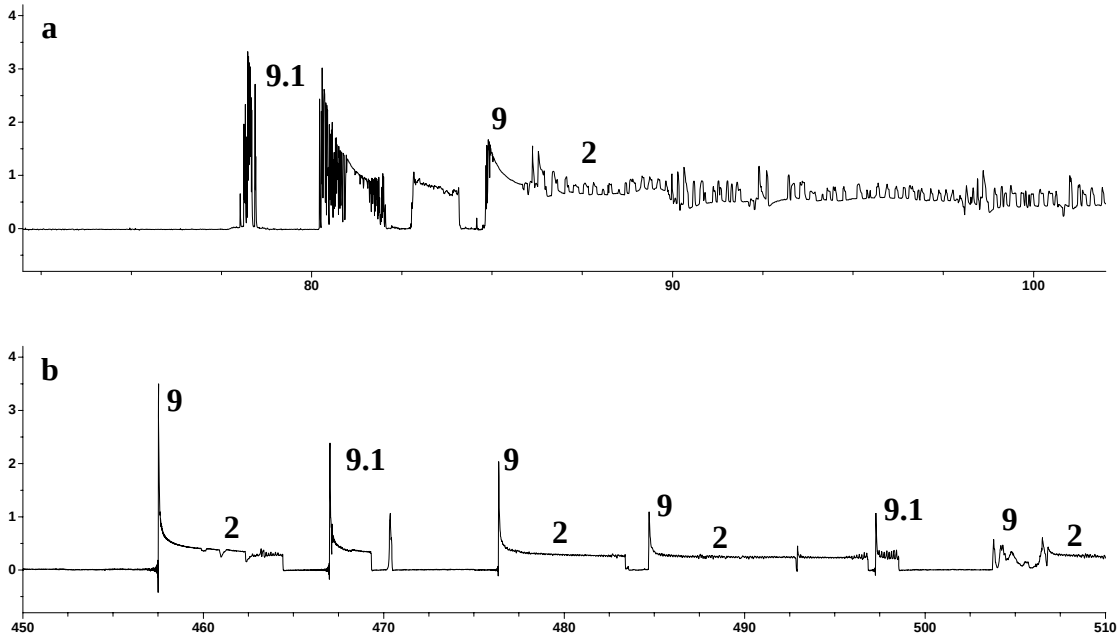


Figure 10. Grape phylloxera probe initiation EPG patterns on susceptible excised roots; a) 30-minute overview showing waveform 9.1 followed by waveform 9 and continual feeding pattern waveform 2, b) 60-second overview showing multiple occurrences of waveform 9.1 and waveform 9 with brief continual feeding patterns, before settling into an extended period of continual waveform 2. Waveform transitions indicated.

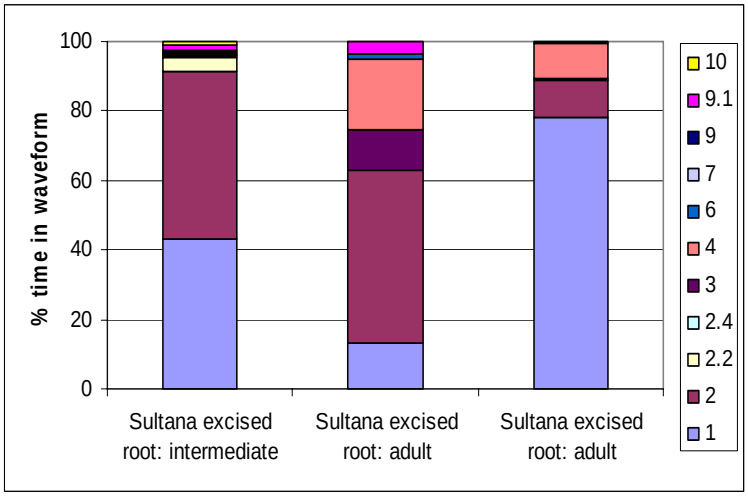


Figure 11. Grape phylloxera probe initiation EPG recordings on susceptible excised roots, waveform distribution as a percentage of total recording time. Individual waveforms indicated by the colour-coded key.

3.5 Rootstock Recordings

For proof-of-concept analysis, 11 EPG recordings were attempted on the root material of resistant *Vitis* species (Table 1). Three of these recordings were successful, however because of differing life stages, root types and EPG plant systems (first instar on Börner excised root, intermediate instar on Ramsey excised root, and adult on Börner tissue culture), there were no replicated recordings. Due to the low sample number, no conclusions could be made regarding grape phylloxera feeding behaviour on resistant roots in comparison with susceptible root material. However these recordings have still been analysed for the initial interpretation of feeding behaviour.

The most common continual feeding patterns, waveforms 2 and 3, were observed in the intermediate instar-Ramsey excised root and adult-Börner tissue culture plant-insect combinations (Table 7). Waveforms 8 and 10 also occurred in both recordings. Waveform 4 was only observed in the intermediate-Ramsey recording, while an undefined waveform 7 was identified in the adult-Börner recording. Both of these recordings displayed 'percent of total recording time in waveform' values similar to susceptible root material (Figure 12).

The first instar-Börner excised root EPG recording was dominated by waveforms 1 and 10 (Figure 12). This variation of the high amplitude waveform 10 appeared visually similar to the probe waveform 9.1 (Figure 13a). Waveform 7 represented a possible feeding attempt (Figure 13b) which occurred for less than 1% of the total recording time. The high voltage patterns observed in the first instar-Börner excised root recording are in contrast to the continual feeding patterns observed in the adult-Börner tissue culture recording (Figure 13c).

Table 7. Comparison of waveform parameters of grape phylloxera resistant rootstock EPG recordings from the excised root and tissue culture plant systems. Root type and plant system combinations (n = 1), absolute parameters (total duration time, frequency, and percentage of total recording time) are presented as totals; mean parameters (mean duration time) are the mean ± standard deviation. Only one life stage presented per plant system; Börner excised root: first instar, Ramsey excised root: intermediate, Börner tissue culture: adult.

wave form	resistant rootstock	total time (sec)	frequency	mean t (sec)	% recording time
1	Börner excised root	8400	115	73 ± 139	47
	Ramsey excised root	not observed	not observed	not observed	not observed
	Börner tissue culture	not observed	not observed	not observed	not observed
2	Börner excised root	not observed	not observed	not observed	not observed
	Ramsey excised root	11939	68	176 ± 188	67
	Börner tissue culture	2649	28	95 ± 243	75
3	Börner excised root	not observed	not observed	not observed	not observed
	Ramsey excised root	4535	84	54 ± 149	26
	Börner tissue culture	428	31	14 ± 32	12
4	Börner excised root	not observed	not observed	not observed	not observed
	Ramsey excised root	1208	20	60 ± 82	6.8
	Börner tissue culture	not observed	not observed	not observed	not observed
7	Börner excised root	74	6	12 ± 7.4	0.41
	Ramsey excised root	not observed	not observed	not observed	not observed
	Börner tissue culture	91	6	15 ± 8.4	2.6
8	Börner excised root	not observed	not observed	not observed	not observed
	Ramsey excised root	12	1	12 ± 0	0.068
	Börner tissue culture	11	2	5.4 ± 2.3	0.31
10	Börner excised root	9522	121	79 ± 115	53
	Ramsey excised root	36	2	18 ± 12	0.20
	Börner tissue culture	345	21	16 ± 21	9.8

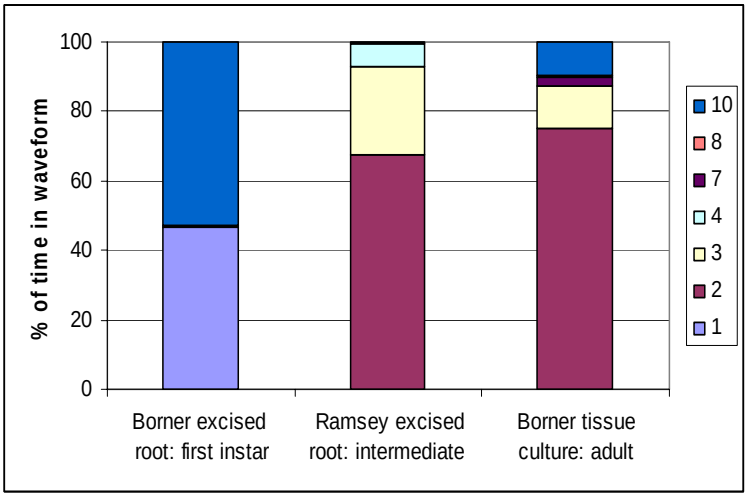


Figure 12. Grape phylloxera resistant rootstock EPG recordings waveform distribution as a percentage of total recording time. Individual waveforms indicated by the colour-coded key.

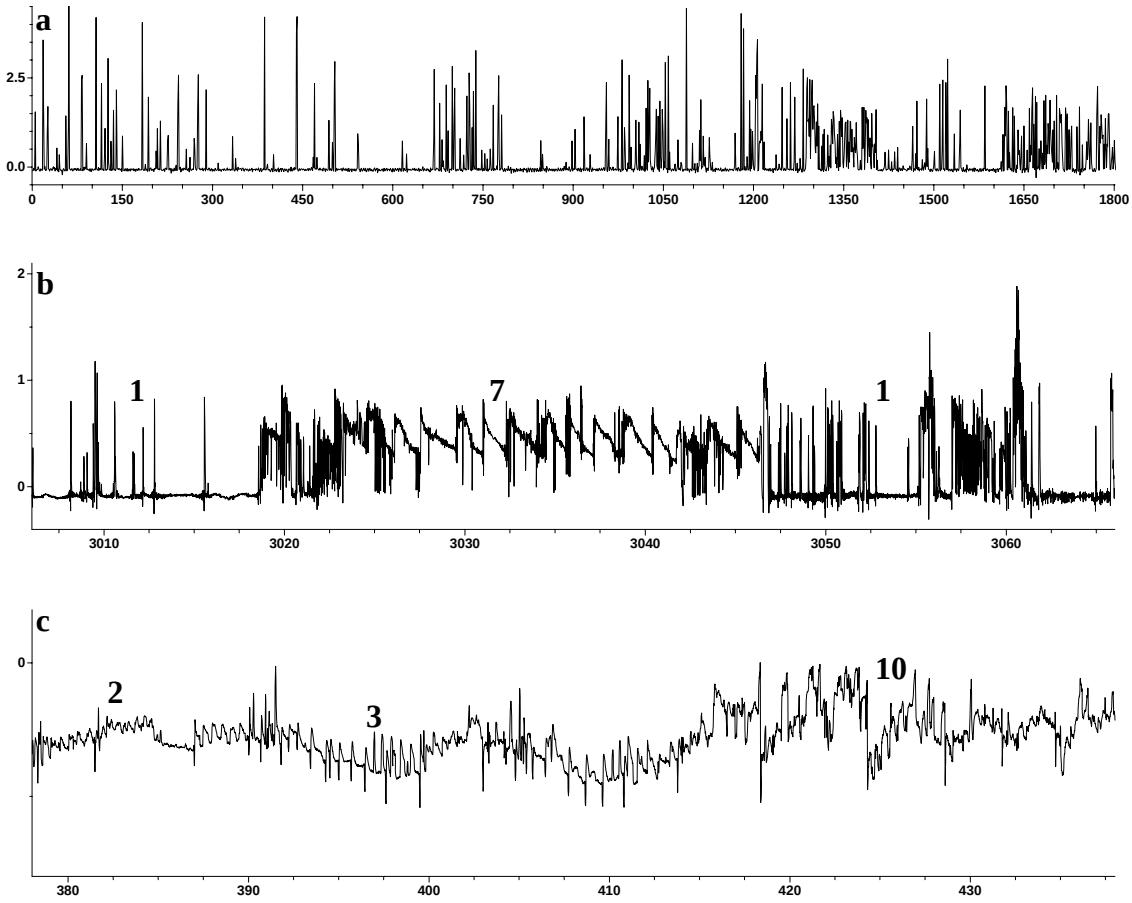


Figure 13. Grape phylloxera EPG patterns on Börner rootstocks; a) 30-minute overview of a first instar recording on Börner excised root, b) 60-second overview of a first instar possible feeding attempt on Börner excised root, and c) 60-second overview of an adult feeding pattern on Börner tissue culture. Waveform transitions indicated.

4 DISCUSSION

EPG recordings were successfully performed on all feeding life stages, represented as first instar, intermediate instars and adult, of radicicolae grape phylloxera. EPG recordings of grape phylloxera have not previously been documented. Data acquisition was achieved using both the excised root and tissue culture recording systems, and with insects in both the active feeding and probe initiation positions. These results validate the EPG technique as an application suitable for grape phylloxera feeding behaviour studies, overcoming several anticipated difficulties highlighted earlier.

Grape Phylloxera Constraints

Radicicolae grape phylloxera are relatively small insects; adults measure ~900 μm in length, and first instars ~300 μm (Davidson and Nougaret, 1921). However insect life stage (and size) did not impact on EPG recording success rate. It is important to obtain EPG recordings from life stages of grape phylloxera that display biologically different associations with the grapevine food source. First instars are important for insect distribution and establishment, being active along the grapevine root surface before becoming sessile feeders once a feeding site is established. Feeding by first instars initiates gall development, which acts as a nutrient sink, and sustains the development of the other life stages. The adult life stage does not move during the course of parthenogenetic egg development (De Klerk, 1974), limiting the available nutrition to the current feeding site.

The root feeding habit of grape phylloxera presented challenges in the set up of the plant electrode system. Potted plants would be the optimal set up as they provide a whole plant response in a natural soil environment, however side-roots and bare soil cause the EPG signal to short-circuit in contact with the gold wire of the insect electrode (Cole *et al.*, 1993). Although limitations have been reported to occur when using an excised plant system in place of a whole, intact plant (Van Helden and Tjallingii, 2000), the excised root and tissue culture plant electrode systems were the preferred options for grape phylloxera. The use of excised roots and tissue culture systems for grape phylloxera laboratory studies has previously been validated in a number of population dynamics and rootstock screening experiments (Granett *et al.*, 1985; Forneck *et al.*, 1996; Kellow *et al.*, 2002). Both plant recording systems obtained successful data acquisition, and sufficient EPG signal strength for waveform analysis.

Grape phylloxera are sessile feeders, resulting in uncertainty that insects would re-establish feeding after being disturbed from their original food source. The success rate (and sample number) for insects in the probe initiation position was reduced in comparison with insects maintained in an active feeding position, however successful data acquisition was obtained. EPG recordings of the complete insect feeding behaviour, acquired from the probe initiation position, are important for the interpretation of grape phylloxera-grapevine interactions. Probe initiation EPG recordings are required to understand the processes of site selection and feeding establishment, therefore the validation of this insect EPG recording position is an important advancement for the future application of EPG technology to investigating grape phylloxera feeding behaviour.

Intermediate and adult life stages analysed for probing patterns indicated repeated probing attempts prior to settling on a feeding site. These recordings also confirmed that intermediate and adult life stages could re-establish feeding if disturbed from an

established location. It is generally reported that first instars initiate grape phylloxera feeding sites and grapevine galling, but confirming that later life stages are also able to establish new feeding locations increases the risk of these life stages posing a contamination risk for non-infested vineyards.

Insect and Plant Electrode Comparisons

The aim of this project was to apply the EPG technique to the grape phylloxera-grapevine model. Multiple recording systems were trialled, and comparisons are able to be made between the active feeding and probe initiation insect positions on the excised root plant system, and between the excised root and tissue culture plant system with insects in the probe initiation position.

The excised root plant system was more successful in obtaining useable EPG data acquisition than the tissue culture plant system. However this direct comparison is misleading as all insects used for the tissue culture recordings were in the probe initiation position. By comparing probe initiation recordings, there are similar success rates between the excised root and the tissue culture plant system, therefore validating both procedures for application to the grape phylloxera-grapevine model.

On the excised root plant system, the active feeding recording position obtained a higher percentage of successful data acquisitions than the probe initiation position. Insect tethering to the gold wire of the insect electrode is known to influence insect feeding behaviour, especially impacting upon the insects' ability to walk over the plant surface (Van Helden and Tjallingii, 2000). Insects in the active feeding position typically did not move from the feeding site during EPG recording, and were therefore less impeded by attachment to the insect electrode. Although grape phylloxera in the probe initiation position were observed to walk along the root surface, the impact of tethering to the insect electrode may have had an impact on the success rate of these EPG recordings.

Grape Phylloxera Waveforms

The waveforms presented in Table 3 appear visually similar to waveforms that have been correlated to biological activity for the phloem-leaf feeding Aphididae (Reese *et al.*, 2000). However EPG waveforms generated from grape phylloxera represent data based on a different insect family (Phylloxeridae), with a putative different primary food source (parenchyma cell contents) and at a different feeding location (roots). In a review written by Van Helden and Tjallingii (2000), they stress that correlation studies must be completed on each new taxon group studied in order to be able to assign biological meaning to the EPG data. Previous research involving *Phylloxera coccinea* Heyden (Hemiptera: Phylloxeridae) does present similar EPG waveforms from a taxon closely related to grape phylloxera (Harrewijn *et al.*, 1998), however no correlation studies were performed in the study to apply definition of biological meaning to the current EPG data.

Correlation studies relate insect activity (penetration, stylet pathway, ingestion, and oviposition) to the EPG waveforms using a number of established techniques (Walker, 2000). Many of these correlation techniques may not be applicable to grape phylloxera, and this area requires further investigation before correlation studies can commence. Therefore all waveform analysis presented in this report are based on visual characterisation and not biological function.

Waveform 3 appears visually similar to waveform 4, but occurs at a reduced repetition rate. These waveforms occurred in sequence and may be involved in the same biological function, with waveform 4 occurring at a more rapid rate. Both waveforms were recorded when the EPG amplifier was adjusted to near 0V, where waveforms resulting from insect muscle contractions and fluid streaming potentials are detected (Harrewijn *et al.*, 1998). Potentially, a change in the rate of insect ingestion could result in an increase in the frequency of either of these factors, leading to the increased repetition rate observed in waveform 4.

The differential occurrence of the 11 continual feeding waveforms suggests that there may be variable feeding requirements for different grape phylloxera life stages. There were significant differences between life stages in the percentage of total time spent in waveforms 2 and 4. The potential ability of EPG waveforms to differentiate between the feeding behaviour of life stages highlights the value of the EPG technique for studying grape phylloxera-grapevine interactions.

The high voltage probe waveform 9 represented the initial electrical contact between in the insect-plant biological system following the non-penetration pattern (waveform 1). The repeated occurrence of waveform 9.1 suggested that the insect was involved in pre-probing and stylet re-positioning activity. Waveform 9.1 may represent the intracellular stylet pathway to the feeding cell within the grapevine root. Pollard (1973) displayed the pathway of the grape phylloxera stylet as the continual puncturing of cells (and their walls) until the insect reached the parenchyma cell feeding site. The occurrence of waveform 9.1 within continual feeding waveforms suggests that a biological activity similar to the puncturing of cell walls during stylet pathway, with the occasionally short feeding attempt, may be the cause for this waveform.

Resistant Rootstock Recordings

Grape phylloxera EPG recordings on resistant roots indicated continual feeding patterns on Ramsey and Börner root material. Börner is reported as being immune to grape phylloxera feeding (Schmid and Ruhl, 2003), and the first instar insect displayed only high voltage, probing-like activity with very limited evidence of feeding waveforms on excised root material. However the adult insect on the tissue culture root displayed feeding patterns. This establishment may have been assisted by the fleshier, softer roots of the tissue culture vine compared with the lignified excised root, and by life stage. Resistant rootstock trials (Powell *et al.*, 2006) involve placing grape phylloxera eggs onto the root material of grapevines, which then hatch into first instars. If the first instars do not successfully establish (as is the case with Börner), then the survival of adult insects is not tested. The EPG results presented here highlight the possibility that non-first instar grape phylloxera insects may be capable of establishing feeding sites on the 'immune' rootstock Börner.

As implied by EPG analysis, resistant rootstock trials indicate that grape phylloxera do establish feeding sites on Ramsey root material. Insect development was capable of reaching the adult life stage with the production of fertile eggs. Differential insect population numbers were observed for different grape phylloxera genotypic classes on Ramsey (Powell *et al.*, 2006). The G1 genotypic class generally out performed the G4 genotypic class used in this study. Only one genotypic class of grape phylloxera was used for these EPG experiments to limit the biological variation displayed in the EPG patterns. Future EPG studies comparing feeding behaviour of different grape phylloxera genotypic

classes may provide insight into the observed differential survival rates of grape phylloxera genotypic classes in resistant rootstock trials.

5 CONCLUSION

Grape phylloxera-grapevine interaction studies have been successfully completed, and reported for the first time, with the application of the EPG technique. Continual feeding and probing EPG waveforms have been identified, with grape phylloxera life stage able to be differentiated by waveform occurrence within an EPG recording. Grape phylloxera successfully re-established feeding after being disturbed from an established feeding site, highlighting the potential quarantine risk of intermediate and adult life stages, along with the already recognised first instar 'crawlers'. The potential of EPG to improve knowledge of grape phylloxera-resistant rootstock interactions is highlighted by the differential feeding behaviour the first instar and the adult insect on Börner root material. Current resistant rootstock studies rely upon the establishment of the first instar life stage in order to determine grape phylloxera survival rates. In future resistant rootstock trial, EPG could be used to determine the feeding potential of all life stages on the resistant root material, removing the reliance upon the initial egg hatching and first instar survival rates. Once a feeding site has been established on resistant rootstocks by a later life stage, earlier life stages may experience higher establishment rates and feeding success, leading to a grape phylloxera population becoming established on the resistant rootstock food source.

6 RECOMMENDATIONS

EPG analysis can be used to identify the mechanical and chemical resistance response mechanisms displayed by plants during pest-insect feeding (Van Helden and Tjallingii, 2000). Resistant rootstocks are currently the only long-term management option for the viticulture industry, and knowledge of the feeding behaviour of grape phylloxera on rootstock varieties will improve our comprehension of resistance mechanisms. Grape phylloxera genetic variation is also a factor affecting rootstock resistance that can be addressed with the application of the EPG technique.

This study has shown that the EPG technique can be applied to radicicolae grape phylloxera to investigate feeding behaviour characteristics on both susceptible and resistant grapevine varieties. Fourteen waveforms were characterised for grape phylloxera feeding on susceptible *Vitis vinifera.* Further studies are now required to correlate these waveforms with biological activity of the insect and its interaction with the host-plant. Once these correlation studies on susceptible grapevines have been completed, the EPG system offers the potential to develop resistance ratings for rootstocks based on active feeding behaviour and establishment ability. Potentially this would increase the processing time of resistant rootstock screening, particularly where a diverse range of both rootstocks and grape phylloxera genotypic classes need to be reviewed.

In particular, future EPG studies should compare of the feeding behaviour of grape phylloxera genotypic classes such as G1, G4 and G20 to provide insight into why survival and development rates of these genotypes differ so markedly on susceptible and resistant host-plants. Other factors that could influence the feeding behaviour of grape phylloxera

genotypic classes, such as humidity and temperature, could potentially be examined using a modified EPG procedure.

As evidenced from the limited studies with Börner presented in this report, future studies on resistant rootstocks should consider screening both first instars and more mature life stages to examine the risk of grape phylloxera establishment.

Although not used in this study, the EPG system could be linked to artificial diet systems (Sub-project 1.1) to examine what influence nutritional or anti-metabolic components within a artificial diet formulation have on grape phylloxera feeding behaviour.

In addition to grape phylloxera, EPG system could be adapted to examine the feeding behaviour and viral transmission/acquisition interactions of other pests of grapevine, such as leafhoppers, thrips and mites.

SUB-PROJECT 2: CHARACTERISATION OF DIGESTIVE AND SALIVARY SYSTEM OF GRAPE PHYLLOXERA USING MORPHOLOGICAL, BIOCHEMICAL AND GENETIC APPROACHES

SUB-PROJECT 2.1. A REVIEW OF THE DIGESTIVE SYSTEM OF GRAPE PHYLLOXERA

SUB-PROJECT 2.2. INVESTIGATING BACTERIAL SYMBIOTIC RELATIONSHIPS IN GRAPE PHYLLOXERA

SUB-PROJECT 2.1

A REVIEW OF THE DIGESTIVE SYSTEM OF GRAPE PHYLLOXERA

1 INTRODUCTION

To fully understand the nutritional interactions between grape phylloxera and its *Vitis* species host-plant, we need to understand not only the nutritional requirements of the insect (Sub-project 1.2), but also its internal and external morphology, and how this impacts on biology.

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, Hemiptera: Phylloxeridae), has been a significant pest of the viticulture industry since the late 1800's. Despite being the focus of research for over 100 years, there are still a number of uncertainties pertaining to the biology of the insect. Understanding the biology of grape phylloxera is fundamental to developing a sustainable approach to managing the pest insect. Grape phylloxera is closely related to the Aphididae and is often described as an "aphid-like" insect. However the two families differ in a number of feeding and digestive characteristics, and the terminology "aphid-like" has resulted in a number of false assumptions regarding the biology of grape phylloxera.

Feeding Site and Dietary Requirements

Grape phylloxera feeding induces the development of galls on the leaves and roots of grapevines. Gall development is associated with a localised increase in sugar, protein and lipids (Gullan and Cranston, 2005). Grape phylloxera is reported to feed on the cell contents of non-vascular, or parenchyma, tissue via intracellular penetration (Petri, as cited in Ponsen, 1997). This diet contains less fluid and less carbohydrates than phloem sap, the main diet of the Aphididae; however the high level of complex proteins provides continual nutrition for the sessile feeding grape phylloxera (Ponsen, 1997). Sectioning of grape phylloxera induced galls, or nodosities, have tracked the stylet of the insect on a path through the cortex, with the tip of the stylet settling within a single parenchyma cell around five cells below the epidermis (Kellow *et al.*, 2004). In contrast with Aphididae, the phloem was never penetrated, and cells surrounding the feed cell appeared not to be damaged. However, the level of resolution obtained from Kellow's study was unable to determine if the stylet tip was positioned within the cytoplasm or the vacuole of the feeding cell. This lack of detail leaves definition of grape phylloxera dietary intake and feeding site unresolved.

Digestive System Characteristics

The digestive system of grape phylloxera has previously been investigated, with most researchers concluding that the insect does not have a complete digestive system, and therefore lacks the capacity for anal waste excretion. Evidence supporting the lack of anal excretion included the intestinal tract, or midgut, ending blindly (Federov, 1959), and the absence of an anal opening (Dreyfus, as cited in Ponsen 1987). Alternative proposed methods of waste excretion have included the use of modified salivary glands (Schaller,

1960; Sobetskiy and Derzhavina, 1973), waste excretion during oviposition (Rilling, as cited in Forneck, et al. 2001) and the use of extra-intestinal digestion removing the need for waste excretion (Federov, 1959). The absence of sugary honeydew production (Riley, 1870) is associated with the absence of dorsal anal muscles, and is cited as evidence for an incomplete digestive system (in Ponsen, 1997). However a more recent light microscopy study by Ponsen (1997) indicates the presence of an anal opening and a complete digestive system. Diagrams presented by Breider (1952) also support this conclusion, although through secondary referencing (Ponsen, 1997), Breider's research has been interpreted as evidence for a blind-ending midgut. Given this background, the structure of the grape phylloxera digestive system, and the method of waste excretion, remains unclear.

Reproductive Capacity

Grape phylloxera reproduce by parthenogenesis, and have a high reproductive capacity which results in elevated population levels of the insect in vineyards during the summer months. Adult grape phylloxera lay 3-6 eggs per day during a reproductive period of 1-2 months (Granett *et al.*, 1983). Following the established of a feeding site, the sessile feeders remain attached to the same feeding location during life stage development, and accommodate for the continual production of eggs by repositioning the posterior end during oviposition (De Klerk, 1974). The energy requirements for the continual egg production would be high, and the rate of food intake would need to equal the rate of energy and water loss with egg oviposition. The contradictions in the literature regarding the digestive morphology and feeding location of grape phylloxera therefore require reinvestigation to put the energy requirements of the high reproductive output into perspective.

Research Aims

This project reinvestigated the internal morphology of radicicolae grape phylloxera using both light and electron microscopy techniques to resolve some of the existing inconsistencies regarding the insects digestive system. The external morphology of radicicolae grape phylloxera was also investigated using scanning electron microscopy (SEM), to build upon and clarify existing knowledge regarding the feeding location and method(s) of waste excretion for the insect. Few publications exist on the external morphology of grape phylloxera, and these investigations were limited to light microscopy technology (Davidson and Nougaret, 1921; Buchanan, 1990). Prior to this report, electron microscopy investigations of grape phylloxera characteristics have not been presented.

2 MATERIAL AND METHODS

2.1 Insect Material

Radicicolae grape phylloxera were originally collected from infested vineyards in Victoria, Australia, and maintained at the Department of Primary Industries – Rutherglen Centre on excised grapevine (*Vitis vinifera* L.) root pieces. Excised root pieces (approximately 1 cm width x 10 cm length) were prepared using a protocol slightly modified from Granett, et al. (1985). Modifications were: (1) roots washed clean of attached soil with a soft brush under running water; (2) roots soaked for 5-minutes in Ridomil® Gold Plus systemic fungicide solution (2.3g/L); (3) roots triple rinsed with sterile distilled water prior to air drying; and (4) the size of the petri dish was increased to 15 cm, with the increase in area reducing the levels of condensation. Grape phylloxera populations were incubated in the dark at a constant temperature of 25 ±3°C.

Only apterous radicicolae grape phylloxera were used for this study. Developmental life stages were determined by comparative increases in size. The presence of nearby eggs indicated adult reproduction. If present, neighbouring moulted cuticles were counted to confirm the insect life stage. For collection, insects were handled using a moist sable-haired paintbrush to avoid damage.

2.2 External Morphology

Observations of the external body features of grape phylloxera were made using both light and electron microscopy techniques. Light microscopy enabled measurements to 10 μm degree of accuracy, while electron microscopy enabled measurements to 1 μm degree of accuracy. Comparison of external body measurements from live insects via light microscopy and fixed insects via electron microscopy assisted in validating the use of the electron microscopy technique, and to determine the level of artefacts introduced by preparative techniques. All life stages were observed.

2.2.1 Light Microscopy

Insects were collected from excised root pieces and placed onto moist filter paper in a 3.5 cm petri dish. Measurements (±10 μm) were immediately taken of the length, width and depth of each live insect using an Olympus BH2 microscope and a graduated eyepiece.

2.2.2 Scanning Electron Microscopy

Several preparation techniques were trialled for viewing grape phylloxera with scanning electron microscopy (SEM). SEM was performed in a Cambridge Instruments Stereoscan 360 SEM. The external morphology of the insects were examined using a 15kV electron beam, with images captured digitally.

Air Drying

Insects were collected from excised root pieces and stored in 100% ethanol. Insects were mounted onto a SEM stub with clear nail polish and air dried overnight, prior to 20 nm gold plate coating.

Critical Point Drying

Insects were collected from excised root pieces and fixed either with 2.5 % glutaraldehyde or 100% ethanol. Insects were post-fixed with 1% osmium tetroxide, placed in a porous vial and immersed in 100% ethanol overnight before being critical point dried using dry ice. Insects were mounted onto the SEM stub with double-sided tape and gold plated (20 nm).

Cold Stage

Insects were collected from excised root pieces into cryo-tubes, snap frozen in liquid nitrogen and stored at -80°C until required. Insects were fixed onto cold stage stubs with a mounting paste of 50:50 graphite and cryo-protectant, and then frozen in liquid nitrogen. Insect stubs were coated with 10 nm of gold and immediately transferred to the cold stage SEM. Measurements of external body features (±1 μm) were made using ImageJ public domain software (Rasband, 2005). Stylet and labium length were measured as an indicator of possible feeding locations within the root structure, and examination was made of the posterior end of the insect for evidence of an anal opening. Antennae length was also measured.

Sample numbers used for each life stage measurement and observation were variable due to the loss of insects during preparation. The small size of grape phylloxera and the speed of preparation required for cold stage SEM prevented the correct orientation of all insects prior to gold coating. Incorrect orientation prevented the measurement of all morphological features on some individuals. Some insects were physically damaged during preparation, and not used for morphological descriptions.

2.3 Egg Oviposition Duration

Adult grape phylloxera were observed *in situ* on the excised root piece to determine the time required to complete egg oviposition. Insect activity was viewed with a stereo microscope and JVC camera (TK-C1381), and simultaneously video captured with by a VHS recorder (LG-FC930W). Videotapes were reviewed to observe the time requirement to completely oviposit an egg, and the time period between egg oviposition. The beginning of egg oviposition was defined by the extension of the posterior end of the insect and visualisation of the egg surface. Egg oviposition was considered complete when the egg was fully emerged and detached from the posterior end of the insect.

2.4 Internal Morphology

Observations of the internal structure of the digestive system of grape phylloxera were made using both light and electron microscopy techniques. Longitudinal and transverse light microscopy thin sectioning provided an understanding of the structure of the midgut,

hindgut and reproductive organs of the insect. Cold stage SEM provided an *in situ* representation of these features. Fourth instar and adult life stages were used for both studies to compare changes as the insect developed into the reproductive life stage. Adult insects were defined by the internal presence of developing eggs.

2.4.1 Light Microscopy

Insects were collected from excised root pieces and rinsed with sterile distilled water. Samples were immersed in 2.5% glutaraldehyde solution and fixed either overnight at 4°C or for a few hours on ice to allow penetration of the insect body parts. The glutaraldehyde solution was removed and the insects rinsed several times with buffer solution. Insects were stored in 70% ethanol until required, then prepared for sectioning with a series of ethanol washes (1 x 95%, 2 x 100%) prior to embedding in LR White Resin. A series of ethanol:resin washes (2:1, 1:2, 100% resin) occurred on ice under vacuum for 30 minutes. Samples were set in a resin cap at 60°C under nitrogen gas overnight. Longitudinal and transverse sections (1 μm) were cut using a glass knife on a Reichert microtome (Austria OmU2). Sections were heat dried onto glass slides, stained with 0.05% toluidine blue solution, and mounted under a cover slip with Permount® solution.

Sections were viewed using an Olympus AX70 Provis microscope and digital images captured with a SPOT RT™ (Diagnostic Instruments) camera and software. Measurements (length and width) were made of the maximum dimensions of the insects external body, digestive system (midgut and hindgut) and developing eggs (adults only). Light microscopy measurements (±10 μm) were made using an Olympus BH2 microscope and a graduated eyepiece. Area measurements were made from the digital images using ImageJ public domain software (Rasband, 2005).

2.4.2 Scanning Electron Microscopy

Insects were collected from excised root pieces into cryo-tubes, snap frozen in liquid nitrogen and stored at -80°C until required. Insects (7 fourth instars, 8 adults) were fixed onto cold stage stubs with a mounting paste of 50:50 graphite and cryo-protectant, and re-frozen in liquid nitrogen. Individual insects were fractured using a triangular glass knife cryomicrotome (Reichert FC4) prior to placing the stub into the cold stage SEM. The SEM was held at -196°C using liquid nitrogen. The specimens were initially etched with the electron beam to improve visualisation, prior to coating with ~20 nm of gold. Measurements (length, width and area) were made using ImageJ public domain software (Rasband, 2005).

2.5 Statical Analysis

GenStat-Eighth Edition© (Lawes Agricultural Trust) was used to calculate the mean and standard deviation of all measurements. Statistical differences in external body measurements from both light and electron microscopy techniques were determined by ANOVA. The unbalanced sample number for SEM external body measurements was resolved by the inclusion of missing values to ensure a standard sample number (n = 7).

3 RESULTS

3.1 External Morphology

Video observation of the grape phylloxera oviposition process determined that the time required for a developed egg to pass through the gonopore, and separate from the posterior end of the insect, was 43 ± 34 minutes (mean ± standard deviation, n = 6), with subsequent egg laying activity observed within 180 minutes (n = 1). Although the egg was moist at the time of oviposition, honeydew excretion was not observed in live insects.

3.1.1 SEM Technique Comparisons

Air drying and critical point drying preparation for SEM resulted in the compression and shrinkage of the external cuticle of grape phylloxera (Figure 1a,b). The splitting of the stylet was damage caused by an induced artefact of these techniques (Figure 1c). The cold stage SEM provided the optimum preparation for grape phylloxera (Figure 1d), with no evidence of cuticle compression or induced artefacts.

3.1.2 Light Microscopy External Measurements

Light microscopy measurements of live grape phylloxera indicate an approximate 20% increase in both length and width between each life stage (Table 1). There was a significant increase in size with life stage for all external body measurements, except for lateral growth between eggs and first instars, and dorsal-ventral growth between first and second instars. Adult grape phylloxera were on average 690 μm in length (anterior-posterior) and 387 μm in width (lateral). Fertile eggs were 42% the size of the reproductive adult lengths.

3.1.3 SEM External Measurements

The external body measurements presented in Table 2 are comparable to measurements from live insects (Table 1), indicating that sample preparation by cold stage SEM did not induce cuticle shrinkage or distortion. Statistical analysis of measurements of the external body features of grape phylloxera from cold stage SEM indicated an increase in body size (length and width) between the fourth instar and adult life stage (Table 2). Third instars were smaller than fourth instars, but there was no significant increase in size between the first to third instars. Eggs were the same size as first instars. External body measurements did not vary from live insect measurements for egg, first instars, second instars and adults. SEM measurements were reduced in size for third and fourth instar insects. SEM images of adult grape phylloxera were on average 701 μm in length and 426 μm in width. Fertile eggs were 41% the size of the reproductive adults length, with an apparent area of 34082 μm^2 (Table 3).

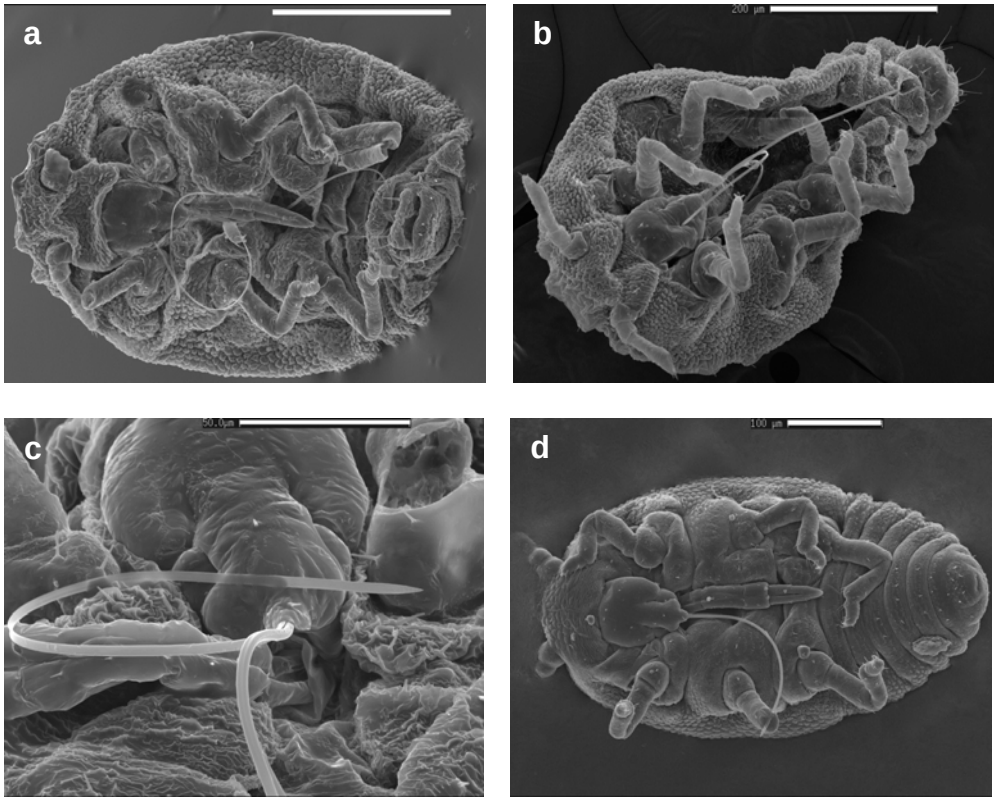


Figure 1. Grape phylloxera comparative structural damage caused by SEM sample preparation techniques. a) cuticle compression observed with air drying, scale bar 200 μm; b) cuticle compression caused by critical point drying, scale bar 200 μm; c) stylet splitting experienced with both air and critical point drying procedures, scale bar 50 μm; d) no cuticle compression observed by cold stage, scale bar 100 μm.

Table 1. Grape phylloxera external body measurements (all life stages) from light microscopy observation of live insects.

life stage	anterior – posterior (μm)	lateral (μm)	dorsal – ventral (μm)
adult	690 ± 61 [a]	387 ± 23 [a]	297 ± 55 [a]
fourth instar	591 ± 42 [b]	310 ± 22 [b]	218 ± 20 [b]
third instar	491 ± 20 [c]	267 ± 19 [c]	178 ± 16 [c]
second instar	394 ± 30 [d]	199 ± 17 [d]	131 ± 18 [d]
first instar	326 ± 31 [e]	160 ± 13 [e]	107 ± 11 [d]
egg	288 ± 9 [f]	150 ± 13 [e]	–
ANOVA p	<0.001	<0.001	<0.001
l.s.d.	34.29	14.78	27.16

Insect orientation indicated, mean values ± standard deviation, n = 10. ANOVA 'p' and l.s.d. values indicated, superscript letter indicates significance groupings.

3.1.4 SEM External Morphology Observations

The external body size of grape phylloxera increased with developmental life stage, but there was limited change in the external morphology of grape phylloxera between instars (Figure 2). The largest increase in size was between the life stages fourth instar and adult. Adult grape phylloxera displayed a visual increase in size, and an expansion, or swelling, of the posterior region (Figure 2e). This expansion was due to the development and oviposition of relatively large eggs (Figure 2f).

The gonopore was ventrally positioned at the posterior end of grape phylloxera. An additional posterior opening was identified as an indentation with a lateral orientation in a dorsal position to the gonopore (Figure 3a). Limited measurements were recorded for the posterior opening of the adult life stage due to the presence of the gonopore and insect orientation on the SEM stub inhibiting the view. A droplet of fluid present at the posterior end of two adult grape phylloxera insects (Figure 3b) also inhibited observation of the posterior opening. The fluid droplet was 14215 μm^2 in area (Table 3).

The size of the posterior opening did not correlate with life stage development (Table 2). Third instars had the smallest posterior opening, and there was no significant difference in size among the first instar, fourth instar or adult life stages.

Sensory pits were observed at the end of the antennae (Figure 4). Table 2 indicates a general increase in the length of the antennae with life stage development; however the ratio of antennae length to insect length decreased as life stage increased. Adult antennae length was significantly larger than all instar life stages.

Stylet length increased with life stage, doubling in length from first instars (124 μm) to adults (282 μm), although there was no change in the ratio of stylet length to external body length. There were limited stylet length measurements for first instar insects as these insects were collected prior to feeding, and due to the lack of probing activity, the stylet was still enclosed within the labium structure. There was no significant difference in the length of the labium with life stage.

Table 2. Grape phylloxera external morphology measurements from cold stage SEM images, all life stages. Some body features are not applicable (NA) to developed eggs.

life stage	external body (µm)		posterior opening	mouthparts (µm)		antennae (µm)	
	length[1]	width[2]	(µm)	labium	stylet	right	left
adult	701 ± 155 [a] (6)	426 ± 122 [a] (7)	29 ± 5.0 [a] (2)	187 ± 54 (4)	282 ± 50 [a] (4)	124 ± 20 [a] (3)	114 ± 4.6[a] (3)
fourth instar	495 ± 33 [b] (7)	300 ± 33 [b] (7)	24 ± 12 [ab] (5)	136 ± 16 (5)	211 ± 40 [b] (6)	95 ± 12 [b] (3)	95 ± 9.0 [b] (3)
third instar	414 ± 37 [c] (4)	242 ± 2.1 [c] (4)	15 ± 5.0 [c] (2)	133 ± 26 (3)	162 ± 28 [bc] (4)	100 ± 2.9 [bc] (3)	87 ± 14 [b] (3)
second instar	361 ± 33 [c] (5)	183 ± 8.6 [cd] (5)	18 ± 2.5 [b] (3)	164 ± 57 (2)	149 ± 19 [c] (3)	90 ± 2.5 [c] (3)	91 ± 6.4 [b] (2)
first instar	324 ± 42 [cd] (6)	154 ± 19 [de] (6)	22 ± 2.6 [ab] (6)	158 ± 27 (6)	124 ± 0 [d] (1)	84 ± 16 [c] (6)	91 ± 4.2 [b] (5)
egg	284 ± 21 [d] (2)	126 ± 22 [e] (2)	NA	NA	NA	NA	NA
ANOVA p	<0.001	<0.001	0.007	0.232	<0.001	0.003	0.002

Mean values ± standard deviation, n value in parenthesis. ANOVA 'p' value indicated, superscript letter indicates significance groupings.

[1] anterior – posterior measurement, [2] lateral measurement

Table 3. Grape phylloxera internal and external body area and dimension measurements from cold stage SEM, adult life stages.

measurement		internal features		external features	
		developing egg	mid-hindgut junction	developed egg	posterior fluid droplet
area (μm^2)		20,920	205	34,082	14,215 ± 902
dimensions (μm)	*l*	215	26	298	111 ± 14
	w	125	10	141	143 ± 18

Values of maximum dimensions, n = 1; except for posterior fluid droplet n = 2, mean ± standard deviation. Length (l) and width (w) dimensions of each feature indicated.

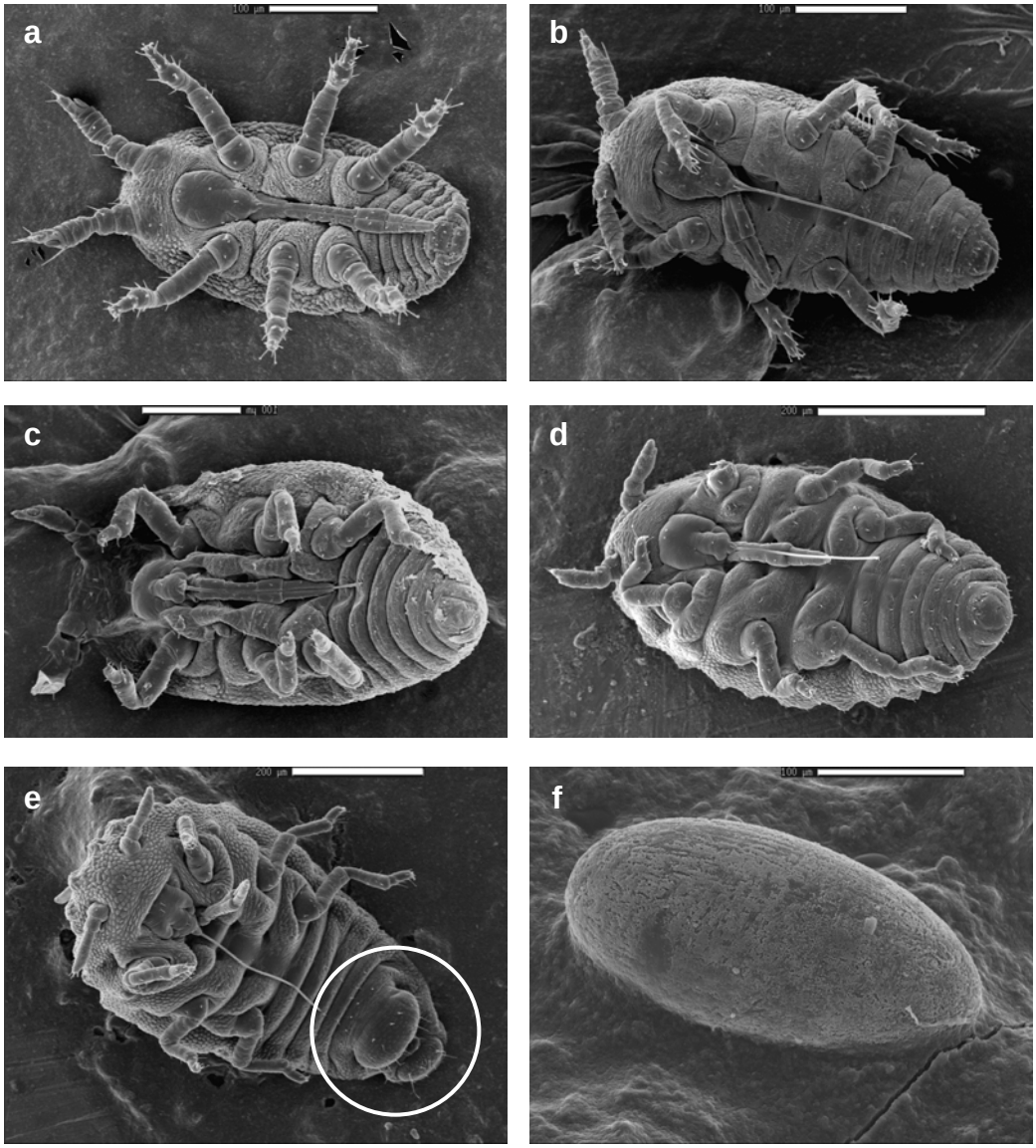


Figure 2. Cold stage SEM images of all life stages of grape phylloxera. a) first instar, scale bar 100 µm; b) second instar, scale bar 100 µm; c) third instar, scale bar 100 µm; d) fourth instar, scale bar 200 µm; e) adult, the circle highlights the swelling of the posterior region, scale bar 200 µm; f) egg, note that the base is sunken into the stub mounting paste, scale bar 100 µm.

In images a-e, the stylet was positioned in a medial anterior-posterior orientation, in the first instar (a) only the labium was visible; the stylet was positioned within the labium in images c) and d), however the labium was displaced in images b) and e).

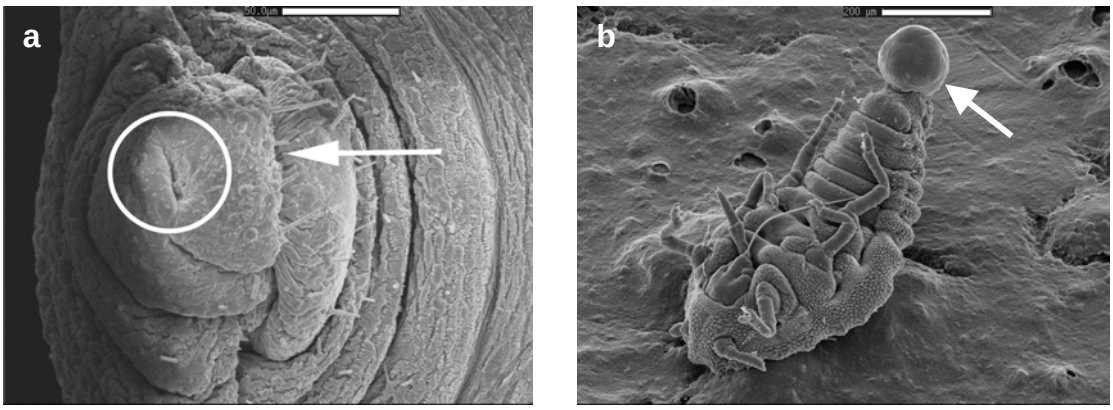


Figure 3. Cold stage SEM images of adult grape phylloxera. a) the posterior opening measured as potential evidence for waste excretion through an anal opening; the posterior opening is circled, the gonopore is indicated by the arrow, scale bar 50 μm; b) droplet of fluid (indicated by the arrow) at the posterior end of the insect, scale bar 200 μm.

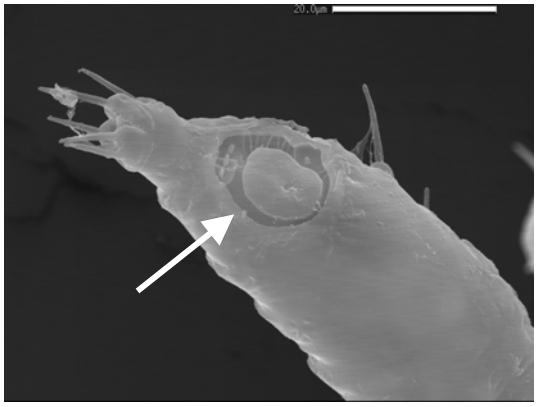


Figure 4. Cold stage SEM image of the sensory pit (indicated by the arrow) at the end of the antennae of all life stages (first instar-adult) of grape phylloxera, scale bar 20 μm.

3.2 Internal Morphology

3.2.1 Light Microscopy Internal Morphology

Longitudinal sectioning of grape phylloxera highlighted a specialised digestive system. The main digestive and reproductive features of grape phylloxera are represented schematically in Figure 5. Food passes from the stylet food canals into a short foregut before entering a compartmentalised midgut. The dimensions of the midgut are largest at the medial position of the insects (Figure 6a). The midgut ended blindly in the posterior region and the hindgut was positioned dorsal to the midgut. The midgut connected to the hindgut in a dorsal position forming a separation in the midgut (Figure 6b). The ovaries were also dorsal to the midgut, the hindgut passed between the ovaries and dorsal to the oviduct toward the posterior end of the insect. The position of the oviduct inhibited a posterior connection between the midgut and hindgut (Figure 6c). The hindgut extended to the posterior end in a dorsal position to the gonopore, however the presence of an anal opening was not been confirmed. The midgut was asymmetrical and reduced in capacity on the side of the insect where the ovaries are located, allowing for the development of eggs in the adult life stage (Figure 6d).

Adult grape phylloxera displayed multiple egg development, resulting in the compression of the midgut (Figure 7a). The hindgut was still present in the posterior region of the insect (Figure 7b) in a dorsal position to the oviduct. The simultaneous development of eggs by the ovaries caused the midgut to be asymmetrical in structure.

Transverse sectioning of adult grape phylloxera confirmed the interpretation of the longitudinal sections. In a medial position of the adult insect, the body cavity was dominated by the midgut and developing eggs, which are positioned in dorsal and ventral positions (Figure 8a). The hindgut appeared in close proximity to the midgut in a dorsal position. Egg size increased towards the posterior region of the insect, and caused the compression of the posterior region of the midgut where extensive folding was present (Figures 8b-8d). The hindgut was constantly located in a dorsal position to the midgut, with the oviduct blocking a posterior connected between the two gut cavities (Figure 8c). At the posterior end of the midgut (Figure 8e), the midgut and hindgut did not connect. The hindgut passed between the developing eggs (Figure 8d), and remained in a dorsal position to the gonopore (Figure 8e). The hindgut could be tracked to the posterior end of the insect (Figure 8f), although an external opening in the cuticle of the insect was not observed in light microscopy sections.

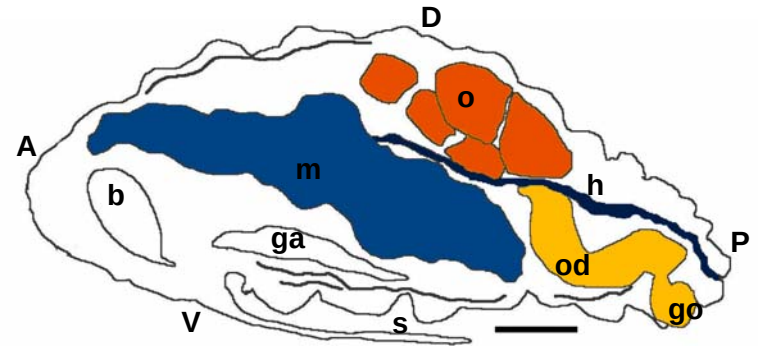


Figure 5. Schematic diagram of the digestive and reproductive features of grape phylloxera, longitudinal section. Scale bar 100 µm.

Abbreviations: **s** stylet, **b** brain, **ga** ganglion, **m** midgut, **o** ovaries, **h** hindgut, **od** oviduct, **go** gonopore; insect orientation indicated: **A** anterior, **P** posterior, **D** dorsal, **V** ventral

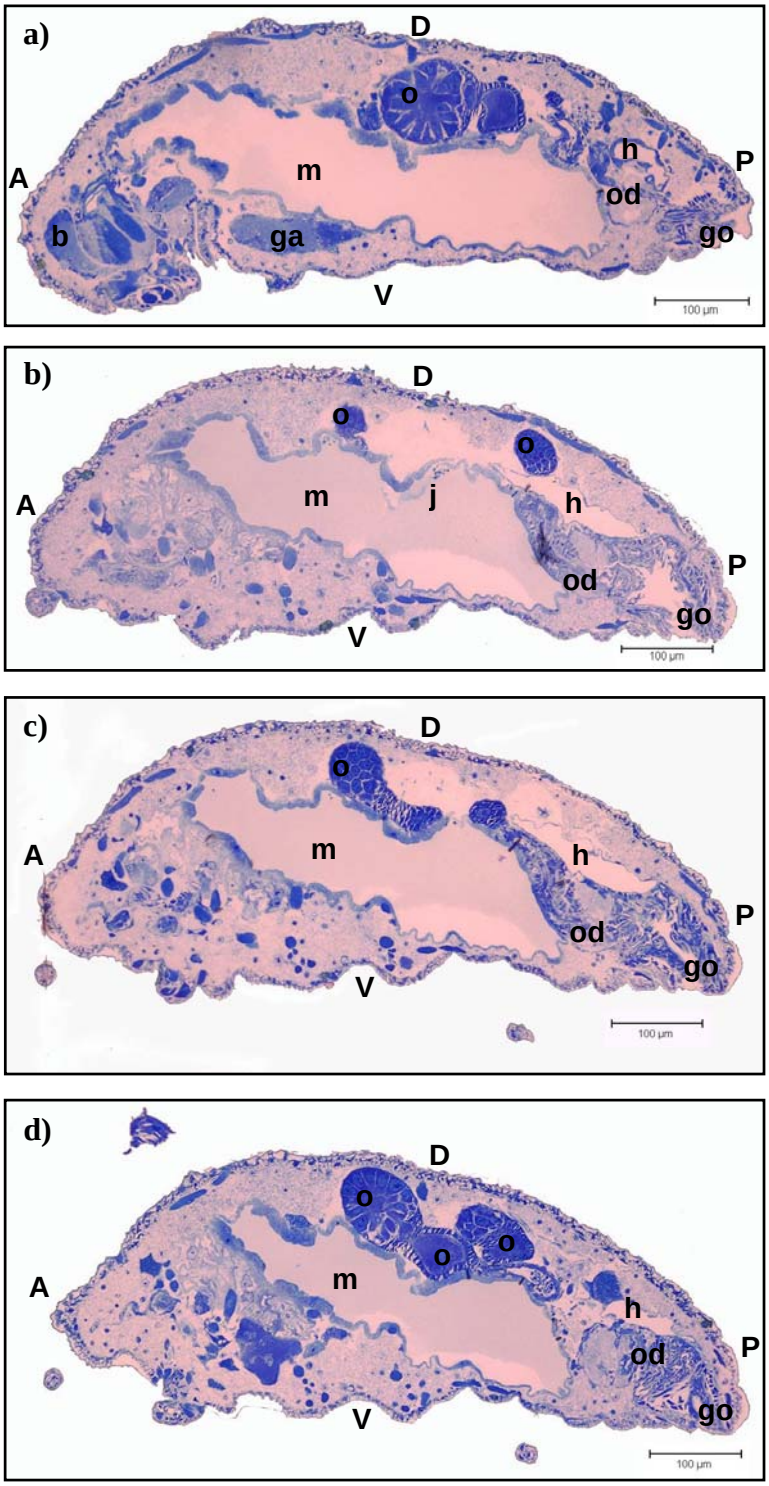


Figure 6. Series of longitudinal sections of fourth instar grape phylloxera. a) the medial position of the insect with the head visible in the anterior region, the midgut is the main component of the body cavity; b) the hindgut, oviduct and gonopore are more evident, the hindgut connects with the midgut in a dorsal position at the mid-hindgut junction; c) the hindgut remains dorsal to the midgut in all sections, the position of the oviduct inhibits a posterior connection between the gut cavities; d) the midgut is reduced in size, the ovaries are positioned dorsal to the digestive system, although egg development has also been observed in a ventral position. Scale bar 100 µm. Abbreviations: **b** brain, **ga** ganglion, **m** midgut, **o** ovaries, **h** hindgut, **od** oviduct, **go** gonopore, **j** junction between midgut and hindgut; insect orientation indicated: **A** anterior, **P** posterior, **D** dorsal, **V** ventral

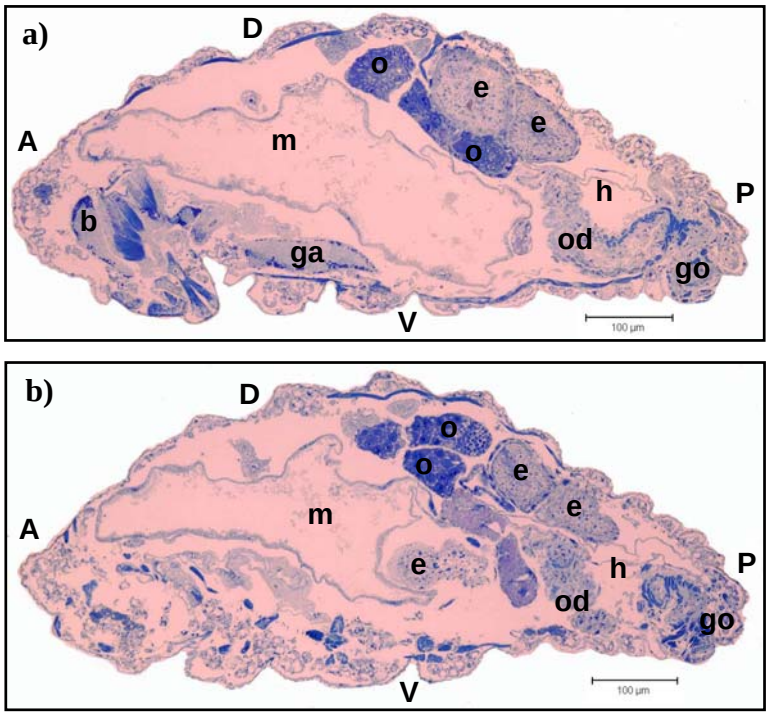


Figure 7. Longitudinal sections of adult grape phylloxera. a) the medial position of the insect with the head visible in the anterior region, multiple eggs are developing simultaneously dorsal to the midgut, the hindgut passes between the oviduct and the development of multiple eggs compresses the size of the midgut compartment and may block the hindgut, which is intertwined with the oviduct; b) the midgut is reduced in size, the hindgut intertwines through the ovaries and developing eggs and is evident at the posterior region of the insect, egg development is present in both a dorsal and ventral position causing the structure of the midgut to be asymmetrical. Scale bar 100 µm.

Abbreviations: **b** brain, **ga** ganglion, **m** midgut, **o** ovaries, **e** egg, **h** hindgut, **od** oviduct, **go** gonopore; insect orientation indicated: **A** anterior, **P** posterior, **D** dorsal, **V** ventral

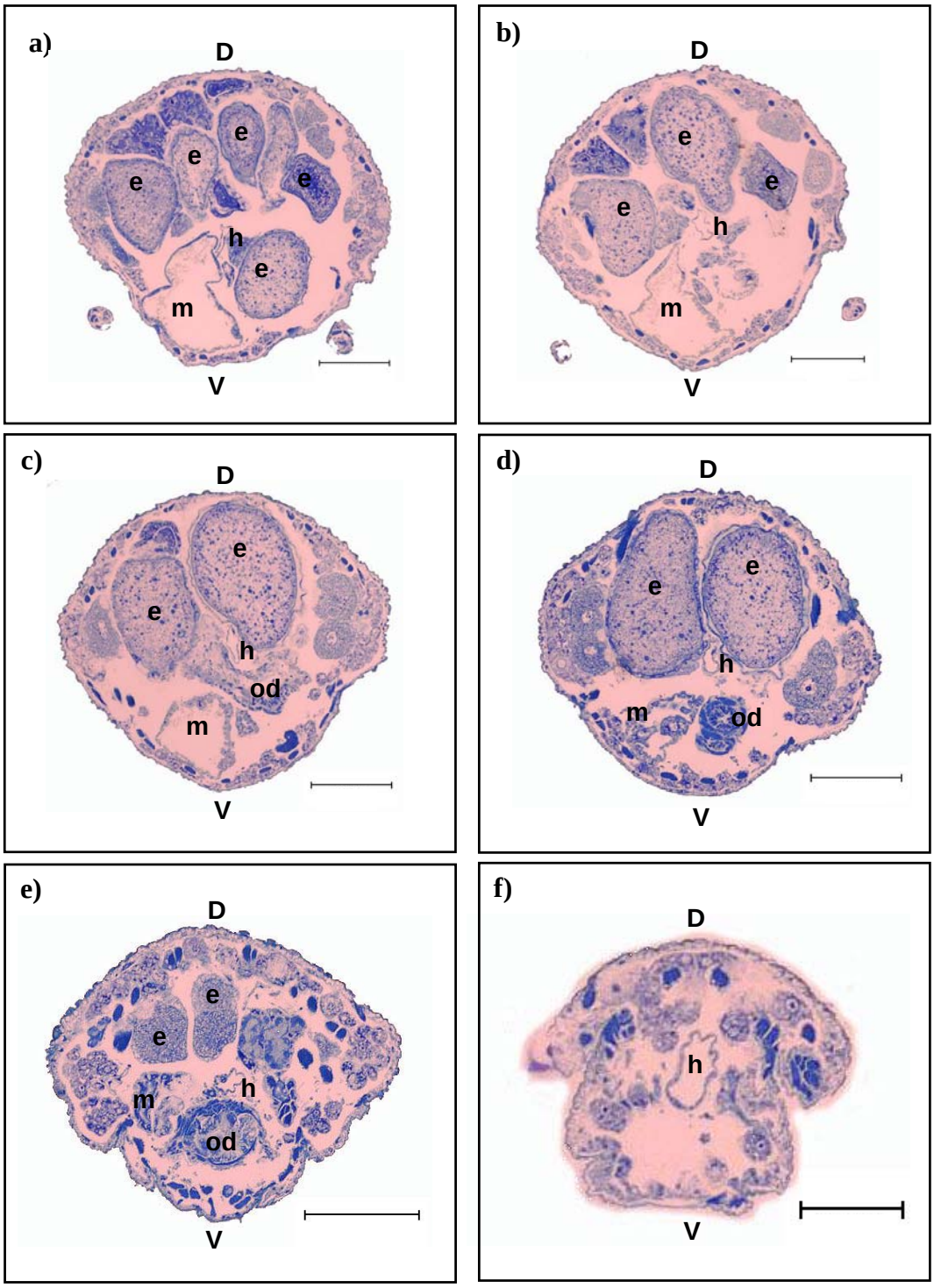


Figure 8. Series of transverse sections of adult grape phylloxera highlighting progressive changes in the digestive and reproductive systems from the medial position of the insect (a) towards the posterior end (f). a) the body cavity is dominated by the midgut and developing eggs, eggs are positioned in dorsal and ventral positions, the hindgut is dorsal to the midgut; b) the hindgut remains dorsal to the midgut, the two gut cavities are more distant; c) the oviduct is present between the midgut and the hindgut, inhibiting a posterior connection between the gut cavities; d) the midgut is reduced in size, the hindgut is positioned between two large, developing eggs; e) the hindgut and midgut are still separated at the posterior end of the midgut, the oviduct has increased in size; f) the hindgut is present at the posterior end of the insect. Scale bar a) - e) 100 µm, scale bar f) 50 µm. Abbreviations: **m** midgut, **e** egg, **h** hindgut, **od** oviduct; insect orientation indicated: **D** dorsal, **V** ventral

3.2.2 SEM Internal Morphology

Developing eggs, an open lattice digestive system and tubules were identified by sectioning grape phylloxera and viewing via cold stage SEM (Figure 9). The positioning of the open lattice structure corresponded with the location of the midgut and hindgut chambers from light microscopy sectioning. An opening located ventral to the developing egg, and adjacent to the midgut in a dorsal position (Figure 9 insert), was in the approximate location of the midgut-hindgut junction. The indentation in the egg, in line with the midgut-hindgut junction, may have been caused by the hindgut chamber en route to the posterior end of the insect.

The hindgut open lattice structure was observed in the posterior region of the insect (Figure 10). The gonopore was ventral to the hindgut position, and aligned with the light microscopy sections.

3.2.3 Internal Morphology Measurements

Measurements of grape phylloxera internal features from light microscopy sectioning indicated adult insects to be larger than fourth instar insects in maximum dimensions of all features, except the width of the midgut (Table 4). The hindgut widths were the same dimensions. The ratio of the length and width of the digestive system to the external body dimension was however reduced in reproductive adults. Developing eggs were 29% the size of the adult life stage external body length dimensions.

Area calculations of the same external and internal features of grape phylloxera from light microscopy sections indicated an increase in the external body cavity of adults compared with fourth instar insects (Table 5). The area of both the midgut and hindgut reduced in size in adult insects. Developing eggs had an average maximum area of 25224 μm^2.

Internal cold stage SEM images were reviewed for area measurements. Internally developing eggs were 20920 μm^2 in size (Table 3). The mid-hindgut junction observed in Figure 9 was 26 x 10 μm in size, with an area of 205 μm^2. Measurements of a similar mid-hindgut junction in a light microscopy transverse section were 42 x 9 μm in size, with an area of 315 μm^2.

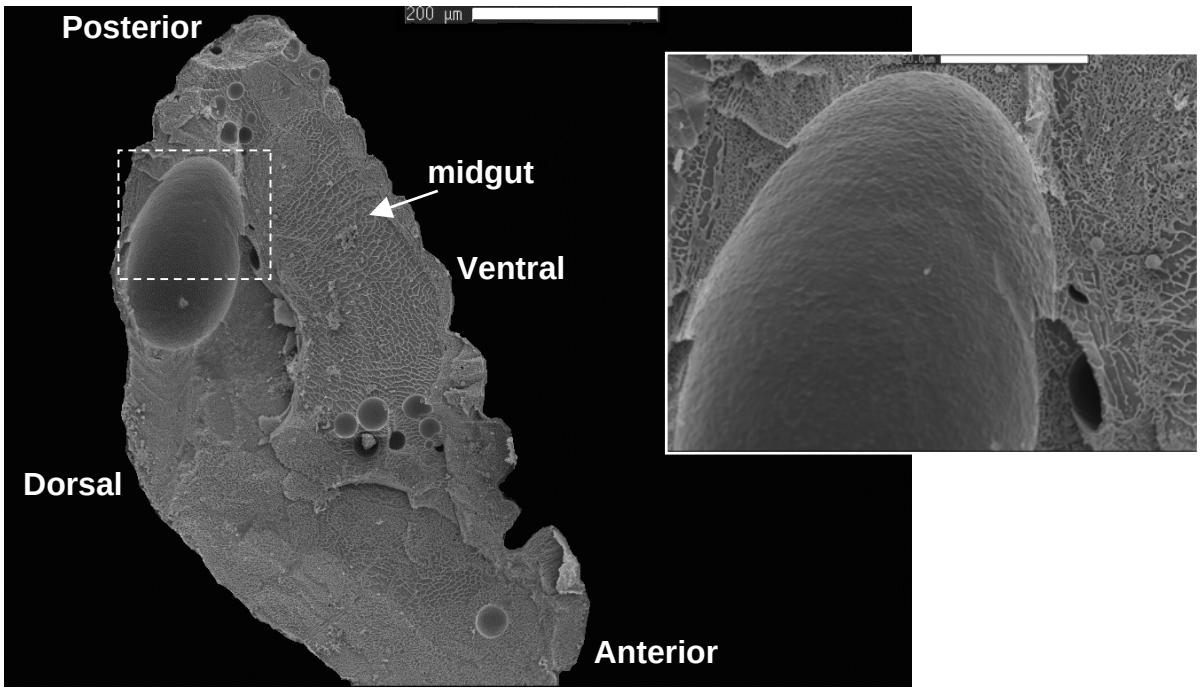


Figure 9. Cold stage SEM images of the internal structure of adult grape phylloxera, insect orientations labelled. Insert box (indicated on main image) is a close up view of a developing egg, with a tubular opening ventral to the position of the egg. The open lattice structure ventral to the egg (indicated by the arrow) is the midgut of the insect. Scale bar on main picture 200 µm, on insert picture 50 µm.

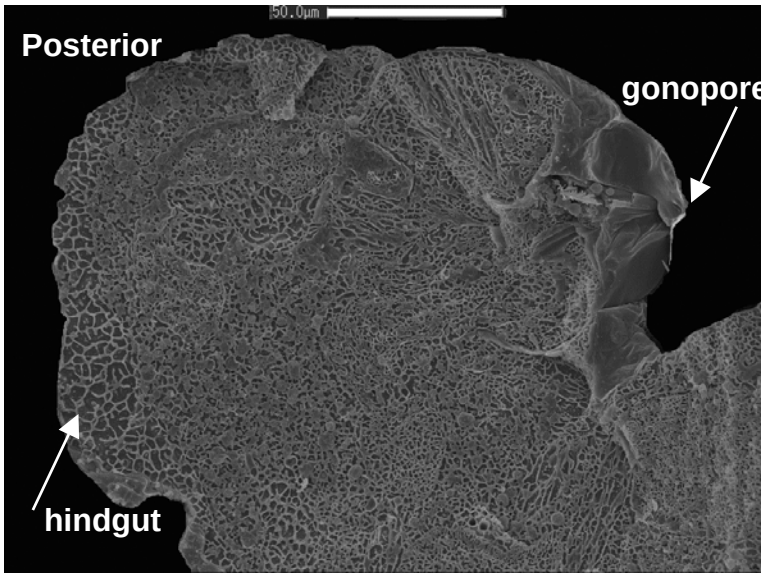


Figure 10. Cold stage SEM image of the internal structure of fourth instar grape phylloxera, insect posterior orientation labelled. The gonopore is situated in a ventral position, the open lattice structure of the hindgut (indicated by the arrow) is in the dorsal position. Scale bar is 50 µm.

Table 4. Grape phylloxera external body dimensions and internal body measurements (digestive system and developing egg) from longitudinal sections, fourth instar and adult life stages.

life stage		external	digestive system		developing
		body (µm)	midgut (µm)	hindgut (µm)[1]	egg (µm)
adult	*l*	803 ± 46	488 ± 92	233 ± 117	233 ± 12
(n = 4)	*w*	308 ± 61	79 ± 35	28 ± 3.5	134 ± 14
fourth instar	*l*	680 ± 170	463 ± 124	180 ± 0	–
(n = 2)	*w*	225 ± 78	98 ± 3.6	28 ± 3.5	–

Mean values of maximum dimensions ± standard deviation, n value in parenthesis. Length (l) and width (w) of each feature indicated.

[1] adult n value = 2

Table 5. Grape phylloxera internal body area measurements (digestive system and developing egg) from longitudinal sections, fourth instar and adult life stages.

life stage	external	digestive system		developing
	body (μm^2)	midgut (μm^2)	hindgut (μm^2)[1]	egg (μm^2)[2]
adult	180,834	25,260	2,898	25,224
(n = 4)	± 43,217	± 14,804	± 1,961	± 6,053
fourth instar	173,604	45,169	3,596	–
(n = 1)	± 0	± 0	± 0	

Mean values of maximum dimensions ± standard deviation, n value in parenthesis.

[1] adult n value for hindgut area measurement = 2, [2] adult n value for developing egg measurement = 3

4 DISCUSSION

The similarity in external body measurements for insects via light microscopy and SEM techniques validated the use of SEM to investigate the fine structures present on grape phylloxera. The development of grape phylloxera from egg to adult involves four nymphal moults, resulting in four instar life stages prior to the reproductive adult. SEM external body measurements of radicicolae grape phylloxera displayed a continual increase in size with each life stage development; however the transition from fourth instar into adult resulted in the largest size increase. This increase in size may be representative of the observation by Davidson and Nougaret (1921) of a growth phase during the fourth instar life stage. Between the third and the fourth moult (indicating the fourth instar life stage), the fourth instar was observed to increase in size by 25%. Adult insects used for measurement were typically the largest insects within the population; smaller insects were observed that could be classified as adults, but these were not collected. Selecting the larger insects, along with the possibility of collecting early moult fourth instars, would exacerbate the variation in size between the fourth instar and adult life stage.

In comparison with previous research using light microscopy technology (Davidson and Nougaret, 1921; Buchanan, 1990), SEM external body measurements of first instar and adult insects are of comparable size, however second, third and fourth instars were smaller in this study. This variation may be due to an unavoidable distortion of the insect with angle placement on the SEM stub, and also the quality of the food source. As noted by Davidson and Nougaret (1921), adult insects feeding on fleshy and succulent roots measured 1000 μm in length and 550 μm in width, while insects feeding on other regions of the grapevine root were smaller in size. The insects used for this study were collected from excised root populations maintained in the laboratory. These insects are smaller in size to insects collected from potted vine populations (K.B. Kingston, pers. comm.), and are not expected to grow to a size comparable with field collected insects as environmental and nutritional factors are limiting. Egg measurements have previously been reported with an average length of 299 μm and width of 149 μm (De Klerk, 1974). The measurements presented in this study are within range of these averages, although the width dimension was reduced by a portion of the egg being sunken into the SEM stub mounting paste, therefore affecting the accuracy of the values (Fig. 2f).

Evidence for Waste Excretion

The location of a posterior opening was investigated by SEM as evidence for the presence of anal waste excretion. All instar life stages displayed one posterior opening, which was variable in size. Adult grape phylloxera have an enlarged gonopore posterior opening for egg oviposition. In limited SEM images (2) the presence of a second posterior opening, dorsal to the gonopore, was visible. The location of this second posterior opening aligned with the expected termination point of the hindgut from longitudinal and transverse light microscopy sections, and is also supported by the internal SEM data.

An absence of honeydew production (Riley, 1870) has been cited as evidence that grape phylloxera does not have a system of anal excretion for waste (Ponsen, 1997). Honeydew was not visible when observing insects actively feeding on grapevine roots, however the possible presence of honeydew was observed under SEM as a droplet of fluid at the posterior end of two individual adult grape phylloxera. The droplet of fluid was potentially the result of pressure applied to the abdomen during preparation, although the exact exit

point was unable to be determined. Area measurements of the droplet of fluid and the hindgut did not align, however the area measurement for the hindgut cavity is expected to be an underestimation due to inaccuracies caused by not viewing the entire hindgut structure within the one light microscopy section for measurement. The actual volume capacity of the hindgut was unable to be determined. It is also possible that the fluid in the SEM image is from the gonopore passage, and is fluid used to assist the oviposition of eggs. Further investigation is required to resolve the origin of the fluid droplet.

Reported observations in the closely related *Phylloxera coccinea* Heyden (Hemiptera: Phylloxeridae), oak phylloxera, may however provide support for the theory that the droplet of fluid observed in grape phylloxera is associated with waste excretion. *Phylloxera coccinea* feeding on their oak host-plant do not display visible levels of honeydew production, however a similar droplet of 'honeydew' has been observed with the application of pressure to the abdomen (Ponsen, 1997). Ponsen inferred that Phylloxeridae feeding on parenchyma cell contents have a reduced fluid intake in comparison to phloem feeding, and honeydew generating, Aphididae. The waste excretion levels generated by grape phylloxera may be too low for visible detection under standard conditions.

Evidence of Feeding Site Location

The feeding location of grape phylloxera has been reported to be a single parenchyma cell within the cortex tissue of the grapevine root (Kellow *et al.*, 2004). Stylet length was measured as an indicator of the possible feeding location of grape phylloxera within the grapevine root system. Stylets were difficult to measure accurately due to possible distortion of the SEM image, and uncertainty if the stylet was fully extended at the time of insect collection. However the values presented in this study are similar to earlier data by Davidson and Nougaret (1921), and can imply possible feeding locations within the grapevine root. By comparing stylet length with the distance to parenchyma and phloem cells, it may be possible to determine where grape phylloxera can feed in the host-plant.

Following examination of sections of several *Vitis* species, the thickness of the cortex tissue of grapevine roots was determined, and compared with the depth of stylet penetration required for phloem feeding. The thickness of the cortex in *V. vinifera* ranges from 200-300 μm, or 7-12 cell layers deep (Kellow, 2000). Stylet penetration from the root surface to the food source is generally indirect, involving trial-and-error sampling of potential food sources before settling in a satisfactory location (Tjallingii and Hogen Esch, 1993). However, even if the stylet of grape phylloxera did penetrate the root surface and travel through the cortex in a straight line, the stylet measurements recorded indicate that it is unlikely that grape phylloxera can reach phloem cells. Only fourth instar and adult life stages recorded a stylet length in excess of 200 μm. These measurements therefore support the expectation that grape phylloxera feed primarily on grapevine root cortex tissue, unlike species of the closely related Aphididae who feed predominantly on phloem tissue.

Dispersal and Reproductive Adaptations

The ratio of grape phylloxera antennae length to external body length was greatest for the first instar active life stage. The function of the sensory pore located at the end of each antenna was unknown, but may be important during dispersal, host-plant displacement and the establishment of new feeding sites. Hemipteran antennae detect olfactory cues from plants to assist in long-distance orientation and plant surface exploration during host plant settling (Backus, 1988). Grape phylloxera feeds exclusively on *Vitis* species (Granett *et al.*, 2001); therefore a highly specific sensory system would be important for the location of a new host-plant. The antennae measurements presented observed using SEM are similar to those presented by Davidson and Nougaret (1921) from light microscopy.

Digestive System Internal Morphology

The internal structure of the grape phylloxera digestive system is designed to provide energy for the insect during periods of low food intake due to dispersal to a new *Vitis* food source and/or the compression of the midgut cavity during egg development. The midgut is compartmentalised by the junction of the hindgut in a dorsal position. Enzyme histochemistry studies (Kemper, 2004) confirm the separation in midgut function, with the anterior region of the midgut staining heavily for alkaline phosphatase, with minimal staining in the posterior region of the midgut. This implies that the anterior midgut is actively involved in the transport of nutrients across the membrane surface, and the posterior midgut is a storage system to sustain the insects survival when active feeding on grapevines is not possible.

The midgut-hindgut junction observed in longitudinal and transverse light microscopy sectioning was also observed in internal cold stage SEM studies. The dimensions and area of these two junctions are of comparable size providing evidence for the structure, which has not previously been reported.

The multiple development of eggs 40% the size of adult grape phylloxera results in the compression of the digestive system. This compression is highlighted by the reduction in area of the digestive chambers in adults compared with fourth instar insects. The developing egg measured for this study were approximately 30% the size of the adult insect, however this is the size of a single egg, and does not represent the entire area utilised by egg development in the ovaries. Egg development in both dorsal and ventral positions compresses the midgut chamber, forcing food from the posterior storage system into the anterior midgut for absorption and energy to support the development of egg production.

The positioning of the hindgut between the oviduct and the developing eggs may result in the blocking of the hindgut passage during egg oviposition. Waste excretion may be occur due to the pressure caused by this blockage and the movement of the developed egg down the oviduct towards the gonopore. The moist egg observed at the time of oviposition may be due to oviduct fluids, or partially due to the excretion of waste. The internal SEM image of the indentation in the developing egg provided visible evidence for the compression of the hindgut during oviposition.

Comparison with Previous Research

The structure of the digestive system reported in this study is similar to previous studies that concluded grape phylloxera to have a complete digestive system (Breider, 1952; Ponsen, 1997). However both Breider and Ponsen interpreted the midgut-hindgut junction to be a twist in the cavity of the digestive system, with the midgut connected to the hindgut in the posterior end. Breider identifies the gonopore position to be in a similar posterior-ventral position, with the anal opening positioned dorsal to the gonopore. Both studies concluded grape phylloxera to have a complete digestive system. Federov (1959) concluded grape phylloxera to have a blind ending midgut, and used this to support a lack of anal excretion. This study does show the midgut of grape phylloxera to be 'blind-ending' in the posterior region, although the complete digestive system is not blind-ending due to the dorsal midgut-hindgut junction. The hindgut is tracked to the extreme posterior of the insect, although an anal opening was not been identified through light microscopy sectioning. However, in a related study by Kemper (2004), light microscopy sectioning evidence for a second external opening dorsal to the gonopore was presented.

5 CONCLUSION

Although the location of an anal opening is not definitive, evidence of an external posterior opening in all life stages, and the internal tracking of the hindgut to the posterior region of the insect, is indicative of the potential for anal waste excretion. Alternative methods of waste excretion have not been discussed, however with grape phylloxera reported to feed on a single parenchyma cell (which is supported by stylet length measurements and no visible honeydew detection), excretion of waste by the salivary glands back into the food source seems an unlikely mechanism. No internal morphological adaptation in the adult life stage, for a connection between the digestive system and the oviduct allowing waste to be excreted during oviposition, was identified. In comparison with phloem sap, the food source of grape phylloxera is high in protein (nutrition) and low in sugar and water (waste products). The slow digestion of this food source in the compartmentalised midgut provides the energy requirements to sustain continual egg production during the adult life stage. Now that the internal components of the digestive system of grape phylloxera have been clearly identified, future research to characterise the biochemistry of the digestive system will be able to proceed. Some preliminary studies conducted by Kemper (2004), in collaboration with the authors of the report, highlighted that the anterior region of the midgut was predominantly involved in nutrient absorption. This would be the region of focus for future digestive enzyme studies.

6 RECOMMENDATIONS

Both the internal and external morphology of different life-stages of radicicolae grape phylloxera has been extensively studied, and for the first time using a range of light and electron microscopy techniques. This allows for the provision of future recommendations, and assists in identifying gaps in research knowledge.

Comparison of insect preparation for SEM highlighted that cold stage is a superior technique when compared to both air drying and critical point drying, as it produces less cuticular shrinkage and no artefacts. This technique should be used in further studies on external morphology of grape phylloxera. In particular, further studies on both the mouthparts (stylet) and antennae are recommended. By studying the size of the stylet in different life stages of the grape phylloxera, and linking this to histochemical studies of *Vitis* plant tissue, this would provide a definitive answer regarding the feeding site of each developmental instar. This would also allow better interpretation of feeding physiology and EPG correlation studies.

In this project, sensory pits were identified on the antennae of grape phylloxera. The role of these sensory pits is assumed to involve a chemosensory location function that allows insects to locate a suitable feeding site based on chemical cues. Further studies on the role of the sensory pits for the dispersive life stages of grape phylloxera (particularly first instars), in association with studies on chemosensory cues (such as host-plant volatiles or exudates) would assist in identifying resistance mechanisms and the level of influence soil type has on grape phylloxera establishment.

The complexity of the internal morphology of the digestive system of grape phylloxera has highlighted the impact of egg production on gut morphology and function. Whilst we have moved closer to determining that the insect has a complete digestive system, the method of waste excretion is still not fully determined. Although a posterior opening was found near the gonophore, whether this is used as a point of honeydew excretion still needs to be determined. Some evidence of a droplet was seen using SEM but this requires further investigation to determine if in fact the droplet was waste excretion. Further SEM studies, or studies using labelled dyes, would assist in determining if the insect excretes honeydew.

As the internal components of the digestive system have now been clearly identified further studies need to be conducted to characterise the biochemistry of the digestive system. Identifying the major digestive enzymes present within grape phylloxera will allow for the screening of particular digestive enzyme inhibitors (using the diet system developed in Sub-project 1.1) which may interfere with the insects normal digestive functions and provide a future management option.

SUB-PROJECT 2.2

INVESTIGATING BACTERIAL SYMBIOTIC RELATIONSHIPS IN GRAPE PHYLLOXERA

1 INTRODUCTION

Several insect groups, including cockroaches, beetles, whiteflies, scale insects, psyllids, leafhoppers, cicadas and aphids, have associations with intracellular micro-organisms (endosymbionts). Typically, the symbiotic relationship develops due to the insect diet being deficient in an essential nutrient, which is biosynthesised by the micro-organism (Gullan and Cranston, 2005). All members of the Hemipteran superfamily Aphidoidea contain the bacterial endosymbiont *Buchnera*, except for members of the Adelgidae and Phylloxeridae families (Douglas, 1998). *Buchnera* provide Aphididae with essential amino acids not found in their diet of phloem sap, resulting in an essential, mutualistic relationship that is maternally inherited. The bacterial endosymbiont can not be cultured outside of the host insect, and the growth of the aphid is inhibited in the absence of the endosymbiont (Martinez-Torres *et al.*, 2001).

Members of the Aphididae family may contain bacterial symbiotic relationships additional to the primary endosymbiont *Buchnera*. Primary endosymbionts are spherical in shape and located in specialised cells (termed mycetocytes) within the hemocoel of the insect. Secondary endosymbionts are rod-shaped bacteria located in the tissue surrounding the mycetocytes, and tertiary endosymbionts are rod-shaped bacteria located free within the hemolymph (Chen *et al.*, 1996). Unlike the primary endosymbionts, secondary (and tertiary) endosymbionts are transient, and are generally not considered to offer nutritional advantage to the aphid (Douglas, 1998), although there is evidence for beneficial impacts on reproduction. The presence of secondary (and tertiary) endosymbionts relies upon repeated infection by the bacteria (Sandstrom *et al.*, 2001).

Evolutionary divergence between the Aphididae and the Phylloxeridae families occurred prior to infection of the Aphididae with *Buchnera* around 100-250 mya (Martinez-Torres *et al.*, 2001). It is generally considered that insects feeding on plant parenchyma cell contents, like the Phylloxeridae, do not require bacterial symbiotic relationships to supply the insects essential nutritional requirements (Buchner, 1965). A review of previous research included claims of culturing bacterial endosymbionts from *Phylloxera vastatrix* Pl. (synonym of *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae), grape phylloxera), and rod-shaped bacteria being identified in the closely related *Phylloxera quercus* (oak phylloxera). The identification of the bacteria proved transient, and it was concluded that grape phylloxera contain no endosymbionts (Buchner, 1965). More recent research (Vorwerk *et al.*, 2006), identified the presence of bacterial DNA in gallicolae grape phylloxera.

Research Aims

It is theoretically possible that members of the Phylloxeridae (including grape phylloxera) were infected with a (non-essential) bacterial species, similar to the secondary and tertiary endosymbionts in the Aphididae, post evolutionary divergence. There has been limited (Vorwerk *et al.*, 2006) re-investigation of a bacterial symbiotic relationship with grape phylloxera since the advent of molecular techniques. In this project we have used DNA technology to determine if bacterial endosymbionts are associated with radicicolae grape phylloxera. If bacterial endosymbionts exist, then they may play an important role in the nutritional physiology and development of grape phylloxera, and may influence host-plant interactions.

2 MATERIAL AND METHODS

2.1 Insect Material

Radicicolae grape phylloxera were originally collected from infested vineyards in the King Valley, Victoria, Australia. Grape phylloxera from this region have been identified as a single genotypic class, G4 (Corrie *et al.*, 2002). Populations were maintained at the Department of Primary Industries – Rutherglen Centre on excised *V. vinifera* cv. Sultana root pieces (approximately 1 cm width x 10 cm length), prepared using a protocol slightly modified from Granett, *et al.* (1985). The modifications were: (1) roots washed clean of attached soil with a soft brush under running water; (2) roots soaked for 5 minutes in Ridomil® Gold Plus systemic fungicide solution (2.3 g/L); (3) roots triple rinsed with sterile distilled water prior to air drying; and (4) the size of the petri dish was increased to 15 cm, with the increase in area reducing the impact of water condensation. Grape phylloxera populations were incubated in the dark at a constant temperature of 25 ±3°C.

Only fourth instar and adult apterous radicicolae grape phylloxera were used for this study. Insects used for bacterial endosymbiont screening were collected directly from the excised root pieces, or from populations of grape phylloxera that had been transferred to tissue culture or potted vines. Micro-propagated grapevines were prepared as outlined by Kellow, *et al.* (2002). Slight modifications included: (1) agar medium was not supplemented with benzyl aminopurine or napthaleneacetic acid; and (2) tissue culture plantlets were not transferred to a second agar medium prior to the perlite-based medium. Potted vines were maintained under glasshouse conditions with a 12 hour max-min temperature cycle of 18°C-26°C and 18°C-22°C. Naturally lighting was supplemented with artificial growth lights for a 14 hour day period, and an additional 1 hour exposure at night (Korosi *et al.*, 2006).

2.2 DNA Extraction

In preparation for DNA extraction, groups of 10 grape phylloxera were collected into a sterile 2 ml tube for sterilisation. The insects were mixed with 70% ethanol for 30 seconds or 5 minutes, and then rinsed with sterile water. The insects were aseptically transferred to a new sterile 2 ml tube for DNA extraction.

Grape phylloxera DNA extractions were performed using a QIAGEN® DNeasy® Tissue DNA purification kit. Specifically, the QIAGEN® DNeasy® DNA purification procedure for the isolation of genomic DNA from insects with microtube pestles was used. DNA extractions were also performed on a group of 5 Monterey pine aphids (*Essigella californica* Hemiptera: Aphidoidea: Lachnidae: Cinarinae) as a positive control (insects supplied by Dr Trudy Wharton, Australian National University). The Monterey pine aphid was expected to contain *Buchnera* as closely related species (Aphidoidea: Lachnidae: Cinarinae) have been confirmed to contain the primary endosymbiont (Martinez-Torres *et al.*, 2001). Monterey pine aphids were stored in 100% ethanol at -20°C until required. A DNA extraction was performed with no insects to confirm the sterility of the procedure.

2.3 PCR Conditions

All DNA extractions were amplified with insect nuclear SSU rDNA and bacterial 16S rDNA primers in a BioRad® iCycler® Thermal Cycler. Polymerase chain reactions (PCR) contained 1.5 µl 50mM $MgCl_2$, 2.5 µl 10x reaction buffer (BIOLINE™), 0.5 µl 20mM dNTP, 0.5 µl 10µM each forward and reverse primer, 1 µl 1U BIOTAQ™ RED DNA polymerase, 2 µl of template and nuclease free sterile water to final volume of 25 µl. All PCR mixtures were prepared with a negative (sterile water) and positive (Monterey pine aphid DNA) control. Cells from a bacterial colony plate were used as a negative control for the SSU rDNA primers, and as a positive control for the 16S rDNA primers. PCR reagents were sourced from BIOLINE™ and primers from GeneWorks.

Amplification with nuclear SSU rDNA primers 2880 (5'-CTGGTTGATCCTGCCAGTAG-3') and B- (5'-CCGCGGCTGCTGGCACCAGA-3') (Cook *et al.*, 2002) confirmed that DNA extractions contained no PCR inhibiting compounds. The SSU rDNA primers amplify insect DNA but do not align with bacteria DNA for amplification. PCR mixtures were incubated in a preheated (94°C) thermal cycler at 94°C for 3 minutes (initial denaturation); followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds (annealing) and 72°C for 1 minute, the time for each step increased by 0.02 seconds per cycle; followed by a final extension period of 7 minutes at 72°C. The SSU rDNA primers produced an amplification product approximately 650 bp in length.

Bacterial specific 16S rDNA forward primer 27F (5'-AGATTTGATCMTGGCTCAG-3') and universal reverse primer 1492R (5'-GGYTACCTTGTTACGACTT-3') were used to investigate the presence of bacterial genes within grape phylloxera DNA extractions. PCR mixtures were incubated in a preheated thermal (94°C) cycler at 94°C for 3 minutes (initial denaturation); followed by 30 cycles at 94°C for 30 seconds, 50°C for 45 seconds (annealing) and 72°C for 1.5 minutes; followed by a final extension period of 7 minutes at 72°C (modified from Dojka, *et al.*, 2000). The 16S rDNA primers produced an amplification product approximately 1.5 kb in length.

PCR products (10 µl) were detected by electrophoresis on a 1% agarose gel stained with ethidium bromide. DNA samples were ran with a 100 bp (0.5 µg) DNA ladder and either a 1 kb (0.5 µg) or a 2-Log: 0.1-10 kb (1.0 µg) DNA ladder (New England Biolabs®). DNA bands were visualised with a BioRad® Molecular Imager Gel Doc™ System. Images were captured digitally using BioRad® Quantity One® 1-D Analysis Software.

3 RESULTS

DNA extractions were performed on grape phylloxera collected from three different root sources (tissue culture, excised root and potted vine) and at two different sterilisation times (30 second and 5 minute). All insect DNA extractions amplified with the SSU rDNA primers (Table 1), confirming that the DNA was not contaminated with PCR inhibiting compounds. The bacterial colony DNA, DNA extraction negative control, and the PCR negative control samples did not amplify the SSU rDNA primers (Figure 1).

Inconsistent product amplification of grape phylloxera DNA extractions was obtained with the 16S rDNA primers (Table 1). All grape phylloxera DNA extractions from the potted vine root source amplified a 1.5 kb product, independent of sterilisation time. There was no amplification of grape phylloxera DNA extractions from the excised root or tissue culture root sources. All negative amplification results were confirmed with (at least) a second PCR attempt. The Monterey pine aphid DNA amplified with the 16S rDNA primers, although only genomic DNA (>10 kb) was present in the bacterial colony DNA lane (Figure 2). The DNA extraction negative control, and the PCR negative control samples did not amplify the 16S rDNA primers.

Table 1. Grape phylloxera DNA extractions identifying the insect root collection source and 70% ethanol sterilisation time. The results of PCR amplification with SSU rDNA and 16S rDNA primers are indicated, including positive (Monterey pine aphid) and negative (water) PCR controls.

phylloxera DNA extraction	insect root source	ethanol sterilisation	amplification	
			SSU rDNA	16S rDNA
1	tissue culture	5 minutes	✓	×
2	excised root	5 minutes	✓	×
3	potted vine	30 seconds	✓	✓
4	excised root	30 seconds	✓	×
5	potted vine	5 minutes	✓	✓
6	potted vine	30 seconds	✓	✓
Monterey pine aphid		5 minutes	✓	✓
water (negative control)		NA	×	×

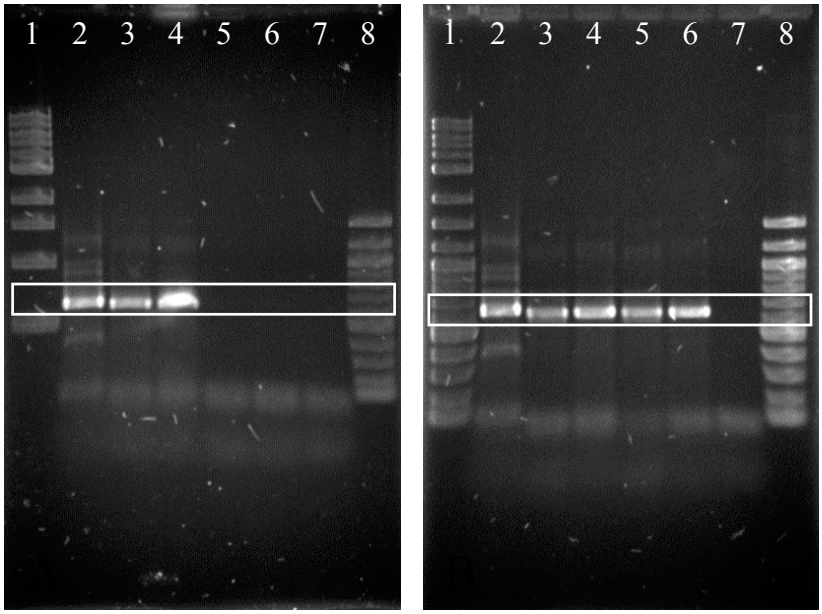


Figure 1. PCR amplification products with SSU rDNA primers, the 650 bp products are boxed. Lane numbers 1-8 are indicated, grape phylloxera DNA extractions denoted by insect root collection source, and ethanol sterilisation time in parenthesis.

Agarose gel 1a) lane 1: 1 kb DNA ladder, lane 2: Monterey pine aphid, lane 3: potted vine (30 sec), lane 4: potted vine (5 min), lane 5: bacterial colony DNA, lane 6: DNA extraction negative control, lane 7: PCR negative control, lane 8: 100 bp DNA ladder.

Agarose gel 1b) lane 1: 2-Log DNA ladder, lane 2: Monterey pine aphid, lane 3: excised root (30 sec), lane 4: potted vine (30sec), lane 5: excised root (5 min), lane 6: tissue culture (5 min), lane 7: PCR negative control, lane 8: 100 bp DNA ladder.

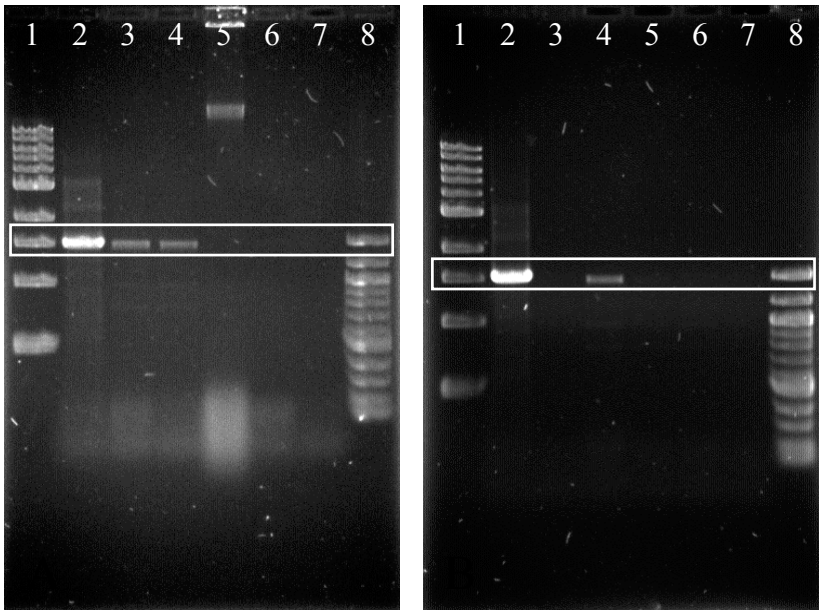


Figure 2. PCR amplification products with 16S rDNA primers, the 1.5 kb products are boxed. Lane numbers 1-8 are indicated, grape phylloxera DNA extractions denoted by insect root collection source, and ethanol sterilisation time in parenthesis.

Agarose gel 2a) lane 1: 1 kb DNA ladder, lane 2: Monterey pine aphid, lane 3: potted vine (30 sec), lane 4: potted vine (5 min), lane 5: bacterial colony DNA, lane 6: DNA extraction negative control, lane 7: PCR negative control, lane 8: 100 bp DNA ladder.

Agarose gel 2b) lane 1: 1 kb DNA ladder, lane 2: Monterey pine aphid, lane 3: excised root (30 sec), lane 4: potted vine (30 sec), lane 5: excised root (5 min), lane 6: tissue culture (5 min), lane 7: PCR negative control, lane 8: 100 bp DNA ladder.

4 DISCUSSION

The positive amplification of the bacterial 16S rDNA primers from grape phylloxera DNA was variable. DNA quality was not the cause of this variation as all DNA extractions amplified with the insect SSU rDNA primers. No DNA extractions from the excised root or the tissue culture collection sources amplified the 16S rDNA primers, although previous DNA extractions (using a non-QIAGEN extraction method) from both root sources did amplify the primers (data not shown).

Insect sterilisation time was initially considered a possible factor for the non-amplification of the 16S rDNA primers, with the 5 minute 70% ethanol sterilisation time potentially having a negative impact on bacterial DNA detection. However the positive amplification of grape phylloxera DNA extractions from the potted vine collection source at 30 second and 5 minute 70% ethanol sterilisation times confirmed that sterilisation time was not a factor. The 16S rDNA amplification of grape phylloxera DNA following the 30 second 70% ethanol sterilisation step was confirmed for the potted vine collection source with a second DNA extraction (Table 1).

The possibility of external contamination during the DNA extraction procedure (causing the transient positive amplification results) was eliminated by the non-amplification of the DNA extraction negative control in both the SSU rDNA and the 16S rDNA PCR. Non-amplification of all PCR negative controls eliminated the possibility of contamination during the PCR set up.

The Monterey pine aphid DNA extraction was a positive control for both the SSU rDNA and the 16S rDNA PCR, with amplification of the correct product size occurring in both reactions. The successful amplification of the Monterey pine aphid confirmed that both primer combinations were working. PCR of the bacterial colony failed in both the SSU rDNA and the 16S rDNA PCR, although amplification of the 1.5 kb product was expected for the 16S rDNA PCR. The presence of the large (>10 kb) genomic DNA band on the agarose gel suggested that the PCR was loaded with too many bacterial colony cells, resulting in the inhibition of the PCR. The positive amplification of the bacterial colony DNA with the 16S rDNA primers was confirmed in a later PCR (data not shown).

The amplification of 16S rDNA primers in grape phylloxera DNA extractions sourced from potted vines suggests that the root material from where the insects are collected may impact upon the presence of the amplified bacteria. The potted vine provided the most 'natural' environment for grape phylloxera, with the insect surrounded by soil and feeding on a relatively mature whole plant system. Excised roots, although initially collected from a soil environment, are removed from a soil environment prior to insect infestation, and are restricted from a whole plant response. Tissue culture vines provide a whole plant response, although the root system is relatively immature, and in a sterile soil-less environment.

If the bacteria detected in the potted vine DNA extractions 'infected' the grape phylloxera from the external environment, then this would not be present in the sterile tissue culture system, explaining the non-amplification result for the tissue culture DNA extraction. The previous amplification of the 16S rDNA primers with grape phylloxera tissue culture DNA extractions (data not shown) may represent a contaminated tissue culture environment, or the retention of the bacterial association from a previous infection. Vorwerk, *et al.* (2006)

identified bacterial DNA in field collected adult gallicolae grape phylloxera, and in their parthenogenetically produced eggs. The presence of bacterial DNA in the eggs if the insect suggests internal transfer of the micro-organism by transovarial transmission. Investigation of the presence of bacterial DNA in the eggs of radicicolae grape phylloxera was not attempted in this project, leaving the issue of internal transference unresolved, however the absence of bacterial DNA in the excised root samples places doubt on this possibility.

The excised roots used for collection of grape phylloxera for DNA extraction were nutritionally poor, with the grape phylloxera population observed to have a reduced fecundity and growth rates compared with 'typical' excised roots described in earlier studies (Powell *et al.*, 2006). The causes for this poor population growth were unknown, but interestingly, previous DNA extractions of grape phylloxera collected from typical excised roots were amplified with the 16S rDNA primers (data not shown). The absence of the bacterial association with grape phylloxera on poor quality excised root pieces highlights the potential that there may be a correlation between vine health, insect development and the presence of bacteria.

The transient detection of bacterial DNA in grape phylloxera has similarities to the observation of rod-shaped organisms in *Phylloxera quercus*. These organisms were concluded to be of low significance with only occasional occurrence (Buchner, 1965). Grape phylloxera do not have an association with the Aphididae primary endosymbiont *Buchnera*, and previous molecular studies have failed to locate the presence of secondary or tertiary Aphididae endosymbionts (Chen *et al.*, 1996; Chen and Purcell, 1997). Speculatively however, there remains the possibility that a non-essential, transient bacterial relationship developed in grape phylloxera after the evolutionary divergence from the aphid family. Transient bacteria may be ingested with food, and either lost with the passing of waste, or during life stage moulting (Douglas, 1998). The transient detection of the bacterial DNA in radicicolae grape phylloxera indicates that it is not essential for the survival of the insect, although the bacterial infection of an insect is expected to have some physiological and ecological impacts (Sandstrom *et al.*, 2001).

The possible bacterial association with radicicolae grape phylloxera presented here potentially represents a different bacterial association to the one with gallicolae grape phylloxera identified by Vorwerk *et al.* (2006). The sequencing of the 16S rDNA PCR product is required to classify the bacterial species associated with radicicolae grape phylloxera. With the genetic identification of the bacterial species, relationships with the bacterial association found in the gallicolae grape phylloxera (Vorwerk *et al.*, 2006) can be interpreted.

5 CONCLUSION

Radicicolae grape phylloxera collected from potted vines amplify with general bacteria 16S rDNA primers. The identity of this bacteria is unknown, but may represent a bacterial association with grape phylloxera. Grape phylloxera do not contain Aphididae bacterial endosymbionts, however there is the possibility that an alternative bacterial endosymbiont might exist. The impact or function of the bacterial endosymbiont is unknown, but the transient identification of bacterial DNA in grape phylloxera DNA extractions suggests that the association is not essential. The presence of the bacterial endosymbiont under potted vine conditions needs to be investigated further as this could potentially impact on grape phylloxera development under field conditions, and could potentially impact on the reliability of rootstock screening under laboratory conditions.

6 RECOMMENDATIONS

With the exception of a recent study on gallicolae grape phylloxera (Vorwerk *et al.*, 2006), most references suggest that symbiotic associations do not occur in grape phylloxera. Grape phylloxera do not contain the bacterial endosymbionts previously identified in Aphididae, however there is the possibility that an alternative bacterial association may exist. This area requires further investigation for radicicolae grape phylloxera to identify the bacteria by sequencing the 16S rDNA PCR product, and to determine the level of the association (primary or secondary endosymbiont) that exists. If a transient bacterial relationship exists, as suggested by this study, it may not play an essential role in insect nutrition, but the impacts of the relationship on the development and nutritional physiology of grape phylloxera still need to be examined. Studies using symbiotic and aposymbiotic grape phylloxera could assist in determining the relative importance of the bacteria to the insect, and the bacterial relationship to its host. A comparison of bacterial endosymbiont relationships in radicicolae and gallicolae grape phylloxera is also warranted to assist in understanding the underlying mechanisms of rootstock resistance.

A molecular investigation for bacterial DNA in grape phylloxera eggs produced by adults feeding on excised roots, potted vines and tissue culture would confirm the method of transfer for the endosymbiont, and determine if transovarial transmission occurs. The method of transmission will provide insight into the necessity of the bacterial association for grape phylloxera development. Maternal inheritance (via transovarial transmission) would imply that the symbiotic relationship was essential.

This study has purely focused on one grape phylloxera genotypic class, G4. It has also not taken into account the potential impact of soil conditions on the bacterial association. Once a bacterial endosymbiont has clearly been identified, further studies need to be conducted using a range of grape phylloxera genotypic classes collected from grapevines grown under different soil conditions. An investigation into the impact of genotypic class and soil type on the occurrence of the bacterial association may assist in determining the impact of the bacterial endosymbiont on grape phylloxera development.

OVERSEAS TRAVEL REPORT

The financial budget for this project included expenditure for overseas travel to meet with researchers for discussions and training to extend the capabilities of experimental techniques required for the successful completion of the project. This overseas travel coincided with attendance at an international conference for a poster presentation. Below is an overview of the meetings undertaken during travel between 13 June – 19 July 2005.

UNIVERSITY OF VIENNA (AUSTRIA)

Dr Astrid Forneck leads a laboratory researching grape phylloxera host-plant adaptation and grapevine quality control. Discussions with Dr Forneck highlighted a number of scientific approaches undertaken by the group that parallel research undertaken at DPI Rutherglen, including the areas of symbiont detection and artificial diet development. An exchange program between the research laboratories was initiated with a 6-month placement of a Masters student (Karin Treder) in October 2005 to work on the development of rootstock screening protocols using selected clonal lineages of grape phylloxera. Ms Treder was based to DPI Rutherglen under the joint supervision of Dr Forneck and Dr Kevin Powell (DPI Rutherglen). This component of research is reported in the GWRDC Final Report – Project 03/03. The visit to the laboratory of Dr Forneck strengthened relationships between the two research groups. Dr Forneck also visited Australia in October 2005 to present two papers at the 3rd International Phylloxera Symposium in Fremantle, Western Australia. Knowledge gained during the visit to the laboratory of Dr Forneck assisted with the reporting in Sub-project 1.1 and 2.2.

GEISENHEIM RESEARCH INSTITUTE (GERMANY)

Geisenheim Research Institute is the location of various grapevine-breeding programs, including the development of Börner, the only rootstock reported immune to grape phylloxera damage. Professor Ernst Ruhl shared knowledge of the development of the rootstock breeding programs, highlighting differences between grape phylloxera damage under European conditions in comparison with Australia. Dr Tatjana Wolf is researching the hypersensitive response of Börner to grape phylloxera damage to isolate the genetics responsible for the resistance mechanisms. Dr Bernd Loskill and Dagmar Heibertshausen are investigating early warning environmental management systems to reduce the impact of fugal diseases on grapevine production. This research is linked with the reduced application of sulphur and copper sprays in vineyards. Current European regulations are limiting applications of these treatments due to environmental pollution concerns. Knowledge regarding the molecular identification of rootstock material was forwarded onto GWRDC Project 03/03.

UNIVERSITY OF MAINZ (GERMANY)

Dr Lars Huber is researching a range of alternative grape phylloxera management options, including the application of vineyard mulches and utilisation of biological control agents. Mulch application has recorded dramatic improvements in vine health on grape phylloxera damaged vineyards, although similar results are yet to be observed under Australian conditions. Successful laboratory and field based trials have occurred for the biological control of grape phylloxera. No biological control system is currently available commercially. The application of this approach to Australian conditions may prove to be a valuable management option for the limitation of grape phylloxera damage. There is currently no research conducted in Australia on the potential biological control of grape phylloxera.

In the past 10 years Germany has witnessed a number of dramatic changes in the occurrence of pest and disease populations, with the main influence expected to be due to climate change. 'Wild', abandoned vineyards have also contributed to pest and disease outbreaks. As the viticulture industry in Australia becomes saturated, the occurrence of wild vineyards may also emerge as an issue in Australia. Wild vineyards, together with climate change, may influence (and increase) pest and disease numbers within Australian viticulture. Dr Huber supplied a poster for presentation at the 3rd International Phylloxera Symposium in Fremantle, Western Australia, in October 2005.

UNIVERSITY OF HOHENHEIM (GERMANY)

Sonja Vorweck is completing a PhD investigating possible grape phylloxera host-plant adaptation to grapevine rootstocks. Another component of this research as been the discovery of a bacteria species within the salivary glands of gallicolae (leaf-galling) grape phylloxera populations. Previous to this research grape phylloxera had been documented to be absent of all symbiont relationships. The direct association is still being investigated, but the possible presence of these bacteria in Australian radicicolae (root-galling) grape phylloxera populations highlights a possible area for future research. Knowledge gained during the visit with Sonja Vorweck assisted with the reporting in Sub-project 2.2.

UNIVERSITY OF WAGENINGEN (THE NETHERLANDS)

Dr Freddy Tjallingii (Department of Entomology, Wageningen University) is a leading researcher in the field of electrical penetration graph (EPG). Characterising EPG waveform patterns under the guidance of Dr Tjallingii was invaluable for the continued progress in this area at DPI Rutherglen, and the in-depth technical advice obtained in the Netherlands could not have been acquired within Australia. EPG has not previously been attempted on grape phylloxera, and as a consequence no waveform interpretation or correlation studies have been performed. Following a 2-week training course, a number of grape phylloxera EPG waveforms have now been characterised. Discussions highlighted the limitations of a number of correlation techniques in application for grape phylloxera research. Knowledge gained during the visit to the laboratory of Dr Tjallingii assisted with the reporting in Sub-project 1.2.

SOCIETY OF EXPERIMENTAL BIOLOGY ANNUAL MEETING: BARCELONA (SPAIN)

The Society of Experimental Biology annual meeting (SEB) conference in Barcelona consisted of 27 sessions across a broad range of sciences, and attracted over 400 researchers from across Europe, America and Australasia. The awarding of a competitive student travel grant by SEB supported attendance at this conference. The poster presentation sessions were well attended, receiving a high level of interest in the grape phylloxera research undertaken at DPI Rutherglen. Abstracts from the meeting have been published in a supplementary edition of the Journal of Comparative Biochemistry and Physiology, widening the availability of information beyond those in attendance. The SEB conference created the opportunity to reconnect with a number of researchers met previously at the International Congress of Entomology in Brisbane, August 2004. Continuing discussions with Dr Glenn Powell and Dr Greg Walker allowed for the improvement of a number of current techniques and clarification of ideas for potential projects. Additionally, a number of new contacts were made, which were further developed during the 7th International Symposium on Aphids and 3rd International Symposium on Phylloxera in Fremantle, October 2005.

APPENDIX 1: COMMUNICATION

CONFERENCE PROCEEDINGS PAPERS

Kingston, K, Cooper, P & Powell, K. (2006). "Digestion for reproduction – the grape phylloxera model." Acta Horticulturae. (submitted)

Kingston, K, Powell, K & Cooper, P. (2006). "Characterising the root feeding habits of grape phylloxera using electrophysiological techniques." Acta Horticulturae. (submitted)

INTERNATIONAL CONFERENCE ABSTRACTS – ORAL PRESENTATIONS

Kingston, K, Powell, K & Cooper, P. (2005). "Characterising the root feeding habits of grape phylloxera using electrophysiological techniques." 3rd International Phylloxera Symposium. Perth: 13.

Kingston, K, Powell, K & Cooper, P. (2004). "Development of an in vitro feeding system for root-feeding Hemiptera." 22nd International Congress of Entomology. Brisbane.

Kingston, K, Powell, K & Cooper, P. (2004). "Characterisation of the digestive system of Grape Phylloxera (Hemiptera: Phylloxeridae)." 22nd International Congress of Entomology. Brisbane

Kingston, K. (2004). "Phylloxera and EPG." 5th International EPG Workshop-Training Course. Brisbane.

INTERNATIONAL CONFERENCE ABSTRACTS – POSTER PRESENTATIONS

Kingston, K, Cooper, P, Kemper, D & Powell, K. (2005). "Digestion for reproduction – the grape phylloxera model." 3rd International Phylloxera Symposium. Perth: 28.

Kingston, K, Cooper, P, Kemper, D & Powell, K. (2005). "Digestion for reproduction – the grape phylloxera model." 7th International Aphid Symposium. Perth: 62.

Kingston, K, Powell, K & Cooper, P. (2005). "Investigating the digestive function and feeding behaviour of grape phylloxera." Society of Experimental Biology Annual Main Meeting. Barcelona, Spain: Comparative Biochemistry and Physiology. 141A(3S): S116

NATIONAL CONFERENCE ABSTRACTS – ORAL PRESENTATIONS

Kingston, K, Powell, K & Cooper, P. (2005). "Grape phylloxera digestive function and feeding behaviour." Australian Entomological Society's 36th Scientific Conference. Canberra: 141.

Kingston, K. (2004). "Investigating the nutritional physiology of grape phylloxera (Hemiptera: Phylloxeridae)." Ecology, Evolution & Systematics Postgraduate Student Conference. Australian National University, Canberra

Kingston, K. (2003). "Understanding the fundamental interactions between grape phylloxera and *Vitis* species." The Australian National University School of Botany and Zoology PhD Student Symposium. Canberra.

NATIONAL CONFERENCE ABSTRACTS – POSTER PRESENTATIONS

Kingston, K, Cooper, P, Kemper, D & Powell, K. (2005). "Digestion for reproduction the grape phylloxera way." 3rd Annual Postgraduate and Postdoctoral Symposium: Bring Biosciences Together. Canberra: 38.

Kingston, K, Powell, K & Cooper, P. (2004). "Interactions between grape phylloxera and grapevine species." 12th Australian Wine Industry Technical Conference. Melbourne: 52.

Kingston, K, Powell, K & Cooper, P. (2003). "Morphological studies of grape phylloxera (Hemiptera: Phylloxeridae)". 34th Australian Entomological Society and 6th Invertebrate Biodiversity and Conservation 2003 Scientific Conference. Hobart: 50.

Kingston, K, Powell, K & Cooper, P. (2003). "Understanding the fundamental interactions between grapevine phylloxera and *Vitis* species", DPI Horticulture Conference. Tatura.

Kingston, K, Powell, K & Cooper, P. (2003). "Understanding the Fundamental Interactions between Grapevine Phylloxera and *Vitis* spp". 1st Annual DPI/DSE Entomology Symposium. Rutherglen.

INDUSTRY ORAL PRESENTATIONS

Kingston, K. (2004, 2005, 2006). "Research update." Annual Phylloxera Workshops, Nagambie, Milawa and Rutherglen.

Kingston, K. (2004). "Understanding the fundamental interactions between grape phylloxera and *Vitis* species (DNR 01/3)." GWRDC Research Update presentation to Jim Fortune. Rutherglen.

INDUSTRY ARTICLES

National GrapeGrowers, June 2005. "Study seeks to kick phylloxera in the guts" by Mark Osborne.

The Australian and New Zealand Grapegrower and Winemaker, May 2003. "New research into phylloxera" by Kim Kingston.

OTHER ORAL PRESENTATIONS

Kingston, K. (2004). "Adventure of the phylloxera." Department of Primary Industries – Rutherglen Centre Seminar Series. Rutherglen

Kingston, K. (2003). "Understanding the fundamental interactions between grape phylloxera and *Vitis* species." Department of Primary Industries – Plant Health Statewide Leaders. Rutherglen.

Kingston, K. (2003). "Root-soil environment; natural and introduced constraints." Secretary of Department of Primary Industries. Rutherglen.

Kingston, K. (2003). "Phylloxera – an insect pest for viticulture." Primary Industries Research Victoria Research Directors Strategic Meeting. Rutherglen.

RELATED PUBLICITY

Chronica Horticulturae, volume 45 (3) 2005. "Third International Grapevine Phylloxera Management Symposium" by Kevin Powell.

South Australian ABC Limestone Coast Rural Report, February 24 2005. Radio interview with Kim Kingston. "The latest research on phylloxera using gold filaments" by David Claughton.

The Weekly Times, March 17 2004. "A new look at hungry vine pests" by Kate Fisher.

The Corowa Free Press, March 17 2004. "PhD student recognised".

AWARDS

2006 Finalist: Dr Kevin Powell and the Phylloxera Research team, Department of Primary Industries Daniel McAlpine Outstanding Achievement Award.

2005 Recipient: Kim Kingston, Society of Experimental Biology (SEB) post-graduate and young scientist travel grant (£561).

2005 Recipient: Kevin Powell, Department of Primary Industries Science honours list.

2004 Recognition: Kim Kingston, Department of Primary Industries International Women's Day Women in Science honours list.

APPENDIX 2: INTELLECTUAL PROPERTY

The Corporation and the Research Organisation agree that the Corporation's share of title to all intellectual property and project income will be allocated on a percentage basis according to the proportion of funding for the project provided by the Corporation in relation to the funding provided by the Research Organisation.

APPENDIX 3: REFERENCES

References are presented by sub-project reporting section.

BACKGROUND

Powell, K. S. and Whiting, J. (2000). Proceedings of the International Symposium on Grapevine Phylloxera Management, Melbourne, Department of Natural Resources and Environment. 110.

SUB-PROJECT 1.1

Akey, D. H. and Beck, S. D. (1971). "Continuous rearing of the pea aphid, *Acyrthosiphon pisum*, on a holidic diet." Annals of the Entomological Society of America **64** (2): 353-356.

Auclair, J. L. (1965). "Feeding and nutrition of the pea aphid, *Acyrthosiphon pisum* (Homoptera: Aphidae), on chemically defined diets of various pH and nutrient levels." Annals of the Entomological Society of America **58** (6): 855-875.

Cohen, A. C. (2003). Insect Diets: Science and Technology, Boca Raton, CRC Press. 324.

Debolt, J. W. (1982). "Meridic diet for rearing successive generations of *Lygus hesperus*." Annals of the Entomological Society of America **75** (2): 119-122.

Febvay, G., Delobel, B. and Rahbe, Y. (1988). "Influence of the amino acid balance on the improvement of an artificial diet for a biotype of *Acyrthosiphon pisum* (Homoptera: Aphididae)." Canadian Journal of Zoology **66** 2449-2453.

Forneck, A., Kleinmann, S., Blaich, R. and Anvari, S. F. (2002). "Histochemistry and anatomy of phylloxera (*Daktulosphaira vitifoliae*) nodosities in young roots of grapevine (*Vitis* spp.)." Vitis **41** (2): 93-97.

Forneck, A. and Wohrle, A. (2003). "A synthetic diet for phylloxera (*Daktulosphaira vitifoliae* Fitch)." Acta Horticulturae **617** (Proceedings of the Workshop on Rootstocks' Performance in Phylloxera Infested Vineyards): 129-134.

Gatehouse, A. M. R., Gatehouse, J. A. and Boulter, D. (1980). "Isolation and characterisation of trypsin inhibitors from cowpea." Phytochemistry **19** 751-756.

Gatehouse, A. M. R., Fenton, K. A., Jepson, I. and Pavey, D. T. (1986). "The effects of amylase inhibitors on insect storage pests: Inhibition of amylase *in vitro* and effects on development *in vivo*." Journal of Science, Food and Agriculture **37** 727-734.

Gatehouse, A. M. R., Powell, K. S., Peumans, W. J., Van Damme, E. J. M. and Gatehouse, J. A. (1995). Insecticidal properties of plant lectins: Their potential in plant protection. Lectins: Biomedical Perspectives. Pusztai, A. J. and Bardocz, S. London, Taylor & Francis. 35-57.

Granett, J., Timper, P. and Lider, L. A. (1985). "Grape phylloxera (*Daktulosphaira vitifoliae*) (Homoptera: Phylloxeridae) biotypes in California." Journal of Economic Entomology **78** (6): 1463-1467.

Jancovich, J. K., Davidson, E. W., Lavine, M. and Hendrix, D. L. (1997). "Feeding chamber and diet for culture of nymphal *Bemisia argentifolii* (Homoptera: Aleyrodidae)." Journal of Economic Entomology **90** (2): 628-633.

Kellow, A. V. (2000). A study of the interaction between susceptible and resistant grapevines and phylloxera. Department of Horticulture, Viticulture and Oenology. Adelaide. 182.

Kellow, A. V., McDonald, G., Corrie, A. and Van Heeswijck, R. (2002). "*In vitro* assessment of grapevine resistance to two populations of phylloxera from Australian vineyards." Australian Journal of Grape and Wine Research **8** (2): 109-116.

Mittler, T. E. and Dadd, R. H. (1962). "Artificial feeding and rearing of the aphid, *Myzus persicae* (Sulzer), on a completely defined synthetic diet." Nature **195** 404.

Mittler, T. E. (1988). Applications of artificial feeding techniques for aphids. Aphids: their biology, natural enemies and control. Minks, A. K. and Harrewijn, P. Elsevier. **B**: 145-169.

Powell, K. S., Gatehouse, A. M. R., Hilder, V. A. and Gatehouse, J. A. (1993). "Antimetabolic effects of plant lectins and plant and fungal enzymes on the nymphal stages of two important rice pests, *Nilaparvata lugens* and *Nephotettix cinciteps*." Entomologia Experimentalis et Applicata **66** 119-126.

R.DevelopmentCoreTeam (2006). "R: A Language and Environment for Statistical Computing", version 2.3.1. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org (11 October 2006).

Ryan, F. J., Omer, A. D., Aung, L. H. and Granett, J. (2000). "Effects of infestation by grape phylloxera on sugars, free amino acids, and starch of grapevine roots." Vitis **39** 175-176.

Sasaki, T., Hayashi, H. and Ishikawa, H. (1991). "Growth and reproduction of the symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum* maintained on artificial diets." Journal of Insect Physiology **37** (10): 749-756.

Srivastava, P. N. and Auclair, J. L. (1971). "Influence of sucrose concentration on diet uptake and performance by the pea aphid, *Acyrthosiphon pisum*." Annals of the Entomological Society of America **64** (3): 739-743.

Stoll, M., Loveys, B. and Dry, P. (2000). "Hormonal changes induced by partial rootzone drying of irrigated grapevine." Journal of Experimental Botany **51** (350): 1627-1634.

van Emden, H. (2003). "Twenty-six years without plant food - an aphid still going strong!" Antenna: Bulletin of the Royal Entomological Society **27** (2): 95-98.

Wohrle, A. (1999). Versuche zur kunstlichen Ernahrung von Reblausen (Dactulosphaira vitifoliae FITCH). Institut fur Obst-, Gemuse- und Weinbau. Stuttgart-Hohenheim. 66.

SUB-PROJECT 1.2

Backus, E. A., Habibi, J., Yan, F. and Ellersieck, M. (2005). "Stylet penetration by adult *Homalodisca coagulata* on grape: Electrical penetration graph waveform characterization, tissue correlation, and possible implications for transmission of *Xylella fastidiosa*." Annals of the Entomological Society of America **98** (6): 787-813.

Boubals, D. (1994). "Phylloxera (*Daktulosphaira vitifoliae*) adaptation to some rootstocks of the grapevine." Brighton Crop Protection Conference - Pests and Diseases **3** 963-966.

Cline, A. R. and Backus, E. A. (2002). "Correlations among AC electronic monitoring waveforms, body postures, and stylet penetration behaviours of *Lygus hesperus* (Hemiptera: Miridae)." Environmental Entomology **31** (3): 538-549.

Cole, R. A., Riggall, W. and Morgan, A. (1993). "Electronically monitored feeding behaviour of the lettuce root aphid *(Pemphigus bursarius)* on resistant and susceptible lettuce varieties." Entomologia Experimentalis et Applicata **68** 179-185.

Corrie, A. M., Crozier, R. H., van Heeswijck, R. and Hoffman, A. A. (2002). "Clonal reproduction and population genetic structure of grape phylloxera, *Daktulosphaira vitifoliae*, in Australia." Heredity **88** 203-211.

Corrie, A. M. (2003). Genetic structure of grape phylloxera populations in Australia. School of Molecular Sciences. Bundoora. 135.

Davidson, W. M. and Nougaret, R. L. (1921). "The grape phylloxera in California." United States Department of Agriculture **Bulletin No. 903** 1-127.

De Klerk, C. A. (1974). "Biology of *Phylloxera vitifoliae* (FITCH) (Homoptera: Phylloxeridae) in South Africa." Phytophylactica **6** 109-118.

Forneck, A., Walker, M. A. and Merkt, N. (1996). "Aseptic dual culture of grape (*Vitis* spp.) and grape phylloxera (*Daktulosphaira vitifoliae* Fitch)." Vitis **35** (2): 95-97.

Granett, J., Timper, P. and Lider, L. A. (1985). "Grape phylloxera (*Daktulosphaira vitifoliae*) (Homoptera: Phylloxeridae) biotypes in California." Journal of Economic Entomology **78** (6): 1463-1467.

Granett, J., Goheen, A. C., Lider, L. A. and White, J. J. (1987). "Evaluation of grape rootstocks for resistance to Type A and Type B grape phylloxera." American Journal of Enology and Viticulture **38** (4): 298-300.

Guo, F. and Zhao, Z. (2000). "Feeding behaviour of omethoate-resistant spider mites (Acari: Tetranychidae): a study using electrical penetration graphs." Systematic and Applied Acarology **5** 3-7.

Harrewijn, P., Tjallingii, W. F. and Mollema, C. (1996). "Electrical recording of plant penetration by western flower thrips." Entomologia Experimentalis et Applicata **79** 345-353.

Harrewijn, P., Piron, P. G. M. and Ponsen, M. B. (1998). "Evolution of vascular feeding in aphids: an electrophysiological study." Proceedings of Experimental and Applied Entomology **9** 29-34.

Jiang, Y. X. and Walker, G. P. (2003). "Electrical penetration graphs of the nymphal stage of *Bemisia argentifolii*." Entomologia Experimentalis et Applicata **109** (101-111):

Kellow, A. V., McDonald, G., Corrie, A. and Van Heeswijck, R. (2002). "*In vitro* assessment of grapevine resistance to two populations of phylloxera from Australian vineyards." Australian Journal of Grape and Wine Research **8** (2): 109-116.

Kindt, F., Joosten, N. N., Peters, D. and Tjallingii, W. F. (2003). "Characteristics of the feeding behaviour of western flower thrips in terms of electrical penetration graph (EPG) waveforms." Journal of Insect Physiology **49** 183-191.

Kingston, K., Powell, K. and Cooper, P. (2005). "Investigating the digestive function and feeding behaviour of grape phylloxera." Comparative Biochemistry and Physiology **141A** (Number 3/Suppl.): S116.

Lei, H., Tjallingii, W. F., van Lenteren, J. C. and Xu, R. M. (1996). "Stylet penetration by larvae of the greenhouse whitefly on cucumber." Entomologia Experimentalis et Applicata **79** 77-84.

McLean, D. L. and Kinsey, M. G. (1964). "A technique for electronically recording aphid feeding and salivation." Nature **202** 1358-1359.

Pollard, D. G. (1973). "Plant penetration by feeding aphids (Hemiptera, Aphidoidea): a review." Bulletin of Entomological Research **62** 631-714.

Powell, K. S., Slattery, W. J., Deretic, J., Herbert, K. and Hetherington, S. (2003). "Influence of soil type and climate on the population dynamics of grapevine phylloxera in Australia." Acta Horticulturae **617** (Proceedings of the Workshop on Rootstocks' Performance in Phylloxera Infested Vineyards): 33-41.

Powell, K. S., Korosi, G. A., Trethowan, C. J. and White, V. (2006). Sustainable long-term strategies for phylloxera management under Australian conditions. Department of Primary Industries. Rutherglen. 158.

Reese, J. C., Tjallingii, W. F., Van Helden, M. and Prado, E. (2000). Waveform comparisons among AC and DC electronic monitoring systems for aphid (Homoptera: Aphididae) feeding behaviour. Principles and Applications of Electronic Monitoring and Other Techniques in the Study of Homopteran Feeding Behaviour. Walker, G. P. and Backus, E. A. Thomas Say Publications in Entomology. 70-101.

Sandanayaka, W. R. M. and Hale, C. N. (2003). "Electronically monitored stylet penetration pathway of woolly apple aphid, *Eriosoma lanigerum* (Homoptera: Aphididae), on apple (*Malus domestica*)." New Zealand Journal of Crop and Horticultural Science **31** 107-113.

Schmid, J. and Ruhl, E. H. (2003). "Performance of *Vitis cinerea* hybrids in motherblock and nursery - preliminary results." Acta Horticulturae **617** (Proceedings of the Workshop on Rootstocks' Performance in Phylloxera Infested Vineyards): 141-145.

Tjallingii, W. F. (2000). Comparison of AC and DC systems for electronic monitoring of stylet penetration activities by Homopterans. Principles and Applications of Electronic Monitoring and Other Techniques in the Study of Homopteran Feeding Behaviour. Walker, G. P. and Backus, E. A. Thomas Say Publications in Entomology. 41-69.

Van Helden, M. and Tjallingii, W. F. (2000). Experimental design and analysis in EPG experiments with emphasis on plant resistance research. Principles and Applications of Electronic Monitoring and Other Techniques in the Study of Homopteran Feeding Behaviour. Walker, G. P. and Backus, E. A. Thomas Say Publications in Entomology. 144-171.

Walker, G. P. (2000). A beginner's guide to electronic monitoring of Homopteran probing behavior. Principles and Applications of Electronic Monitoring and Other Techniques in the Study of Homopteran Feeding Behaviour. Walker, G. P. and Backus, E. A. Thomas Say Publications in Entomology. 14-40.

SUB-PROJECT 2.1

Backus, E. A. (1988). "Sensory systems and behaviours which mediate Hemipteran plant-feeding: A taxonomic review." Journal of Insect Physiology **34** (3): 151-165.

Breider, H. (1952). "Beitrage zur Morphologie und Biologie der Reblaus *Dactylosphaera vitifolii* Shim." Zeitschrift fur angewandte Entomologie **33** 517-543.

Buchanan, G. A. (1990). The distribution, biology and control of grape phylloxera, *Daktulosphaira vitifolii* (Fitch), in Victoria. Department of Zoology, School of Biological Sciences. Melbourne. 179.

Davidson, W. M. and Nougaret, R. L. (1921). "The grape phylloxera in California." United States Department of Agriculture **Bulletin No. 903** 1-127.

De Klerk, C. A. (1974). "Biology of *Phylloxera vitifoliae* (FITCH) (Homoptera: Phylloxeridae) in South Africa." Phytophylactica **6** 109-118.

Federov, S. M. (1959). "The biological basis of Phylloxera (Dactylosphaera vitifolii Schim., Homoptera, Phylloxeridae) control." Entomological Review **38** 74-85.

Forneck, A., Walker, M. A. and Blaich, R. (2001). "Ecological and genetic aspects of grape phylloxera *Daktulosphaira vitifoliae* (Hemiptera: Phylloxeridae) performance on rootstock hosts." Bulletin of Entomological Research **91** 445-451.

Granett, J., Bisabri-Ershadi, B. and Carey, J. (1983). "Life tables of phylloxera on resistant and susceptible grape rootstocks." Entomologia Experimentalis et Applicata **34** 13-19.

Granett, J., Timper, P. and Lider, L. A. (1985). "Grape phylloxera (*Daktulosphaira vitifoliae*) (Homoptera: Phylloxeridae) biotypes in California." Journal of Economic Entomology **78** (6): 1463-1467.

Granett, J., Walker, M. A., Kocsis, L. and Omer, A. D. (2001). "Biology and management of grape phylloxera." Annual Review of Entomology **46** 387-412.

Gullan, P. J. and Cranston, P. S. (2005). The Insects: an outline of entomology, Third edition. Blackwell Publishing. 505.

Kellow, A. V. (2000). A study of the interaction between susceptible and resistant grapevines and phylloxera. Department of Horticulture, Viticulture and Oenology. Adelaide. 182.

Kellow, A. V., Sedgley, M. and Van Heeswijck, R. (2004). "Interaction between *Vitis vinifera* and Grape Phylloxera: Changes in root tissue during nodosity formation." Annals of Botany **93** (5): 581-590.

Kemper, D. (2004). Morphological and physiological analysis of phylloxera, *Daktulosphaira vitifoliae*: Midgut analysis using microscopic and histochemical techniques. Botany and Zoology. Canberra. 45.

Ponsen, M. B. (1987). Alimentary Tract. Aphids: their biology, natural enemies and control. Minks, A. K. and Harrewijn, P. Elsevier. **A**: 79-97.

Ponsen, M. B. (1997). "A histological description of the alimentary tract and related organs of Phylloxeridae (Homoptera, Aphidoidea)." Wageningen Agricultural University Papers **97-1** 1-77.

Rasband, W. (2005). "ImageJ", version 1.34s. National Institutes of Health, USA. URL http://rsb.info.nih.gov/ij/ (11 October 2006).

Riley, C. V. (1870). "The grape-leaf gall-louse (*Phylloxera vitifoliae* Fitch)." The American Entomologist and Botanist **2** (12): 353-359.

Schaller, G. (1960). "The amino acid content of the salivary gland secretion of the vine phylloxera, *Viteus vitifolii* Shimmer, Homoptera." Entomologia Experimentalis et Applicata **3** 128-136.

Sobetskiy, L. A. and Derzhavina, M. A. (1973). "A contribution to the study of the physiology of the feeding of the vine phylloxera, *Viteus vitifolii* Fitch (Homoptera: Phylloxeridae)." Entomological Review **52** 357-361.

Tjallingii, W. F. and Hogen Esch, T. (1993). "Fine structure of aphid stylet routes in plant tissue in correlation with EPG signals." Physiological Entomology **18** 317-328.

SUB-PROJECT 2.2

Buchner, P. (1965). Endosymbiosis of animals with plant microorganisms, Interscience Publishers.

Chen, D.-Q., Campbell, B. C. and Purcell, A. H. (1996). "A new Rickettsia from a herbivorous insect, the pea aphid *Acyrothosiphon pisum* (Harris)." Current Microbiology **33** 123-128.

Chen, D.-Q. and Purcell, A. H. (1997). "Occurrence and transmission of facultative endosymbionts in aphids." Current Microbiology **34** 220-225.

Cook, L. G., Gullan, P. J. and Trueman, H. E. (2002). "A preliminary phylogeny of the scale insects (Hempitera: Sternorrhyncha: Coccoidea) based on nuclear small-subunit ribosomal DNA." Molecular Phylogenetics and Evolution **25** 43-52.

Corrie, A. M., Crozier, R. H., van Heeswijck, R. and Hoffman, A. A. (2002). "Clonal reproduction and population genetic structure of grape phylloxera, *Daktulosphaira vitifoliae*, in Australia." Heredity **88** 203-211.

Dojka, M. A., Harris, J. K. and Pace, N. R. (2000). "Expanding the known diversity of environmental distribution of an uncultured phylogenetic division of bacteria." Applied and Environmental Microbiology **66** (4): 1617-1621.

Douglas, A. E. (1998). "Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*." Annual Review of Entomology **43** 17-37.

Granett, J., Timper, P. and Lider, L. A. (1985). "Grape phylloxera (*Daktulosphaira vitifoliae*) (Homoptera: Phylloxeridae) biotypes in California." Journal of Economic Entomology **78** (6): 1463-1467.

Gullan, P. J. and Cranston, P. S. (2005). The Insects: an outline of entomology, Third edition. Blackwell Publishing. 505.

Kellow, A. V., McDonald, G., Corrie, A. and Van Heeswijck, R. (2002). "*In vitro* assessment of grapevine resistance to two populations of phylloxera from Australian vineyards." Australian Journal of Grape and Wine Research **8** (2): 109-116.

Korosi, G. A., Trethowan, C. J. and Powell, K. S. (2006). "Screening for grapevine rootstock resistance to phylloxera genotypes from Australian vineyards under controlled conditions." Acta Horticulturae **submitted**

Martinez-Torres, D., Baudes, C., Latorre, A. and Moya, A. (2001). "Molecular systematics of aphids and their primary endosymbionts." Molecular Phylogenetics and Evolution **20** (3): 437-449.

Powell, K. S., Korosi, G. A., Trethowan, C. J. and White, V. (2006). Sustainable long-term strategies for phylloxera management under Australian conditions. Department of Primary Industries. Rutherglen. 158.

Sandstrom, J. A., Russell, J. A., White, J. P. and Moran, N. A. (2001). "Independent origins and horizontal transfer of bacterial symbionts of aphids." Molecular Ecology **10** 217-228.

Vorwerk, S., Blaich, R. and Forneck, A. (2006). "Pantoea ssp. an associated bacteria common in grape phylloxera (*Daktulosphaira vitifoliae*) Fitch." Acta Horticulturae **accepted October 2006**

APPENDIX 4: STAFF

PROJECT STAFF

Staff involved in the project activities included Dr Kevin Powell (project supervisor), Kim Kingston (PhD student), based at DPI-Rutherglen, and Dr Paul Cooper (student supervisor), based at ANU.

ACKNOWLEDGMENTS

Acknowledgment is made of the assistance and support provided by past and present colleagues of the grape phylloxera research team at DPI-Rutherglen, including Anna Burns, Annette Blanchfield, Carolyn Trethowan, Ginger Korosi, Karen Herbert, Rob Bederian and Sarah Brown.

Dr Alvin Milner and Sorn Norng (DPI) provided biometric support.

Neda Plovanic (ANU) provided technical support and training for light microscopy sectioning. Electron microscopy images were capture with the use of the facilities at the ANU Electron Microscopy Unit, under the direction and experience of Dr Roger Heady.

Dr Trudy Wharton (ANU) provided the Monterey pine aphids used as a positive control for the detection of bacterial symbionts. Dr Lyn Cook (ANU), Dr Daryl Nelson and Dr Andrew Oxley (DPI) provided assistance and advice in the area of molecular symbiont detection.

Dr Freddy Tjallingii (Wageningen University) provided training in the use and analysis of the EPG technique. Dr Elaine Backus (USDA) and Dr Greg Walker (University of California – Riverside) provided additional support and guidance.

Peter Clingleffer (CSIRO, Merbein) provided grapevine root material for grape phylloxera population maintenance, and EPG trials. Börner rootstock material was used in agreement with Professor Ernst Ruhl (Geisenheim Research Institute, Germany)

APPENDIX 5: RAW DATA

No additional data or material to provide.

APPENDIX 6: BUDGET RECONCILIATION