COMPARATIVE MORPHOGENESIS OF CYNIPID LEAF GALLS INDUCED BY *Diplolepis polita* AND *Diplolepis nebulosa* (HYMENOPTERA: CYNIPIDAE) AND MODIFICATION BY INQUILINES OF THE GENUS *Periclistus* (HYMENOPTERA: CYNIPIDAE)

by

Brandy Fenwick

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Thesis Examiners/Examinateurs de thèse:

Dr. Joseph D. Shorthouse
(Supervisor/Directeur de thèse)

Dr. Yves Alarie
(Committee member/Membre du comité)

Dr. Peter Ryser
(Committee member/Membre du comité)

Dr. Arthur E. Weis
(External Examiner/Examinateur externe)

Approved for the School of Graduate Studies
Approuvé pour l'École des études supérieures
Dr. David Lesbarrères
M. David Lesbarrères
Director, School of Graduate Studies
Directeur, École des études supérieures

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ABSTRACT

Cynipid galls are atypical plant growths induced by wasps in the family Cynipidae that provide larvae with shelter and nutrition. Larvae gain control of attacked plant organs and send them on a new developmental trajectory, with three developmental phases known as initiation, growth, and maturation. Each of the approximately 1400 species of cynipid gall wasps manipulates plant tissues in a slightly different manner such that galls of each species are structurally distinct. Although the means by which cynipids initiate galls has fascinated naturalists for hundreds of years, the basic events in gall induction are still poorly understood.

In an attempt to understand the galling strategies and developmental processes responsible for species-specific galls, I chose to compare the intimate details of life history strategies of two taxonomically related species attacking the same plant organs. The strategies included host specificity, phenology, and oviposition strategies, along with gall development to highlight basic events in gall biology and reveal possible stages in past speciation events. It is argued that differences in phenology and gall development played a role in driving speciation and thus, the patterns observed today are a result of extensive ecological interactions in the past that have influenced the evolution of these complex insect-plant relationships.

Two species of cynipid wasps of the genus *Diplolepis* that occur on the wild roses of central Ontario proved to be ideal candidates for the study. One species, *Diplolepis polita*, induces single-chambered, prickly galls found in clusters on the adaxial surface of leaflets of *Rosa acicularis* whereas, the other species, *D. nebulosa*, induces single-

chambered, smooth-surfaced galls found in clusters on the abaxial surface of the leaflets of *Rosa blanda*. Galls at all stages of development, from freshly oviposited eggs to maturation, were found in large numbers for both species. Leaf tissues from the bud stage to maturity of both species of rose were fixed in FAA, embedded in paraffin, sectioned and stained for histological study. Likewise, leaf tissues with freshly oviposited eggs of both species and galls from immaturity to maturity were fixed, sectioned, and stained.

Comparing the several thousand slides made for the study revealed that galls of *D. polita* and *D. nebulosa* differ in their developmental events as well as the anatomy of their mature galls. Like the galls of all species of cynipids, those of *D. polita* and *D. nebulosa* are composed of distinct layers of gall cells known as nutritive, parenchymatous nutritive, sclerenchyma, cortex, and epidermis. Galls of *D. polita* consist of nutritive cells, parenchymatous nutritive cells, and an epidermis throughout the initiation and growth phases. Larvae remain small in relation to chamber volume until the maturation phase, when a hard layer of sclerenchyma differentiates. In contrast, galls of *D. nebulosa* have a delayed initiation phase, where galls remain nearly undetectable on leaflets for several weeks after oviposition before they enter the growth phase. Freshly-hatched larvae are protected by two layers of bowl-shaped patches of sclerenchyma that differentiates soon after initiation, along with nutritive cells and parenchymatous nutritive cells that surround the larval chambers, but galls of this species develop without an external layer of epidermis. Galls become spherical as they mature and a second layer of sclerenchyma differentiates within the walls of the galls, as does a layer of spongy cortex that appears between the second layer of sclerenchyma and the gall exterior. Larvae of *D. nebulosa* occupy nearly the entire volume of their larval chambers throughout gall development.

The size of each type of cell found within developing galls of both species, from gall initiation to gall maturity were measured and compared. Cells in the galls of both species continue to increase in size throughout development; however, the cells comprising the galls induced by *D. polita* are significantly larger than those induced by *D. nebulosa.* Comparing the biologies and galls of these two species, demonstrates how niche partitioning has occurred. It also shows that striking differences in structures occur within the galls of closely taxonomically related species as a result of differences in adult phenology, oviposition strategies, egg placement, and environmental conditions such as moisture levels. Although it has been proposed by other authors that diversity in cynipid galls results from differentiation of tissues found in the outer parts of galls, such as cortex and epidermis, the present study indicates the reasons are more complex. Furthermore, it is apparent that many aspects of gall development and anatomy have been overlooked by previous researchers and a variety of ecological factors contribute to differences in gall structure.

To further complicate the already complex series of events that occur over the course of gall development, galls of *D. polita* and *D. nebulosa* are inhabited and structurally modified by inquilines of the genus *Periclistus*. These insects are also cynipid wasps, and have evolved a close relationship with *Diplolepis* galls whereby they kill the inducer larvae, feed on gall cells, and change the developmental trajectory of attacked galls. Galls of *D. polita* and *D. nebulosa* are attacked by two undescribed, but gall-specific species of *Periclistus*. Here, the inquiline associated with the galls of *D. polita* is referred to as *Periclistus* 1 and the inquiline associated with galls of *D. nebulosa* is referred to as *Periclistus* 2. The purpose of this study was to histologically examine all phases of

modification by the two species of *Periclistus* to establish the events that are developmentally unique to inquilines.

Periclistus 1 and 2 are phenologically distinct as *Periclistus* 1 oviposits into immature galls of *D. polita* in late May soon after galls are induced, and *Periclistus* 2 oviposits into immature galls of *D. nebulosa* in July. Modified galls of *D. polita* differ from modified galls of *D. nebulosa* as they are significantly enlarged compared to normal galls. *Periclistus* 1 chambers are arranged around the periphery of the inner gall whereas modified galls of *D. nebulosa* are of a similar size to normal galls. Chambers of *Periclistus* 2 are evenly distributed throughout the inner gall. *Periclistus* 1 and 2-modified galls undergo four phases of development identified as the egg phase, gall enlargement, chamber formation, and maturation phases. Both *Periclistus* 1 and 2 oviposit into immature galls, killing the inducer larvae with their ovipositors, and then the presence of *Periclistus* eggs along the inner chamber surface cause changes in gall structure. *Diplolepis*-induced nutritive cells degrade and *Diplolepis*-induced parenchymatous nutritive cells enlarge. Galls become significantly enlarged compared to those inhabited by inducer larvae and then feeding by first-instar *Periclistus* larvae stimulates the differentiation and proliferation of *Periclistus*-induced parenchymatous nutritive cells and nutritive cells. Immature larvae of both species of *Periclistus* initially feed around the inner surface of the *Diplolepis*-induced chamber, and then restrict their feeding to one spot. This results in cell proliferation such that each larva becomes restricted to the centre of a bowl-shaped growth of cells. Continued proliferation causes *Periclistus* nutritive and parenchymatous nutritive cells to rise up and completely encase the larvae. As this is occurring in modified galls of *D. polita*, a layer of sclerenchyma, referred to here as the

inquiline-induced primary sclerenchyma, differentiates and circumscribes the periphery of the entire gall. This does not occur in galls of *D. nebulosa* until maturity. In modified galls of both species, nutritive cells and parenchymatous nutritive cells appear in dense clusters throughout the inside surface of *Periclistus* chambers. Once modified galls enter the maturation phase, inquiline-induced primary sclerenchyma differentiates, circumscribing the periphery of galls of *D. nebulosa.* In addition, *Periclistus* 1 and 2-inhabited galls both develop a second layer of inquiline-induced sclerenchyma, known as secondary sclerenchyma, around each inquiline chamber. Secondary sclerenchyma cells in the walls of *Periclistus* chambers are smaller than primary sclerenchyma cells circumscribing the entire gall.

Gall cells induced by *Periclistus* 1 are larger than those induced by *Periclistus* 2; however, gall cells induced by both species of *Periclistus* are larger than those of their host *Diplolepis* galls. Based on differences in phenology, gall development, and final gall structure, modified galls of *D. polita* and *D. nebulosa* are anatomically distinct with each species of *Periclistus* responsible for gall tissues that are species-specific. In addition, the developmental pattern of *Periclistus*-modified galls is distinct from that of *Diplolepis* galls, illustrating the level of control inquilines have over the tissues of their host galls.

This thesis demonstrates the complex nature of the interrelationships between cynipid wasps of the genera *Diplolepis* and *Periclistus* and their host roses. *Diplolepis* are true gall inducers that have an intimate relationship with the genus *Rosa* and there are many attributes of the genus *Rosa* that have contributed to the success of *Diplolepis* and allowed for their extensive radiation and divergence in their galls. Similarly, *Periclistus*

inquilines have an intimate relationship with *Diplolepis* and the rose hosts. *Periclistus* have evolved the ability to manipulate rose tissues that have previously been under the influence of *Diplolepis.* Based on two species of inquilines examined in this thesis, *Periclistus* have also evolved an array of phenologies and modification strategies and like the developmental patterns of the *Diplolepis*, the developmental trajectories of *Periclistus* are also species-specific. This project has shown that the histological approach to studying cynipid galls, as well as those occupied by *Periclistus* inquilines, is highly rewarding and contributes to our overall understanding of these fascinating insects.

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CHAPTER I: INTRODUCTORY MATERIAL

A. GENERAL INTRODUCTION

The extent to which insects have become specialized to a particular food source is one of the most remarkable features of insect-plant relationships and after more than 100 million years of coevolution, a variety of phytophagous guilds have appeared including chewers, sap feeders, borers, miners, and gall inducers (Kennedy and Southwood 1984). Each of these guilds are amazingly diverse and speciose, where about half the total number of all species are plant-feeding insects (Schoonhoven *et al.* 2005). Most phytophagous insects have become specialized to a particular host plant and often to a particular plant organ making this one of the most remarkable features of the entire natural world (Schoonhoven *et al.* 2005). The most highly specialized feeding strategy is that of the gall-inducers where insects have evolved the ability to stimulate the proliferation of plant cells of their host organs to induce specialized structures known as galls (Raman 2007, 2011). Whereas most phytophagous insects must move about their hosts while feeding, gall inducers are sedentary and feed exclusively on nutrient rich cells they have 'designed' from the tissues of attacked organs (Raman 2007, 2011; Shorthouse 2010). In contrast, all other phytophagous insects such as chewers, leaf miners and stem borers cause structural damage and loss of biomass, but cause no proliferation of plant cells (Schoonhoven *et al.* 2005).

Insect galls are atypical plant growths that provide nourishment, shelter, and protection to the immatures of the inducers (Raman 2007; Shorthouse *et al.* 2005). Although insect galls were once considered the result of defensive encapsulation by the plant in response

to insect feeding (see historical literature in Raman *et al.* 2005; Raman 2007), it is now widely accepted that gall-inducing insects are in control of plant development and redirect the growth of plant tissues to the benefit of the insect (Raman 2011). More remarkably, each of the 13 000 species of gall-inducing insects, which account for approximately 2% of all insects (Shorthouse *et al.* 2005), induce galls with structural features that are species-specific (Stone and Schönrogge 2003; Raman 2011). That is, each species of gall-inducing insect is responsible for such distinct galls that the species of gall-inducer can often be identified without examining the inducers within (Raman 2011). It is common to find galls induced by members of several orders of insects on the same plant and the extent and success of the guild is illustrated by the nearly 15 000 species of vascular plants that are host to gallers of at least one species of insect (Raman *et al*. 2005). Furthermore, when individuals of the same species induce their galls on the wrong host plant, the resulting galls are morphologically consistent with those found on the correct host (Shorthouse 1988). Even more complex; some gall insects induce sexually dimorphic galls where galls induced by females are much larger than those induced by males (Dorchin *et al.* 2009), and others exhibit alternation of generations (sexual and asexual generations) whereby the individuals of one generation induce galls that are structurally distinct galls from those induced by the other generation (Stone *et al.* 2002; Bailey *et al.* 2009).Thus, although galls are composed of tissues belonging to their plant, their development is controlled by the genes of the insect rather than the plant (Stone and Cook 1998; Raman *et al.* 2005; Bailey *et al.* 2009) and as a result, galls are considered an 'extended phenotype' of the galling insect (Dawkins 1982).

The gall-induction strategy has evolved independently through different routes among the insect orders Thysanoptera, Hemiptera, Coleoptera, Diptera, Lepidoptera, and Hymenoptera (Raman 2007). The order Diptera contains the most species of gall-inducers, with the largest adaptive radiation occurring in the family Cecidomyiidae (Dreger-Jauffret and Shorthouse 1992). Second to the order Diptera in gall-inducer diversity is the order Hymenoptera, with the largest adaptive radiation occurring within the family Cynipidae. Cynipids, also commonly known as gall wasps, induce the most differentiated and morphologically complex galls within the gall-inducing guild (Csóka *et al*. 2005; Raman 2011).

Galls range in structural complexity from slight depressions to elaborate growths and one of the oldest classification systems is based on morphological criteria. Organoid galls are induced by aphids and some non-insect organisms (parasitic fungi and mites), and are slight modifications of normal organs, where the attacked organ remains recognizable and internal anatomy is not completely disrupted (Meyer and Maresquelle 1983). Histoid galls are characterized by dedifferentiation and/or redifferentiation of plant tissues to produce novel organ-like structures (Meyer and Maresquelle 1983; Rohfritsch 1992). The latter can be further separated into kataplasmas and prosoplasmas. Kataplasmas have indeterminate growth, lacking a consistent form or period of development (Meyer and Maresquelle 1983). These galls are typically induced by hemipterans and are often less differentiated than the host organ, consisting of several layers of parenchymatous tissue (Rohfritsch 1992). Prosoplasmas have determinate growth, a definitive size and shape, and a brief period of development (Meyer and Maresquelle 1983). These galls are highly differentiated and their tissues are fundamentally different from the host organ; having

undergone a constructive rather than degenerative process (Raman 2011). Prosoplasmas may be monothalmus (single-chambered), having one larva per gall surrounded by layers of specialized gall tissues, or polythalmus (multi-chambered), where several larvae inhabit a gall, each situated within its own larval chamber, surrounded by specialized gall tissues. All cynipids induce prosoplasmic galls (Meyer and Maresquelle 1983; Rohfritsch 1992).

Biologists have long wondered how galls such as those induced by cynipids may have evolved and the question as to why galls develop at all remains challenging and daunting (Raman *et al*. 2005). However, cynipid wasps are thought to be derived from parasitoid ancestors that fed internally on stem-boring hosts (Ronquist 1995; Ronquist and Liljeblad 2001; Csóka *et al.* 2005). Ronquist (1995) suggested that the shift from entomophagy to phytophagy could have occurred as an adaptation to food shortage and proposed that when their hosts died prematurely, these early parasitoids were able to complete their development by feeding on nearby plant tissues. It is then argued that these early cynipids shifted from stem-boring insect-hosts, to those living in more nutritious plant tissues such as fruits, seeds, and flowers in herbaceous plants. As endoparasitoids, they were likely capable of manipulating their insect hosts through female secretions at oviposition or substances produced by the developing larva itself. Once ancestral gall wasps shifted from an entomophagous to phytophagous lifestyle, they next evolved the ability to manipulate the development of their plant hosts, thus producing galls (Ronquist 1995; Csóka *et al.* 2005).

The first cynipid galls are thought to have been simple, single-chambered swellings within reproductive tissues such as fruits, seeds, or flowers of herbaceous plants that

lacked secondary surface adornments (Ronquist and Liljeblad 2001; Csóka *et al.* 2005). Cynipid galls have since evolved into a striking diversity of complex structures comprising multiple, highly differentiated layers of plant tissues supplied by vascular bundles and covered by secondary surface structures such as extra-floral nectaries, spines, sticky resins, and coatings of hair (Stone and Schönrogge 2003). Even though it is understood that cynipid wasps are in control of gall development, the driving force behind diversification of gall morphology remains unclear (Raman *et al*. 2005; Raman 2007).

One approach to understanding how cynipids manipulate their host plants into producing species-specific galls is to examine the developmental patterns associated with their galls using histological techniques (Shorthouse 1993; Brooks and Shorthouse 1998; Leggo and Shorthouse 2006a). The structures of many species of cynipid galls have been studied in the past (Meyer and Maresquelle 1983); however, there are few studies following all events from the deposition of eggs by cynipid females to the maturation of their galls. At the beginning of this thesis project, it was envisioned that a study of the life-history strategies of two closely taxonomically related cynipid gall wasps found sympatrically in central Ontario, would be the best approach to assess the developmental events associated with their galls, and would provide much useful information as to the developmental events responsible for the diverse gall morphologies observed today. To this end, I searched for two species of leaf gallers that appeared to have galls with different anatomy and once they were located, I settled for a detailed study of the galls of *Diplolepis polita* (Ashmead), a species that induces prickly, single-chambered galls on the adaxial surface of leaflets of *Rosa acicularis* Lindl., and *Diplolepis nebulosa* (Bassett), a species that

induces smooth, single-chambered galls on the abaxial surface of leaflets of *Rosa blanda* Ait..

As if the development of new organ-like structures on plants is not complex enough, it is also known that cynipid galls support a number of secondary insect inhabitants that either feed on inducer larvae or the gall tissues (Csóka *et al.* 2005, Shorthouse 2010). Those that feed on the gall inducer are known as parasitoids and are found either in the hymenopteran superfamily Chalcidoidea or the family Ichneumonidae and can be major mortality factors (Csóka *et al.* 2005; Shorthouse 2010). Galls of some species of cynipids are also inhabited by inquiline phytophagous insects which feed on gall tissues to complete their development. Inquilines are defined as tenants or guests from the Latin *Inquilinus* (Yang *et al.* 2000). All tribes within the Cynipoidea are gall inducers, except for the inquilines which belong to the tribe Synergini (Csóka *et al.* 2005). Cynipid inquilines have been termed agastoparasites by Ronquist (1994) because of the close phylogenetic relationship with their hosts. These wasps apparently lost the ability to initiate their own galls but retained the ability to induce larval chambers within galls induced by other species (Ronquist 1994).

Both leaf galls used in this study are attacked by cynipid inquilines of the genus *Periclistus* (Shorthouse 2010). *Periclistus* are known to significantly modify the structures of the galls they inhabit. For example, Brooks and Shorthouse (1998) showed that single-chambered galls induced by *Diplolepis nodulosa* (Beutenmüller) attacked by the inquiline *P. pirata* Osten Sacken are inhabited by an average of 17 inquiline larvae and become three times larger than those inhabited by inducers. Previous studies of inquiline modification in cynipid galls have compared modified galls to those inhabited

by inducer larvae (Shorthouse 1998; Brooks and Shorthouse 1998; Le Blanc and Lacroix 2001); however, none have compared modification strategies and developmental morphologies of inquiline-modified galls of two closely taxonomically related species of inducers. Thus, the objective for this section was to compare the developmental events of galls of *D. polita* and *D. nebulosa* that had been attacked by different species of *Periclistus* to explore the possibility that different species of inquilines have species-specific strategies as they modify the galls in which they inhabit.

Comparing the anatomy of galls of two closely related species of *Diplolepis* presents the opportunity to ask some basic questions about galling insects. For example, what developmental events occur that result in the galls of the two species found on the same host organ, but of different hosts, becoming structurally different? This study presented the opportunity to observe two species of wild roses throughout the growing season, and since it was already known that wild roses are host to 13 species of *Diplolepis* across Canada (Shorthouse 2010), I became curious as to the characteristics of roses that lead to their being a platform for the evolution of an entire genus of gallers. I also came to realize that studying galls on wild roses would contribute to our understanding of shrub biology and my questioning the anatomical and physiological features of roses that make them such successful shrubs. These questions will be answered in Chapter IV.

B. BIOLOGY OF ROSES

I. BIOLOGY AND ECOLOGY OF WILD ROSES

Wild roses belong to the genus *Rosa* (Family Rosaceae) and are mainly found across northern North America, Europe and Asia, between 20 and 70° latitude with the centre of

the genus in central and southwestern Asia (Krüssmann 1981). They are of the most common shrubs across Canada, extending from the U.S.A./Canada border north to the edge of the tree line.

Wild roses are erect, deciduous, perennial shrubs capable of clonal growth from underground rhizomes and tend to form thickets. Stems are usually bristly and armed with dense prickles (Soper and Heimburger 1982). Roses have prickles and not thorns, thorns being modified stems made from the same cells as the stem itself, whereas, prickles are comparable to hairs, sometimes being quite coarse, and are extensions of the epidermis and cortex. Leaves of roses are alternate and pinnately compound with five to seven serrate leaflets fused to the petiole for most of their length. Rose flowers are large, fragrant, and perfect with five parted, pink to red petals (Soper and Heimburger 1982). Flowers are usually borne singly or in groups of two or three, and generally bloom from late spring to early summer and are pollinated by insects. The fruits, known as hips, are red with a fleshy or pulpy receptacle surrounding the seeds, called achenes (Soper and Heimburger 1982). Seeds are hard, have stiff hairs along one side, and are resistant to damage by the digestive systems of the many vertebrates that feed on them. Hips are variable in shape (elliptic, globose, or pyriform) and often persist on plants throughout the winter (Soper and Heimburger 1982).

Wild roses are considered keystone species in many landscapes as they reinforce relationships with many other plants, mammals, birds and insects that inhabit the area (Shorthouse 2010). The fragrant flowers attract bees, flies and many beetles, not because of nectar (roses flowers are without nectaries), but rather the copious amounts of protein-rich pollen. Rose thickets also provide cover, nesting sites and protection for many

species of wildlife. All above-ground parts are eaten by a variety of herbivores ranging from large mammals such as deer, bears, and moose that eat branches and hips, to insects occupying the leaf chewing, leaf sucking, leaf mining, and stem boring guilds (Shorthouse 2010). Seeds do not germinate during the first spring because they must undergo warm stratification during the summer followed by cold stratification the second winter. After cold stratification, seeds germinate over a wide range of temperatures soon after snowmelt, taking advantage of early spring moisture and growing vigorously at low temperatures (Schori 2003).

Roses are well adapted to survival and dispersal in the harsh conditions of north temperate regions of the world (van Groenendael *et al.* 1997). Although the number of seeds is small, large seed size contributes to rapid production of a large root system. Plants then spread vegetatively by rhizomes over a wide area, forming clones that may persist for hundreds of years (van Groenendael *et al.* 1997). Although roses produce many fine roots in the top 20 cm of soil, deep roots may reach 140 cm (Strong and LaRoi 1986). Calmes and Zasada (1982) found rhizomes 20-30 cm below the surface. Rhizomes are protected at this depth allowing them to resprout following fire or other disturbances.

The genus *Rosa* is notoriously difficult and taxonomically complex (Bruneau *et al.* 2007) because of variability within species and the existence of multiple reproductive strategies, ranging from apomixis (replacement of normal reproduction by asexual reproduction) to hybridization and outcrossing (Lewis 1959; Wissemann 2003). In addition, they exhibit different modes of character inheritance such as intra-specific variability and polyploidy (Erlanson-MacFarlane 1966; Bruneau *et al*. 2007). The boundaries between species of

wild roses have always been difficult to define because where some rose taxonomists saw polymorphism, others saw distinct species. Depending on the author, between 14 and 4000 species have been proposed; however, it is generally accepted that the genus consists of 150-200 morphospecies (Bruneau *et al*. 2007).

The taxonomic complexity of roses makes the genus a model in which simple concepts of radiation, speciation and taxonomy come to their limits. For example, the biological species concept is not applicable, as all species are inter-fertile and produce viable and fertile offspring (Wissemann and Ritz 2007). As a result, taxonomists of the genus *Rosa* apply two concepts of classification: a morphospecies-based system that focuses on morphological differentiation, and an evolutionary system which combines numerous sources of evidences including molecular data (Bruneau *et al.* 2007).

The genus *Rosa* has been divided into 10 sections (Wissemann 2003), three of which include roses that are found in Canada. The section Carolinae is composed of 5 species in North America, four of which (*R. carolina* L., *R. nitida* Willd., *R. pulustris* Marsh, *R. virginiana* Herrm.) occur in Canada. The section Cinnamomeae is composed of about 80 species in Asia, Europe and North America. Species found in Canada are *R. acicularis, R. arkansana* Porter, *R. blanda, R. gymnocarpa* Nutt. ex Torr. Et A. Gray, *R. nutkana* C. Presl., *R. pisocarpa* A. Gray and *R. woodsii* Lindl. Another rose in this section, *R. rugosa* Thunb. is endemic to Japan and was intentionally introduced into Canada as a garden plant and has since become feral in various regions. The section Synstylae is composed of approximately 25 species, however, only *R. setigera* Michx. is found in Canada. In addition, the section Caninae is composed only of European species; however, two of the

50 species, *R. rubigenosa* L. and *R. canina* L., were introduced into Canada and have become feral in some areas. Species of wild rose found in Ontario are *R. acicularis*, *R. blanda*, *R. pulustris*, *R. carolina*, and *R. setigera*.

Most of the roses grown in the botanical gardens of the world come from wild roses in Asia, the same area where about 60% of domesticated flowers, fruit trees and vegetables originated (Krüssmann 1981; Cairns 2003). About eight species of Asian wild roses are the source of the domesticated shrubs that were altered by breeding and their flowers turned into the hundreds of varieties enjoyed in botanic gardens throughout North America and Europe, the former since ancient times (Cairns 2003). For the past two centuries, ornamental and cut flowers from such roses have become the centre of a multi-billion dollar horticultural industry of ornamental and cut flowers (Cairns 2003). Domesticated roses are also a major source of essential oils for perfumes and their hips have been used for human food throughout recorded history (Cutler 2003).

II. BIOLOGY OF *Rosa acicularis*

Rosa acicularis, commonly known as the prickly rose, has the widest geographic range of any rose in the world (Lewis 1957, 1959; Shorthouse 2010). It is circumboreal and occurs in all provinces in Canada west of New Brunswick (Shorthouse 2010). It grows in a wide variety of forested and open habitats across its range with various soil and moisture conditions. It is drought and disturbance tolerant, fire-resistant, and is considered an early-successional plant where it readily colonizes habitats soon after disturbance. Seed production may be low, especially in shaded habitats, but plants spread readily by rhizomes, forming large clones that persist for decades and even centuries. Shrubs are

typically found in meadows, clearings, and open woods, and in disturbed areas along roadsides, lakeshores and river banks. It is the most widely distributed rose in Ontario, found from the arctic tundra along the shores of Hudson Bay and James Bay, south to Windsor. In northern Ontario, it is most typically found in ditches along roadsides (Fig. 1.1) or bordering forest edges in abandoned agricultural fields (Fig. 1.2). It is a bushy shrub that grows 1-2 m tall with reddish stems and branches that are densely armed with numerous slender, straight prickles that are 3-4 mm in length (Soper and Heimburger 1982; Shorthouse 2010). Leaves are pinnately compound, typically with five to seven leaflets and a pair of conspicuous stipules that are fused to the petiole for most of their length (Fig. 1.3). Leaflets average two to five centimeters in length and are dull green with an ovoid to elliptical shape. Flowers have five pink petals that are two to three centimeters long, are generally solitary, and grow at the ends of branches of the previous year (Soper and Heimburger 1982). *Rosa acicularis* appears to have a relatively early and shorter blooming period than other roses (Schori 2003). In northern Ontario, flowers of *R. acicularis* bloom from late May to mid June (Fig. 1.7). Hips are ovoid or globular in shape, average 2 cm in diameter, and become bright red and glaborous as they mature from late summer to early fall (Soper and Heimburger 1982).

Figs. 1.1 – 1.3: Habitats of *Rosa acicularis* in Northern Ontario and a representative of a mature leaf. All photographs were made in July. **Fig. 1.1.** Dense patch of *R. acicularis* growing in a ditch along a roadside near Timmins, Ontario. **Fig. 1.2.** *R. acicularis* bordering the edge of a forest in an abandoned agricultural field near Rouyn-Noranda, Quebec. **Fig. 1.3.** Mature leaf of *R. acicularis* showing flat broad leaflets.

III. BIOLOGY OF *Rosa blanda*

Rosa blanda, commonly known as the smooth rose, is a perennial, deciduous species native to North America found from eastern Saskatchewan to Nova Scotia and south from Missouri to Pennsylvania (Bruneau *et al.* 2005). It is a calcareous species; typically found growing in sandy or rocky soils of pastures, meadows, clearings, and along roadsides (Soper and Heimburger 1982); whereas, other species of roses native to northeastern North America are generally associated with acidic soils (Fernald 1918). Plants that are heavily galled by *D. nebulosa* are most commonly found growing in ditches along roadsides (Fig. 1.4) or on sand dunes (Fig. 1.5). Shrubs can reach 1.5 m in height with stems and branches reddish-purple in colour. The common name comes from the rose having a stem with no prickles, or very few at the base of the plant. Leaves are pinnately compound with five, seven, or rarely nine leaflets, with conspicuous stipules (Fig. 1.6). Leaflets are dull green, have an elliptical shape, and average 1 – 4.5 cm in length. Flowers are typically solitary with few in clusters on previous year branches. Petals are pink and broadly wedge-shaped, and average two to three centimetres in length (Soper and Heimburger 1982). Flowers of *R. blanda* bloom in central Ontario from early June to early July (Fig. 1.7). Hips of *R. blanda* are ovoid, 1 – 1.5 cm in diameter and mature in late summer to early fall, becoming red and glaborous (Soper and Heimburger 1982).

From a taxonomic standpoint, *R. blanda* is extremely variable and has been subdivided into many species (Bruneau *et al.* 2005). For example, it has been suggested that *R. blanda* is the same species as *R. woodsii*, which is found only in the grasslands of the prairies where it has stems covered with thick prickles (Joly and Bruneau 2007). Joly and Bruneau (2007) concluded the species is polymorphic and there is a gradient of characters

that are expressed from west to east. Despite this controversy, individuals of *R. blanda* in Ontario are morphologically consistent with the species description and there is little to no variability among individuals. It is important to note that despite confusion within this species, as well as within the entire genus, individuals of *R. blanda* and *R. acicularis* are morphologically distinct in Ontario.

C. ANATOMY AND DEVELOPMENTAL PATTERNS OF CYNIPID GALLS

I. ANATOMY OF CYNIPID GALLS

The general pattern of the anatomy for mature galls of all species of cynipids is a series of well defined cell types in concentric layers around the larval chamber. From the surface of the larval chambers, these layers are known as nutritive tissue, parenchymatous nutritive tissue, vascular tissue, sclerenchyma tissue, cortical parenchyma tissue, and epidermal tissue (Meyer and Maresquelle 1983; Rohfritsch 1992; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001; Sliva and Shorthouse 2005).

Nutritive cells are the sole source of food for developing larvae and are rich in proteins, sugars, and lipids (Bronner 1992; Rohfritsch 1992; Csóka *et al.* 2005). They are large, thin-walled cells characterized by dense cytoplasm, fragmented vacuoles, hypertrophied nucleus/nucleolus, and abundant organelles (Bronner 1992; Raman 2011). Cynipid larvae consume nutritive cells evenly around the larval chamber. They do not consume whole nutritive cells and instead, slice them with their mandibles and imbibe the contents and thus chamber walls become lined with layers of collapsed cells (Roth 1949). Adjacent to the layer of nutritive cells is a layer of vacuolated parenchymatous nutritive cells which

are converted into nutritive cells as they are consumed (Rohfritsch 1992). Parenchymatous nutritive cells contain starch; however, nutritive cells do not as inducer larvae produce enzymes that convert starch in into soluble sugars in the nutritive cells. Thus, cynipid larvae never consume starch (Roth 1949).

A layer of thick-walled, hard sclerenchyma tissue next circumscribes galls. Sclerenchyma cells form by the deposition of lignin in secondary cell walls after cells have completed their growth. Sclerenchyma cells have a reduced lumen and the cytoplasm and nucleus are absent. Sclerenchyma formation in galls is thought to have evolved to provide protection for inducer larvae from ovipositing parasitoids (Stone and Cook 1998; Ronquist and Liljeblad 2001; Stone and Schönrogge 2003; Csóka *et al.* 2005; Bailey *et al.*2009). The sclerenchyma layer may also prevent other herbivores from consuming gall tissues and rupturing the larval chamber because lignified tissues are unsuitable for consumption (Csóka *et al.* 2005) and provide structural support to gall tissues and vascular bundles (Roth 1949). The differentiation of sclerenchyma can determine when a galled plant organ abscises from the host which may be of importance for inducer larvae within galls that abscise and overwinter in the leaf litter (Csóka *et al.* 2005). Sclerenchyma may also serve to protect gall wasp larvae from freezing by reducing moisture and ice nucleation in larval chambers during the winter (Williams *et al.* 2002).

Figs. 1.4 – 1.6: Habitats of *Rosa blanda* on Manitoulin Island, Ontario and a representative of a mature leaf. All Photographs were made in July. **Fig. 1.4.** Dense patch of *R. blanda* in a roadside ditch near Sheguiandah, Manitoulin Island, Ontario. **Fig. 1.5.** *R. blanda* on the sand dunes of Providence Bay, Manitoulin Island, Ontario. **Fig. 1.6.** Mature leaf of *R. blanda* showing curled and folding leaflets characteristic of leaves later in the season and when exposed to dry conditions.

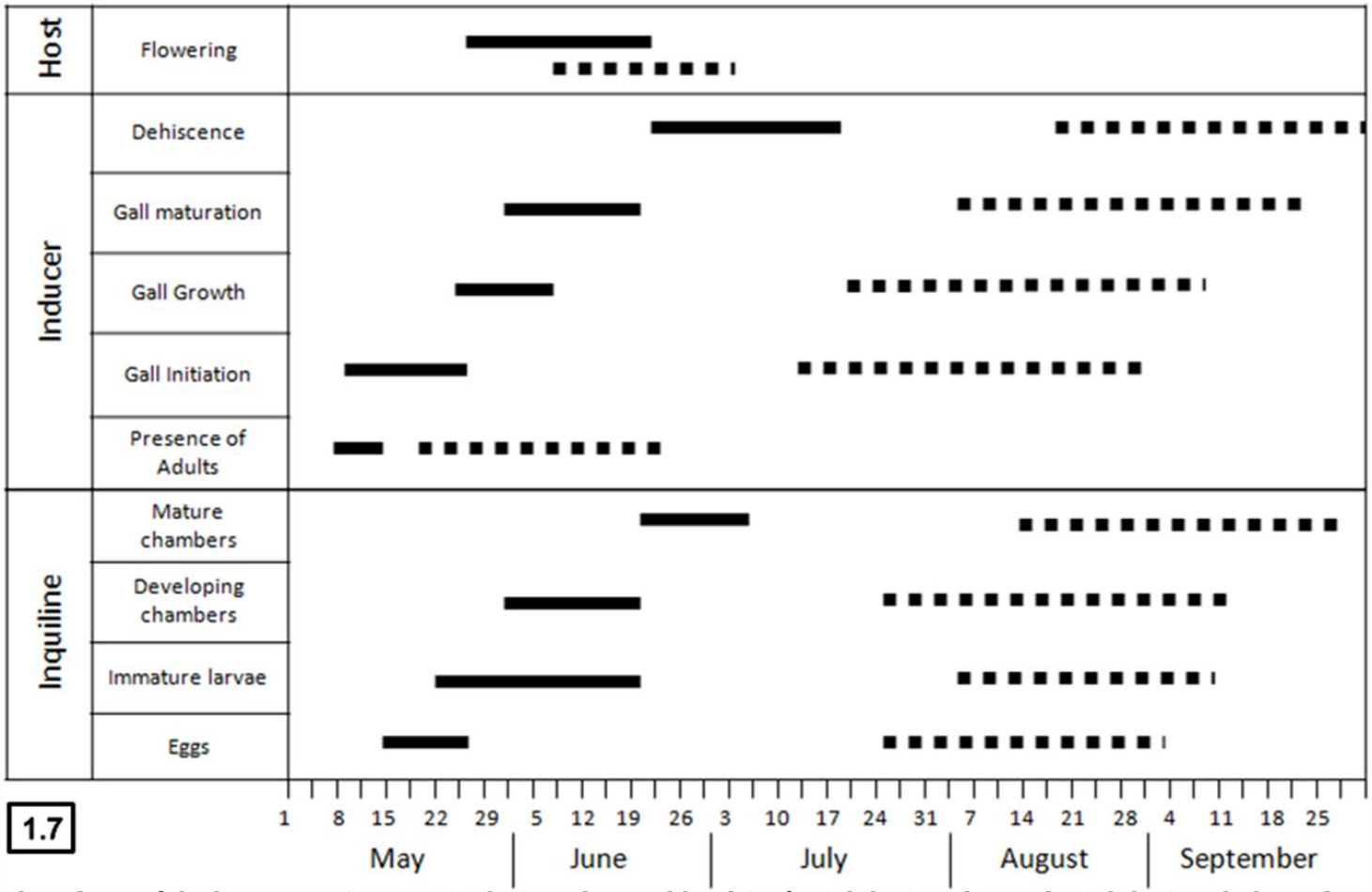


Fig. 1.7: Phenology of the host roses (*Rosa acicularis* and *Rosa blanda*) of *Diplolepis polita* and *Diplolepis nebulosa,* development of galls of *D. polita* and *D. nebulosa,* and the modifications made to each gall by *Periclistus.* Data were collected through field observations, gall dissections, and sweeping rose patches for the presence of *Diplolepis* adults. Solid lines represent data associated with galls of *D. polita* and dotted lines represent data associated with galls of *D. nebulosa.* Data for *D. polita* were compiled over the 2009, 2010, and 2011 field seasons; whereas, data associated with *D. nebulosa* were compiled over the 2010, 2011, and 2012 field seasons.

The layers of nutritive, parenchymatous nutritive, and sclerenchyma cells combined are called the 'inner gall' whereas tissue outside the sclerenchyma layer is referred to as the 'outer gall' (Roth 1949). Beyond the sclerenchyma layer is a multi-cellular layer of cortical parenchyma cells that are bound by a single-cellular epidermis (Csóka *et al.* 2005). These tissues contain a large amount of tannins, but no starch (Roth 1949). It is typically outer gall tissues that are heavily modified and are considered to be responsible for the diversity in external gall features (Stone and Schönrogge 2003; Bailey *et al.* 2009). Secondary surface adornments such as extra-floral nectaries, spines, sticky resins, and coatings of hair are examples of epidermal layer modification (Stone and Schönrogge 2003).

II. GALL DEVELOPMENT

Cynipid gall morphogenesis is divided into three phases: initiation, growth and maturation; each of which is characterized by distinct tissue types that develop in response to inducer larvae (Rohfritsch 1992; LeBlanc and Lacroix 2001). The initiation phase begins at oviposition when plant development becomes under the control of the insect, and ends with the formation of the larval chamber (Rohfritsch 1992; Bronner 1992). The growth phase is characterized by the differentiation of nutritive and vascular tissue and a rapid increase in gall biomass (Rohfritsch 1992; Bronner 1992). Gall maturation is characterized by differentiation of a sclerenchyma layer and cessation of gall growth (Rohfritsch 1992). The duration of each developmental phase varies among species, host plant, and season when galls are induced. For example, galls induced in the spring typically develop more quickly than those induced in late summer or early autumn (Bronner 1992).

A majority of gall-inducing insects initiate their galls through feeding action (Rohfritsch 1992; Raman 2011); whereas, most authors agree cynipid gall initiation begins at oviposition (Csóka *et al.* 2005; Raman 2007; Raman 2011), where wounding plant tissues with the ovipositor, chemicals in the ovipositional fluid, secretions from the eggs, and feeding activity of the recently hatched larvae are all considered contributing factors in gall initiation (Rohfritsch 1992). However, Roth (1949) suggested that eggs do not stimulate the initiation of cynipid galls and only larvae do. Cynipid eggs are deposited in a species-specific manner and location on the host plant that is at a particular stage of development to initiate their galls (Shorthouse *et al.* 2005). Tissues required for gall initiation are typically meristematic or undergo rapid cell division; however, some cynipids have the ability to cause differentiated tissues to revert to a meristematic state (Raman 2007). Plant cells adjacent to eggs lyse, producing cavities that larvae enter upon hatching (Rohfritsch 1992; LeBlanc and Lacroix 2001). Cell proliferation around the larvae follows, completing the formation of the larval chambers (Rohfritsch 1992; Csóka *et al.* 2005). Cells comprising the chamber walls at this phase of development have not yet differentiated, they are cytoplasmically dense, but are not nutritive cells (Roth 1949). All subsequent differentiation of gall tissues is controlled by developing larvae (Csóka *et al.* 2005).

The growth phase involves rapid growth of gall tissues rather than the larva, which usually remains small until the maturation phase because nutritive substances must first appear in the nutritive cells before larvae begin to eat (Roth 1949; Csóka *et al.* 2005). During the growth phase, parenchyma tissue rapidly divides (hyperplasia) and enlarges (hypertrophy), resulting in an increase in gall biomass as well as the volume of larval

chambers (Rohfritsch 1992; Brooks and Shorthouse 1998). Cells lining the larval chambers soon after initiation differentiate into nutritive cells; however, at this early phase, nutritive cells are not well structured and have a patchy distribution (Bronner 1992). Galls act as physiological sinks (references in Csóka *et al.* 2005) during the growth phase and vascular tissues differentiate within the parenchymatous nutritive tissue, eventually connecting with that of the host plant (Brooks and Shorthouse 1998; Sliva and Shorthouse 2005). All assimilates and nutrients are transferred from the host plant to galls via the vascular bundles (Bagatto and Shorthouse 1994). Although larvae only feed minimally during the growth phase, it is important to note that both the growth of the gall as well as the movement of assimilates are under control of the inducer larvae and cease should the inducer be killed (Leggo and Shorthouse 2006b).

The beginning of the maturation phase is marked by a reduction in gall growth and a period when the larvae grow rapidly as they feed on nutritive cells (LeBlanc and Lacroix 2001). Increased larval feeding causes the layer of nutritive cells to thicken around the larval chamber (Rohfritsch 1992). As nutritive cells are consumed, adjacent parenchyma cells develop the cytological features of nutritive cells and are fed upon by the larvae (Rohfritsch 1992). The early maturation phase is also marked by the differentiation of a layer of sclerenchyma that circumscribes the gall (Rohfritsch 1992). Vascular tissues, which join vascular bundles of the host organ, pass through the sclerenchyma layer and carry assimilates to the nutritive layers (Bagatto and Shorthouse 1994; Sliva and Shorthouse 2005).The differentiation of the sclerenchyma layer is also under the control of the inducer larvae and will not form should larvae be killed prior to the maturation phase (Rohfritsch 1992; Leggo and Shorthouse 2006b).

D. BIOLOGY OF *Diplolepis*

I. BIOLOGY OF THE GENUS *Diplolepis* AND THEIR GALLS

Cynipid wasps of the genus *Diplolepis* are Holarctic in distribution, but no single species is naturally found on both the Palearctic and Nearctic continents. Compared to other genera of gall-inducing cynipids, *Diplolepis* is not speciose with approximately 44 species worldwide, 13 of which are native to Canada (Shorthouse 2010). Each species within Canada has a different distribution, and may be found on different host plants throughout their range (based on data associated with the Shorthouse collection of *Diplolepis* at Laurentian University). Here, western and eastern Canada are divided at the Manitoba/Ontario border. One of the most wide-spread species is *D. rosaefolii* (Cockerell) which is found on *R. woodsii* in western Canada, *R. acicularis* from British Columbia east to Quebec, including the Yukon, *R. virginiana* in Prince Edward Island (P.E.I), Nova Scotia, and New Brunswick, and on *R. nitida* in Newfoundland. *D. fusiformans* (Ashmead) is found on *R. blanda* from Alberta to Ontario, and on *R. woodsii* in Southern Alberta. *D. nebulosa* is found on *R. woodsii* across western Canada, *R. blanda* in Ontario, and *R. virginiana* in Prince Edward Island. *D. ignota* (Osten Sacken) is not a widespread species, only occurring in southern Alberta and Saskatchewan on *R. arkansana*. *D. variabilis* (Bassett) also has a limited distribution found only in the Okanagan Valley in British Columbia on *R. woodsii*. *D. bicolor* (Harris) is found on *R. woodsii* in western Canada, *R. blanda* in Quebec and Ontario, and *R. virginiana* in Prince Edward Island. *D. polita* is found on *R. nutkana* west of the Rockies, *R. arkansana* in the Cypress Hills of Alberta (Shorthouse 1991), and most commonly on *R. acicularis* in the boreal forest from Alberta to Ontario, north to the tree line in the Yukon and North West

Territories (N.W.T.). *D. bassetti* (Beutenmüller) is found only on *R. woodsii* in the Okanagan Valley in British Columbia, southern Alberta, and southern Saskatchewan. *D. gracilis* (Ashmead) is found on *R. acicularis* from Alberta to Manitoba, and on *R. blanda* in Ontario and Quebec. *D. nodulosa* is found on *R. arkansana* in southern Alberta, *R. woodsii* from British Columbia to Manitoba, and *R. blanda* in Quebec and Ontario. *D. triforma* is found on *R. acicularis* from British Columbia to Quebec, including the southern Yukon and on *R. woodsii* in Alberta and Saskatchewan. *D. spinosa* (Ashmead) is found on *R. woodsii* in western Canada and on *R. blanda* in Ontario and Quebec. *D. radicum* (Osten Sacken) is found on *R. woodsii* in southern Alberta and southern Saskatchewan, and on *R. acicularis* in Ontario and Manitoba.

All species of *Diplolepis* are restricted to inducing galls on specific organs of roses and are categorized as leaf-, stem-, or adventitious root-gallers. They are also categorized based on the number larval chambers as single- or multi-chambered depending on the inducer species (Csóka *et al.* 2005; Shorthouse 2010). Despite relatively few species in Canada, the diversity of galls is high with single- and multi-chambered galls occurring on leaves, roots, and stems.

Adult *Diplolepis* are small, ranging from three to six millimeters in length with female colouration varying from entirely black, to reddish-brown and black, to entirely orange-red depending on the species, while males of most species are entirely black (Shorthouse 2010). The adult body is short and globular and females have a plowshare-shaped hypopygium (modified eighth metasomal tergite), a character that distinguishes *Diplolepis* from all other cynipids (Csóka *et al.* 2005; Shorthouse 2010). Larvae are hymenopteriform, apodous, and have weakly defined heads. The body has 13 segments

and has cream-coloured, translucent integument. Larvae of species that induce leaf galls are comma-shaped and tapered toward the caudal end, whereas larvae of species that induce stem galls are more cylindrical (Shorthouse 2010).

All species of *Diplolepis* are univoltine and do not exhibit an alteration of generations as seen in other cynipids such as those that induce galls on oaks (Csóka *et al.* 2005). Like other Hymenoptera, sexual reproduction in *Diplolepis* is by facultative arrhenotoky, whereby unfertilized eggs develop into haploid males and fertilized eggs develop into diploid females (haplodiploidy). Males are often rare or absent from populations or entire species of *Diplolepis* due to infection by the bacterium *Wolbachia* which causes females to produce diploid daughters by parthenogenesis from unfertilized eggs (Csóka *et al.* 2005). *Wolbachia* is maternally inherited by the offspring of infected individuals and its effects are widespread with 12 of 19 species examined by Plantard *et al.* (1999).

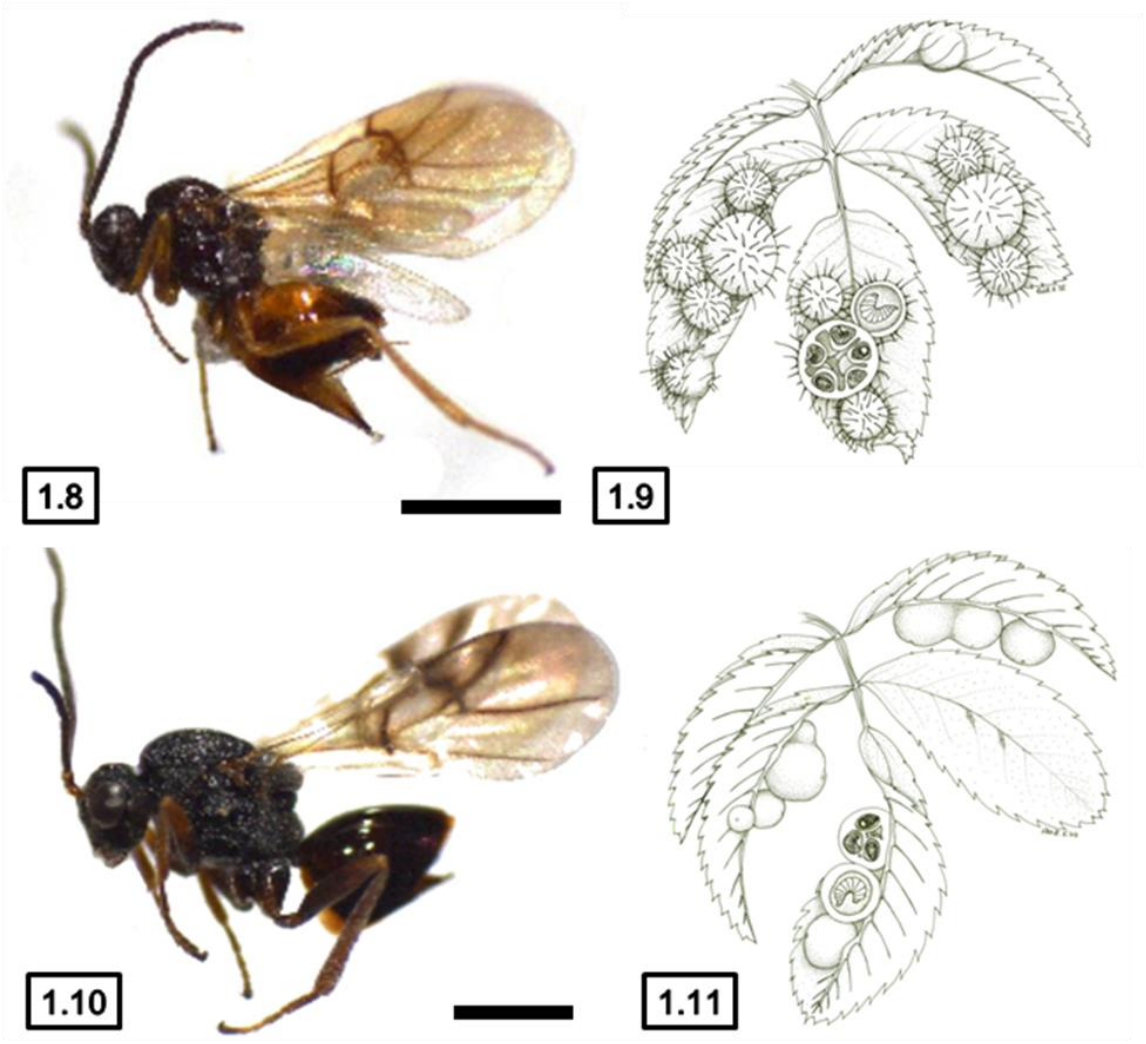
The biology of several species of *Diplolepis* has been studied in detail and all exhibit a similar life cycle (Brooks and Shorthouse 1997, 1998; Shorthouse and Brooks 1998; LeBlanc and Lacroix 2001; Sliva and Shorthouse 2005; Leggo and Shorthouse 2006a). In the spring, adults exit their galls by chewing a tunnel from their larval chambers in synchrony with the availability of host tissues at a suitable stage for gall initiation (Shorthouse 2010). Most species oviposit in the first vegetative buds to appear in the spring; however, some oviposit later in the season in axillary buds (Shorthouse 2010). Larvae develop within individual chambers of their species-specific galls and overwinter as pre-pupae (Leggo and Shorthouse 2002). The pre-pupa is a transition stage between a larva and a pupa in which eyes and reproductive organs have begun to develop; however, the insect maintains its general larval appearance (Leggo and Shorthouse 2002;

Shorthouse 2010). Pupation occurs the following spring for a period of 7-14 days (Shorthouse 1993).

II. BIOLOGY OF *Diplolepis polita* AND ITS GALL

Adults of *D. polita* average two to four millimeters in length (Beutenmüller 1907). Females are black and orange-brown (Fig. 1.8) and induce visually striking galls on the adaxial surface of leaflets of *R. acicularis* (Fig. 1.9) (Shorthouse 1973, 2010). Adults exiting from their galls are closely synchronized with the presence of host tissues at the appropriate stage for oviposition. Adults are present in the field during the second and third weeks of May (Fig. 1.7) and live for approximately 7-10 days (Shorthouse 1973). Freshly initiated galls are found in the field until the end of May and are light green to bright red. Galls become brown and woody from late June to the end of July (Fig. 1.7) and galled leaves abscise about two months before ungalled leaves. Leaf tissue around each gall undergoes senescence, dries out, and turns brown well before fall leaf senescence (Shorthouse *et al.* 2005). Galls drop to the ground and are buried in the leaf litter in the fall and larvae overwinter in their galls in as pre-pupae (Shorthouse 2010).

Mature galls are small, average 3.46 +/- 0.61 mm in diameter (n=48, range= 1.56-4.57), spherical, covered in weak prickles, and are inhabited by one inducer larva, enclosed within a thin-walled chamber (Fig. 1.2). Galls are typically found in clusters, most commonly on the distal leaflets (Fig. 1.2). Gall density is high with an average of 4.45 galls per galled leaf (n=247 leaves, range = 1-24 galls) and 3.97galls per galled leaflet (n=293 leaflets, range = 1-24 galls per leaflet).



Figs. 1.8 – 1.11: Adults and galls of *Diplolepis.* **Fig. 1.8.** Adult *D. polita* female (Photographed by J. Lima). Scale bar = 1 mm **Fig. 1.9.** Schematic drawing of galls of *D. polita*. Note one dissected gall is occupied by an inducer larva in a single larval chamber; whereas, those modified by the inquiline *Periclistus* are significantly enlarged and occupied by several larvae, each enclosed in an individual chamber. Drawing by K. Kivinen. **Fig. 1.10.** Adult *D. nebulosa* female (Photographed by J. Lima). Scale bar = 1 mm **Fig. 1.11.** Schematic drawing of galls of *D. nebulosa*. Note one dissected gall is single-chambered and occupied by an inducer larva; whereas, those modified by *Periclistus* are occupied by several larvae, each enclosed in an individual chamber. Drawing by K. Kivinen.

III. BIOLOGY OF *Diplolepis nebulosa* AND ITS GALL

*Diplolepis nebulosa a*dults are black (Fig. 1.10) and average 2.75 mm in length (Beutenmüller 1907). Galls are induced on the abaxial surface of leaflets of *R. blanda* in Ontario and are typically found singly or in rows along the midrib (Fig. 1.11). Adults of *D. nebulosa* have a lengthy emergence period, exiting galls from the middle of May to late June (Fig. 1.7). Freshly-initiated galls appear as small (<1mm) light green to red dots on the adaxial surface of leaflets and are present in the field from mid July to the end of August (Fig. 1.7). Developmental phases overlap considerably (Fig. 1.7) and as a result, freshly initiated and mature galls are often found on the same plant. Mature galls become brown and woody when mature and abscise from late August to early October (Fig. 1.7). Mature galls average 4.92 +/- 0.64 mm in diameter (n=112, range= 2.12 -6.16) and are spherical with a smooth exterior. Most galls are dull yellow or cream, but those exposed to the sun become partially orange-red when mature. Galls are inhabited by one inducer larva enclosed within a spherical chamber surrounded by a thick, spongy wall. They are typically found in rows along the midrib and are generally evenly distributed among all leaflets on leaves (Figure 1.11) with an average of 3.05 galls per galled leaf (n=160 leaves, range = 1-12 galls) and 1.84 galls per galled leaflet (n=396 leaflets, range = 1-9 galls per leaflet).

E. BIOLOGY OF INQUILINES OF THE GENUS *Periclistus*

The genus *Periclistus* belongs to the cynipid tribe Synergini which is composed of wasps that are phytophagous inquilines of mostly other cynipid galls (Csóka *et al.* 2005). Ronquist (1994) hypothesized that inquilines were once capable of inducing their own

galls and have since lost that ability possibly in response to intense competition for favorable gall induction sites. It is possible that ancestral females increased fitness by arriving late to oviposit and displaced the eggs or larvae of other species or individuals, eventually becoming obligate usurpers (Ronquist 1994). While *Periclistus* inquilines are incapable of inducing their own galls, they maintain the ability to manipulate plant tissues and induce the development of their own larval chambers inside host galls (Brooks and Shorthouse 1998).

Periclistus only attack galls of *Diplolepis* and are considered lethal inquilines as they kill inducer larvae with their ovipositors while ovipositing (Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001). The life cycle of *Periclistus* is similar to that of all cynipid inquilines (Fig. 1.7) where adults exit shortly after their inducer hosts and eggs are deposited on the inner surface of the larval chamber of immature galls. Larvae hatch and consume gall tissues which begin to proliferate, eventually enclosing each larva in an individual larval chamber (Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001; Csóka 2005). Larvae overwinter inside the gall and pupate in the spring (Shorthouse 1998).

A revision of the genus *Periclistus* by Ritchie (1984) concluded that most species attack one or few closely taxonomically related species of *Diplolepis*. Furthermore, it was shown that the species of inquiline that inhabit the two galls used in this study are distinct; however, both remain undescribed. Thus, the *Periclistus* sp. that attacks galls of *D. polita* will herein be referred to as '*Periclistus* 1', whereas the species that attacks galls of *D. nebulosa* will be referred to as '*Periclistus* 2'.

Periclistus 1 is found in leaf galls of *D. polita* and *D. bicolor* (Ritchie 1984). Adults exit from galls in mid May, soon after *D. polita* (Fig. 1.7), and deposit eggs into immature galls. Immature larvae are found in chambers from mid May to late June and galls are larger and display less radial symmetry than inducer-inhabited galls (Fig. 1.9). Chambers develop around each inquiline larva (Fig. 1.9) and mature by late June or early July (Fig. 1.9). Mature inquiline-inhabited galls are significantly larger than those inhabited by inducer larvae with a mean diameter of 4.96 mm (n=66 galls) (Fig. 1.9). Mature galls contain between one and twelve chambers, with a mean of 5.49 (n=55 galls) and chambers are always arranged around the periphery of the inner gall (Fig. 1.9). *Periclistus* 2 is found in leaf galls induced by *D. nebulosa, D. ignota,* and *D .variabilis* (Ritchie 1984). Adults exit from galls from late July to early September, in synchrony with the presence of immature galls of *D. nebulosa* rather than in synchrony with the emergence of the host adults (Fig. 1.7). Females deposit their eggs into immature galls and recently hatched larvae are found in galls between early August and early September (Fig. 1.7). Larvae are surrounded by gall tissues within individual chambers and gall tissues become hard as they mature from mid August until leaf senescence in the fall (Fig. 1.7). Galls containing inquiline larvae are difficult to distinguish from those with inducer larvae in the field as inquiline-modified galls are not significantly enlarged, with a mean diameter of 5.47 mm (n=69 galls) (Fig. 1.11). Mature inquiline-modified galls contain between one and eighteen larval chambers with a mean of 4.85 (n= 40 galls) that are centrally distributed within galls (Fig.1.11).

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CHAPTER II: DIFFERENCES IN THE ANATOMY AND DEVELOPMENTAL MORPHOLOGY OF LEAF GALLS INDUCED BY *Diplolepis polita* (HYMENOPTERA: CYNIPIDAE) ON *Rosa acicularis* (ROSACEAE) AND *D. nebulosa* ON *R. blanda*

A. ABSTRACT

Galls induced by cynipid wasps are structurally and developmentally complex. Each species induces a species-specific, morphologically distinct gall. The means by which cynipids initiate their galls has fascinated naturalists for hundreds of years; however, the basic events in gall induction are still poorly understood. To further the understanding of the galling strategies and developmental processes responsible for species-specific galls, the life history strategies and developmental events associated with galls induced by two closely taxonomically related species attacking the same plant organ were examined. This study included host specificity, phenology, and oviposition strategies, along with gall initiation, and development. Two species of the genus *Diplolepis* that occur on the wild roses of central Ontario were chosen for the study. One species, *Diplolepis polita*, induces prickly, single-chambered, spherical galls on the adaxial surface of the leaflets of *Rosa acicularis*, whereas the other species, *D. nebulosa*, induces smooth-surfaced, single-chambered galls on the abaxial surface of the leaflets of *Rosa blanda*. Galls at all stages of development were examined histologically along with non-galled leaf tissues. All materials were fixed in FAA, embedded in paraffin, sectioned and stained. Galls of *D. polita* and *D. nebulosa* were found to differ in their developmental events as well as the anatomy of their mature galls. Galls of *D. polita* are composed of nutritive cells, parenchymatous nutritive cells, and an epidermis throughout the initiation and growth phases. Larvae of *D. polita* remain small in relation to chamber volume until the

maturation phase, when a hard layer of sclerenchyma differentiates. In contrast, galls of *D. nebulosa* have a delayed initiation phase and galls remain nearly undetectable on leaflets for several weeks after oviposition before they enter the growth phase. Freshly-hatched larvae of *D. nebulosa* are protected by sclerenchyma that differentiates soon after initiation, and are also surrounded by layers of nutritive cells and parenchymatous nutritive cells. Galls of this species develop without an external layer of epidermis and become spherical as they mature, when a second layer of sclerenchyma and a layer of cortex differentiate within the walls of the galls. Larvae of *D. nebulosa* occupy nearly the entire volume of their larval chambers throughout gall development. The size of each type of cell found within the developing galls of both species were measured and compared. Cells in the galls of *D. polita* and *D. nebulosa* increase in size throughout development; however, cells comprising the galls induced by *D. polita* are significantly larger than those induced by *D. nebulosa.* Comparing the biologies and galls of these two species, demonstrates how niche partitioning has occurred and also shows the striking differences in structures within galls of closely taxonomically related species. Cynipid gall diversity has been attributed to differentiation of tissues found in the outer parts of galls, such as cortex and epidermis; however, the present study indicates the reasons are more complex. Furthermore, it is apparent that many aspects of gall development and anatomy have been overlooked by previous researchers.

B. INTRODUCTION

The genus *Diplolepis* includes approximately 44 species, all of which induce galls on wild roses (Shorthouse 2010). Adult wasps are small (typically ranging from three to six millimeters in length) and difficult to distinguish; however, each species induces a

morphologically distinct gall that coupled with host plant identification can serve as an effective method of identifying the inducer to the species level (Shorthouse 1993). Galls induced by *Diplolepis* are occupied by either a single inducer larva (single-chambered) or many inducer larvae (multi-chambered) and are induced on the leaves, stems, and adventitious shoots of their hosts (Shorthouse 2010). *Diplolepis* galls range in external morphology from slight swellings such as those induced by *D. fusiformans* on stems (Shorthouse *et al.* 2005) and *D. rosaefolii* on leaves (LeBlanc and Lacroix 2001), to large structures covered with secondary surface adornments such as the prickle-covered, multi-chambered stem gall (mean diameter of 23 mm (Bagatto and Shorthouse 1994)) of *D. spinosa* and the hairy, multi-chambered leaf gall (mean diameter of 28.8 mm (László and Tóthmérész 2006)) induced by *D. rosae.*

The developmental events responsible for differences in gall morphology remain unknown. Brooks and Shorthouse (1998) suggested that searching for patterns in the developmental anatomy of galls induced by several taxonomically related species could provide clues as to how insects evolved the ability to manipulate their hosts to produce species-specific galls. Additionally, gall anatomy and morphogenesis could prove useful in developing accurate patterns in cynipid phylogeny (Shorthouse 1993). Ideally, the developmental morphology of all galls induced by members of the entire family would be examined.

In order to isolate developmental characters between galls used for developmental study, they should occur on the same host organ and both contain a single or multiple chambers. Two such galls that occur in abundance in central Ontario are induced by *D. polita* on *R.*

acicularis, and *D. nebulosa* on *R. blanda.* These galls were chosen because they are both spherical, single-chambered, and are found on the leaves of their host.

The purpose of this study was to determine how these two closely taxonomically related species manipulate the development of their host plants to produce morphologically distinct galls. It was predicted that a detailed histological examination of all stages of development from oviposition to maturity would reveal the source of these differences. To further the understanding of the differences between the developmental morphologies of both galls, this study also asks the following questions: (1) how does the size of each cell type change throughout development? and (2) are cells comprising galls of *D. polita* and *D. nebulosa* of the same size? It was hypothesized that a) cells of both *D. polita* and *D. nebulosa* galls increase in size (hypertrophy) over the course of development and b) cells of galls of *D. polita* would be the same size as those within galls of *D. nebulosa.*

The anatomy of typical mature leaves of *R. acicularis* and *R. blanda* was also examined to illustrate the influence of each gall inducer on their respective host organs. The anatomy and development of leaf buds of both species of rose has previously been examined (Brooks and Shorthouse 1998; Sliva and Shorthouse 2006; Leggo and Shorthouse 2005; Shorthouse *et al.* 2005) and thus only the anatomy of mature leaves is presented in this study.

C. MATERIALS AND METHODS

i. STUDY GALLS

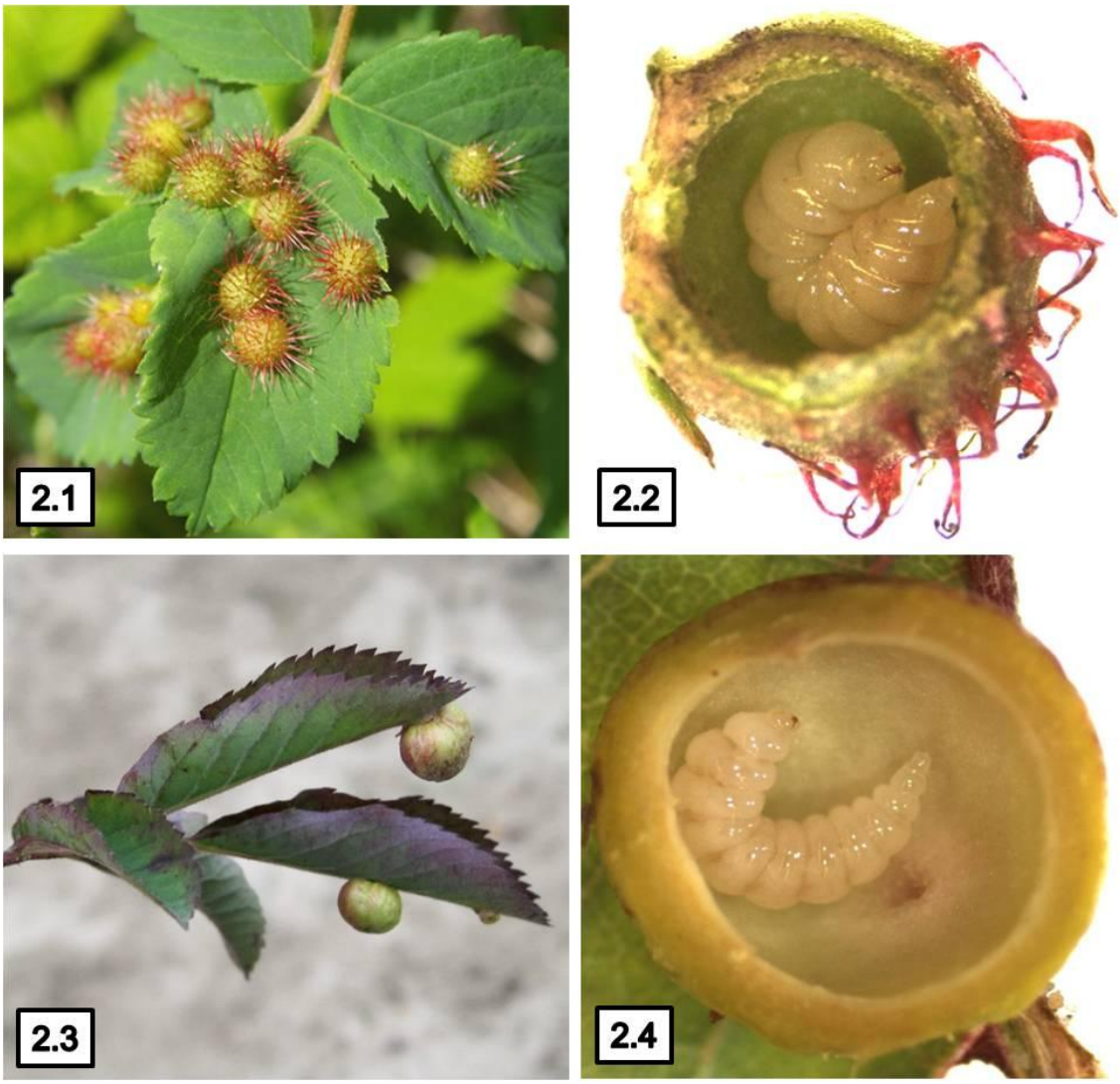
Mature galls of *D. polita* are red and spherical and are covered with prickles (Fig. 1.1). Galls are found in clusters on the adaxial surface of leaflets of *R. acicularis* (Fig. 1.1) and

are inhabited by a single larva that nearly fills the chamber when mature (Fig. 1.2). Mature galls of *D. nebulosa* are cream to red and have a smooth exterior (Fig. 2.3). Galls are induced on the abaxial surface of leaflets of *R. blanda* (Fig. 2.3) and are also single-chambered (Fig. 2.4).

ii. COLLECTION AND FIXATION OF BOTANICAL MATERIAL

Non-galled leaves and galls of both species at different developmental phases were collected between May 2009 and October 2011 from sites previously identified as having large populations of galls. Galls of *D. polita* were collected from sites near Chelmsford and Timmins, Ontario, and La Sarre, Quebec and those induced by *D. nebulosa* were collected from sites near Sheguiandah and Providence Bay, Manitoulin Island, Ontario. Galls of both species were collected by haphazardly walking through rose patches and removing all galled leaves observed which were then placed in Whirl pak® bags and transported to the laboratory.

Freshly-initiated galls, barely visible to the naked eye, were cut whole from leaves with a razor blade and placed in FAA fixative (90 parts 70% ethanol: 5 parts glacial acetic acid: 5 parts 37.5% formalin) and then vacuum infiltrated. Older galls were dissected to determine inhabitants and to estimate the developmental phase prior to fixation. Gall diameter, inhabitant type (inducer, inquiline, or parasitoid), phase of development, and gall density per leaflet and leaf were all recorded. Only galls inhabited by inducer larvae were processed further for this study.



Figs. 2.1 – 2.4: Habitus and dissections of galls of *Diplolepis polita* and *Diplolepis nebulosa*. **Fig 2.1.** Cluster of mature leaf galls of *D. polita* on the adaxial surface of leaflets of *R. acicularis*. **Fig. 2.2.** Single-chambered leaf gall of *D. polita* dissected to show a mature inducer larva. **Fig. 2.3.** Mature leaf galls of *D. nebulosa* on the abaxial surface of leaflets of *R. blanda*. **Fig. 2.4.** Single-chambered leaf gall of *D. nebulosa* dissected to show a mature inducer larva.

Eggs of *D. polita* were obtained by locating an ovipositing female in the field. One female and rose stem clippings were collected north of La Sarre, QC and brought back to the laboratory. The female was placed into a container with cut stems of *Rosa acicularis* and ovipositions were observed. A portion of stem adjacent to each attacked bud was marked with masking tape. Stems were placed in flasks with water for 24 hours, after which the marked buds were harvested and fixed. Additional slides of *D. polita* eggs were made by Rose-Marie DeClerk, a previous student in the Shorthouse laboratory at Laurentian University and were also included in this study.

To obtain eggs of *D. nebulosa*, mature 2010 induced galls were collected in spring of 2011 at Providence Bay, Manitoulin Island, Ontario. Galls were dissected and pupae were transferred into gelatin capsules until adults emerged. Females were provided with immature leaflets on cut stems of *R. blanda* for oviposition and tissues were processed as outlined above.

iii. PREPARATION OF HISTOLOGICAL SECTIONS

Fixed tissues were washed for 12 hours in a stream of tap water to remove the fixative, dehydrated in an ethanol–tertiary butyl alcohol series, and embedded in paraffin. At least 14 leaves and galls at each developmental phase were sectioned at 8-10 µm using a Leica® Jung Biocut 2035 rotary microtome. Tissues were adhered to microscope slides using Haupt's adhesive (Jensen 1962) and stained using safranin – fast green (Sass 1958). This staining technique colours lignified cell walls of sclerenchyma, cytoplasm of provascular and procambial tissues, and nuclei red, the non-lignified cell walls of phloem and parenchyma cells green, and the dense cytoplasm of nutritive cells purple. The

staining protocol differed between the two galls used in this study. Tissues of *D. nebulosa* were more absorptive than those of *D. polita* and thus less time was required in the safranin and fast-green to adequately stain tissues. The staining protocol for this study is as follows: gall tissues of *D. polita* were stained with safranin for about 1 hour and fast-green for 20-25 seconds; whereas, gall tissues of *D. nebulosa* were stained with safranin for 20-30 minutes and fast-green for 6-10 seconds. Slides were made permanent by adhering cover slips using Permount® mounting medium and dried at room temperature.

iv. PHOTOGRAPHY AND QUANTITATIVE ANALYSIS

Photographs of sections were taken using a compound microscope fitted with a Leica® camera. Various types of gall cells circumscribing the larval chambers in mature galls were of consistent size, thus randomly chosen previously photographed histological sections of typical galls of high histological quality were used for cell measurements. The areas, as seen in cross section, of 15 nutritive, parenchymatous nutritive, sclerenchymatous, cortical parenchymatous, and epidermal cells nearest the midpoint of galls were measured from 14 galls at each phase of development using ImageJ software. Analyses included: i) mean areas with standard deviations for cells at each stage of development; ii) analysis of variance (ANOVA) with a Tukey's Post Hoc test, or student's t-test of each cell type across development to determine whether or not the size of cells of each tissue type changes throughout gall development. For example, are nutritive cells lining the chambers of immature, growth and maturation phase galls of *D. polita* the same size?, and iii) student's t-test between cell types of galls of *D. polita* and *D. nebulosa* at similar developmental phases to determine if cells comprising both galls

are of similar size. For example, are sclerenchyma cells in the maturation phase the same size in both galls? All statistical analyses were performed using IBM SPSS statistics 20.

v. ABBREVIATIONS

Throughout the remainder of this chapter when discussing cell types comprising galls of *D. polita* and *D. nebulosa,* abbreviations will be used both in the text and within figure captions. Each of the cell types are listed in alphabetical order as they appear in figure captions throughout the results section of this chapter. For the convenience of the reader, each of the cell types with their respective abbreviations will also be listed at the beginning of the discussion.

D. RESULTS

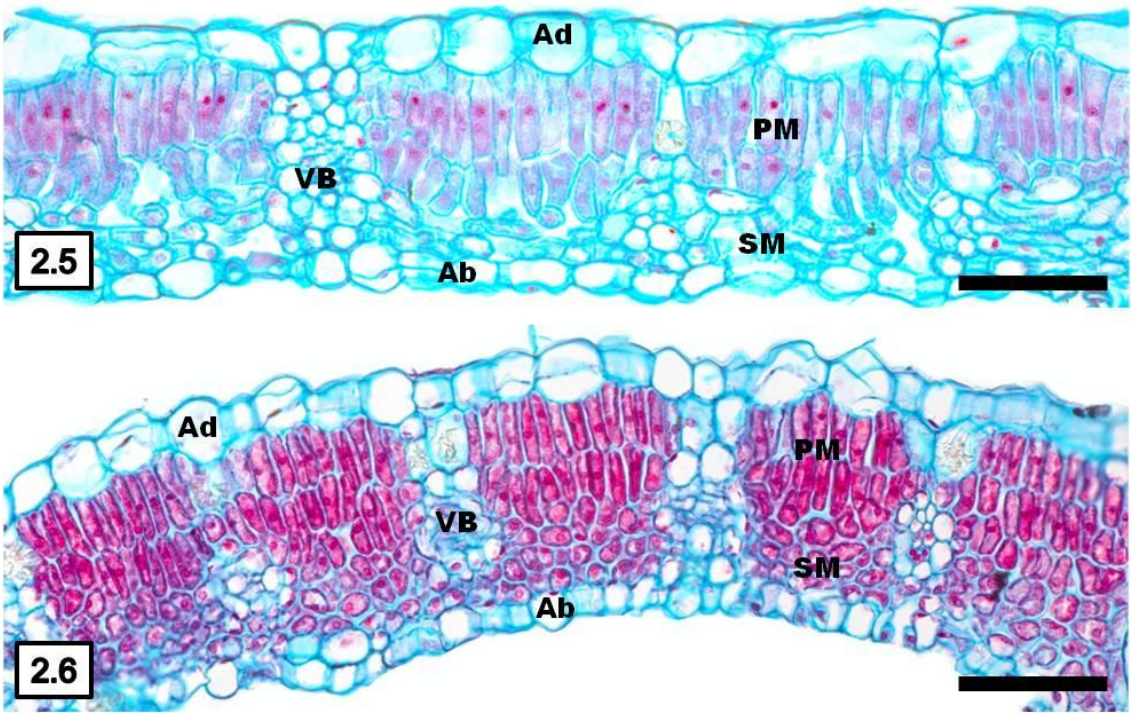
i. LEAVES OF *Rosa acicularis* AND *Rosa blanda*

The epidermis of leaves of both *R. acicularis* (Fig. 2.5) and *R. blanda* (Fig. 2.6) is composed of compactly arranged cells. Epidermal cells of the abaxial (Ab) side of the leaf are smaller than those of the adaxial surface and also contain guard cells (Figs 2.5 and 2.6).

Mesophyll tissue is located between the two epidermal layers and is composed of palisade (PM) and spongy mesophyll (SM). PM is composed of parenchyma cells that are elongated and contain many chloroplasts. They form a loosely arranged layer typically 1-2 cells in thickness in leaves of *R. acicularis* (Fig. 2.5), and a compactly arranged layer that is 2-3 cells in thickness in leaves of *R. blanda* (Fig. 2.6). SM is characterized by the presence of intracellular spaces and cells that are irregular in shape. In leaves of *R.*

acicularis, the layer of SM is 1-3 cells in thickness and contains large intracellular spaces (Fig. 2.5). The layer of SM in leaves of *R. blanda* is typically three cells in thickness and are compactly arranged (Fig. 2.6). Mesophyll of leaves of *R. blanda* typically absorbed more Safranin-Fast green stain than leaves of *R. acicularis*, accounting for the dense appearance of cells shown in Figure 2.6.

Vascular tissue (VT) is found throughout the mesophyll and is in the form of vascular bundles (Figs. 2.5 and 2.6). Vascular bundles are surrounded by a bundle sheath which is one cell layer thick and is composed of large vacuolated cells that resemble those of the epidermis (Figs. 2.5 and 2.6).



Figs. 2.5 – 2.6: Sections of leaves of *Rosa acicularis* and *Rosa blanda.* Scale bars = 50 µ. **Fig. 2.5.** Cross section of a mature leaf of *R. acicularis*. **Fig. 2.6.** Cross section of a mature leaf of *R. blanda*. Ab, abaxial epidermis; Ad, adaxial epidermis; PM, palisade mesophyll, SM, spongy mesophyll; VB, vascular bundle.

ii. GALLS OF *Diplolepis polita*

a. OVIPOSITION

Females of *D. polita* exit galls from early to mid May and search for suitable hosts. Once a host has been located, they walk along the branches searching for buds at the appropriate phase of development for oviposition (Fig. 1.7- please note: any figures beginning with 1 are found in Chapter I of this thesis). They are only able to oviposit into buds within a narrow range of development from when buds have swollen and elongated to when immature leaflets have differentiated at the tips (Fig. 2.7).

Females tap each bud with their antennae to assess oviposition sites and once a bud is selected, they position themselves in an inverted position in the middle or distal portion of buds, lower the hypopygium to a 90° angle, and insert the ovipositor (Fig. 2.7). The ovipositor is guided through folded leaflets without piercing or damaging developing tissues, and several eggs are deposited. Based on the number of galls found on leaves collected later in the season, it is estimated that the number of eggs laid in each bud ranges from 1-24, with a mean of four (n= 247 leaves). Following oviposition, females search for additional suitable buds.

b. INITIATION PHASE

Eggs are deposited with their proximal pole anchored to a single cell of the adaxial epidermis by a ‘plug’ composed of ovipositional fluids, which is in contact with several epidermal cells (Fig. 2.8). Changes in leaf cells adjacent to eggs (leaf cells that would normally differentiate into palisade mesophyll) are first observed in an area that is two to four cells thick. The first cells influenced by *D. polita* are hypertrophic, cytoplasmically

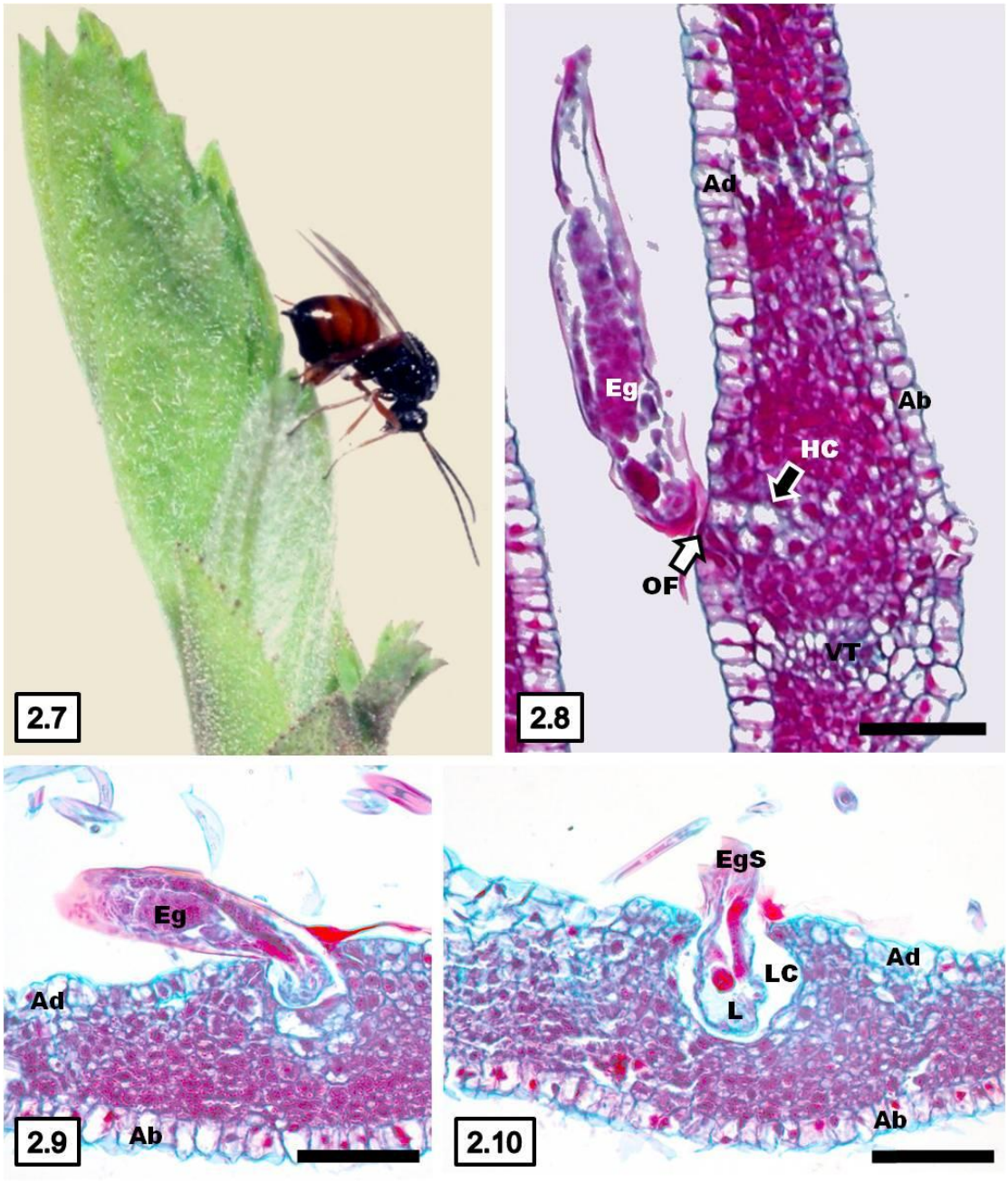
dense, are larger than adjacent leaf cells, causing an increase in leaf thickness (Fig. 2.8), and do not resemble a specific cell type found in galls as they are still undifferentiated.

Leaf cells adjacent to eggs lyse, forming a depression in leaf tissues that larvae enter head first upon hatching (Fig. 2.9). Egg shells split open and the heads of larvae protrude and feed on the hypertrophied, dense cells that were previously stimulated by eggs. Consumption of these cells causes the depressions in the leaves to increase in size as well as the proliferation of adjacent cells (Fig. 2.10).

Once larvae enter their larval chambers, cells within the adaxial portion of the leaves differentiate into parenchymatous nutritive cells (PNC) which are large, cytoplasmically dense cells with enlarged nuclei (Fig. 2.11). These cells rapidly proliferate around each larva above the leaf and form the chamber walls. Opposing chamber walls have not met, and the eggshell remains situated at the opening (Fig. 2.11). Cells of the leaf epidermis divide to accommodate the growth of galls and develop immature prickles. At this phase of development, the margins of galls within leaves are well defined and gall cells can be easily distinguished from adjacent leaf tissue (Fig. 2.11).

All previous developmental events take place on immature leaflets within unforced buds and are thus not seen in the field. Galls at the end of the initiation phase are the smallest that may be located in the field. They are found on unfolding leaflets of expanding compound leaves and are light green or yellow. Once leaflets unfold, galls are exposed to the sun and become bright red and are clothed with many succulent prickles (Fig. 2.12). At this phase in gall development, larvae nearly fill the entire volume of the larval chambers and gall tissues are arranged concentrically around larval chambers (Fig.

2.13a). Egg shells remain at the juncture where opposing chamber walls have met to close the larval chambers and cells at this site are crushed (Fig. 2.14). By the end of the initiation phase, all tissue types that are present in galls until the maturation phase have differentiated. A single layer of cytoplasmically dense, fabiform (bean-shaped), nutritive cells (NC) are differentiated and sparsely line larval chambers (Figs. 2.13b and 2.14). These are the largest gall cells at the end of the initiation phase, with a mean cell area of 412 μ^2 and there is a cellular size gradient from the larval chamber outwards to the epidermis (Fig. 2.48) (details of the data used to construct this figure appear in the appendix). Moving away from the larval chamber, there is a thick layer of PNC (10-15 cells in thickness) which makes up the majority of chamber walls. These polygonal cells are less cytoplasmically dense and smaller than NC (Figs. 2.13b and 2.14), with a mean cell area of 330 μ^2 (Fig. 2.48); however, PNC surrounding the juncture of the opposing chamber walls are particularly dense and undergo rapid cell division (Fig. 2.14). Galls are initiated on immature leaves with weakly defined tissue layers (Fig. 2.8). Thus, as the vascular tissue (VT) differentiates in the host leaf, it also extends into gall tissues (Figs. 2.13b and 2.14) and eventually an entire network of vascular tissue differentiates within the gall that is connected to the vasculature of the host leaf. Galls are covered by a single layer of small cuboidal epidermal cells that average 249 μ^2 (Fig. 2.48), and are generally less cytoplasmically dense than PNC. Prickles, which are essentially protuberances of PNC from the chamber wall, are also bordered by a single layer of epidermal cells (Fig. 2.13b).

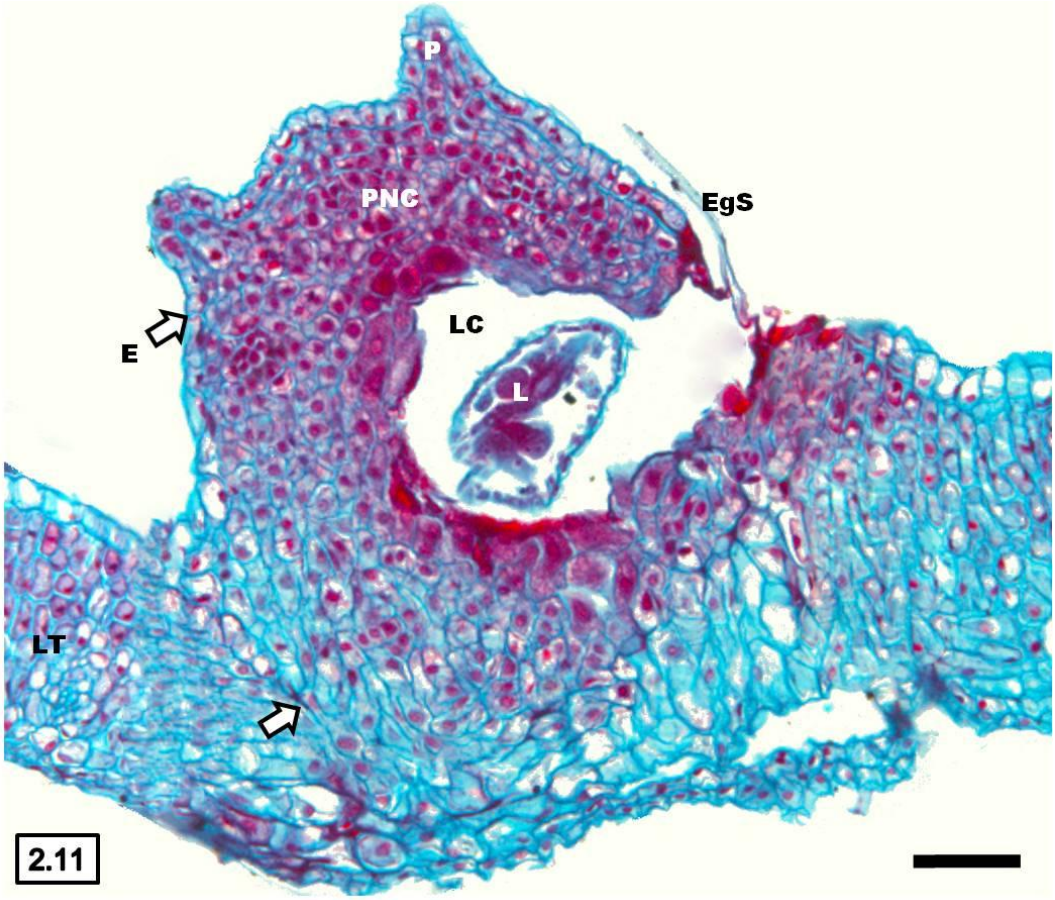


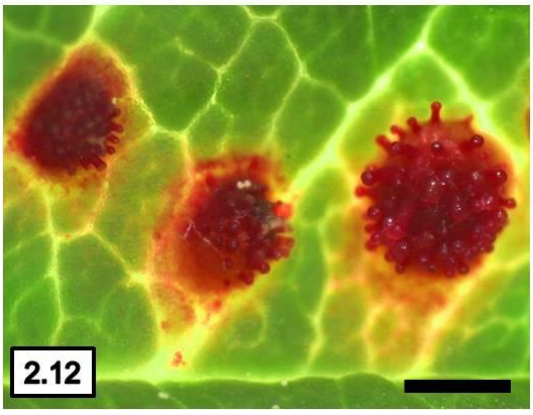
Figs. 2.7– 2.10: Oviposition and early initiation of galls of *Diplolepis polita* on *Rosa acicularis*. **Fig. 2.7.** *D. polita* female ovipositing into a leaf bud of *R. acicularis*. **Fig. 2.8.** Longitudinal section of eggs deposited onto the adaxial surface of immature leaflets. Note the ovipositional fluid securing the egg to the surface of the leaf and the hypertrophic cells beneath the egg (black arrow). Scale bar = 50 µ. **Fig. 2.9.** Longitudinal section of a hatching larva and lysis of leaf cells beneath forming an immature chamber. Scale bar = 70 µ. **Fig. 2.10.** Longitudinal section of a larva entering the larval chamber. Scale bar = 60 µ. Ab, abaxial epidermis; Ad, adaxial epidermis; Eg, Egg; HC, hypertrophic cells; L, larva; LC, larval chamber; LT, leaf tissue; OF, ovipositional fluid; VB, vascular bundle.

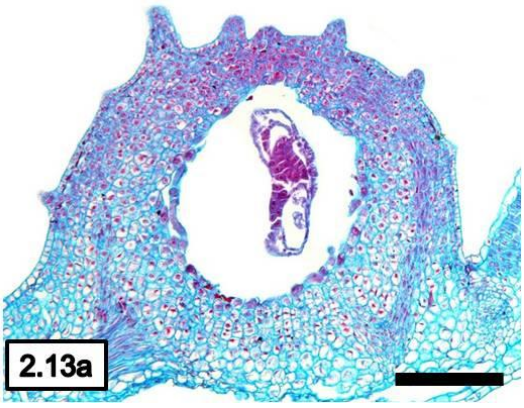
C. GROWTH PHASE

Galls in the growth phase are found in the field typically from the third week of May to mid June (Fig. 1.7). The epidermis of galls is red and covered with elongated and hardened prickles (Fig. 2.15). Chamber walls are firm, but are easily cut with a razor blade for dissections. Larvae are small in relation to the larval chamber throughout the growth phase as it is galls that undergo rapid growth during this phase of development rather than larvae (Fig. 2.16).

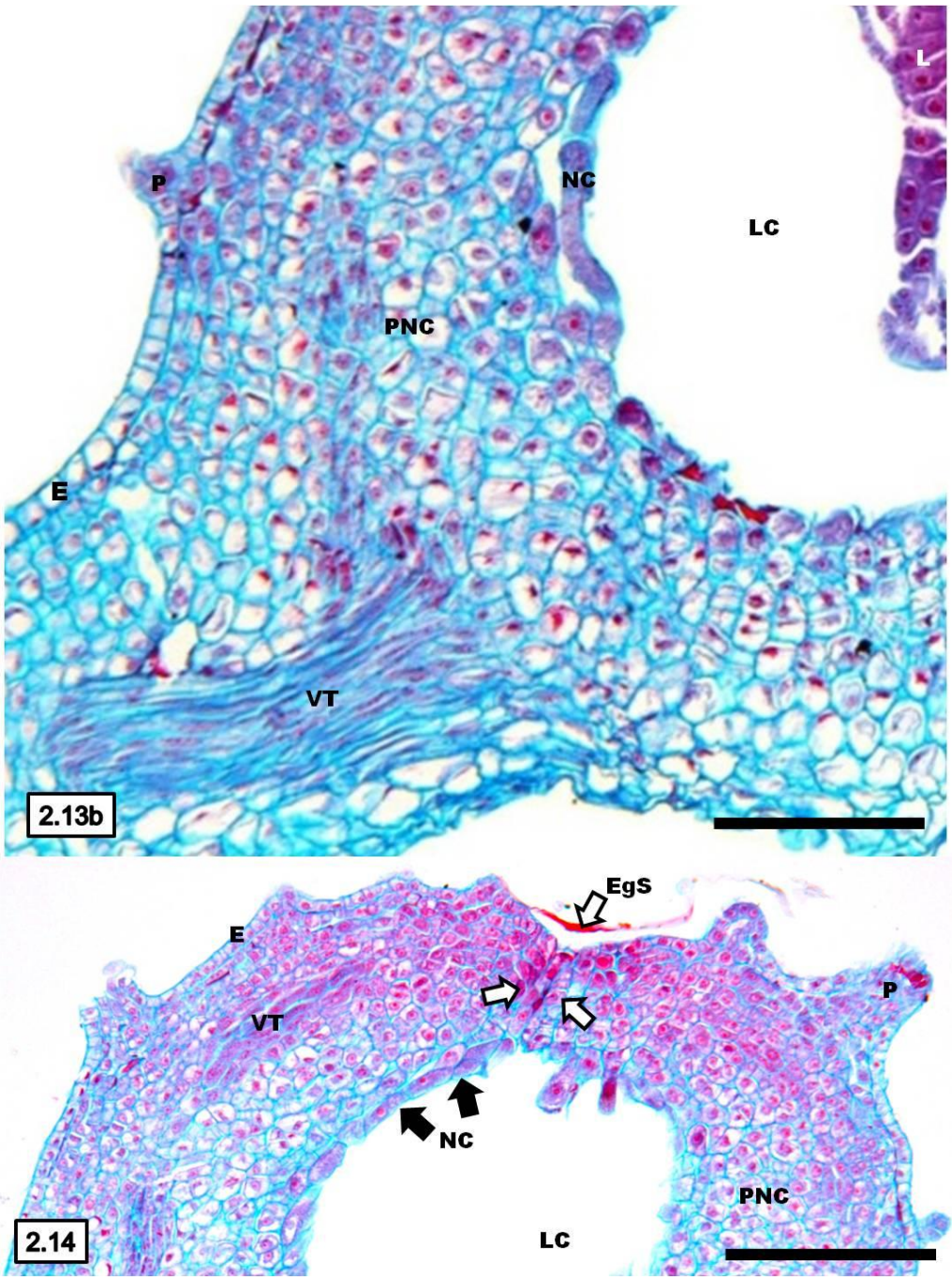
No additional cell types differentiate in the growth phase. That is, all of the types of cells found in galls at the end of initiation are found in growth phase galls. The layer of NC remains one cell in thickness and cells are more sparsely arranged around the larval chamber (Fig. 2.17). There is little to no sign of larval feeding over the course of the growth phase; all nutritive cells appear whole and there are no collapsed cells lining the larval chamber. Nutritive cells have a mean area of 465 μ^2 early in the growth phase and average 880 μ^2 by the late growth phase (Fig. 2.48). All cell types increase in size over the course of the growth period (Fig. 2.48). The layer of PNC is typically 12-17 cells in thickness, and contains the largest cells in growth phase galls (Fig. 2.17) averaging 447 μ^2 in the early growth phase and 1129 μ^2 by the end of the growth phase (Fig. 2.48). PNC at this phase are less cytoplasmically dense (Fig. 2.18) than those found in galls at the end of initiation (Fig. 2.13b). VT further proliferates and differentiates within the chamber wall (Figs. 2.17 and 2.18). In addition, epidermal cells significantly increase in size (Fig. 2.48), with a mean area of 265 μ^2 early in the growth phase and 824 μ^2 by the end of the growth phase (Fig. 2.48). Epidermal cells are less cytoplasmically dense (Fig. 2.18) than



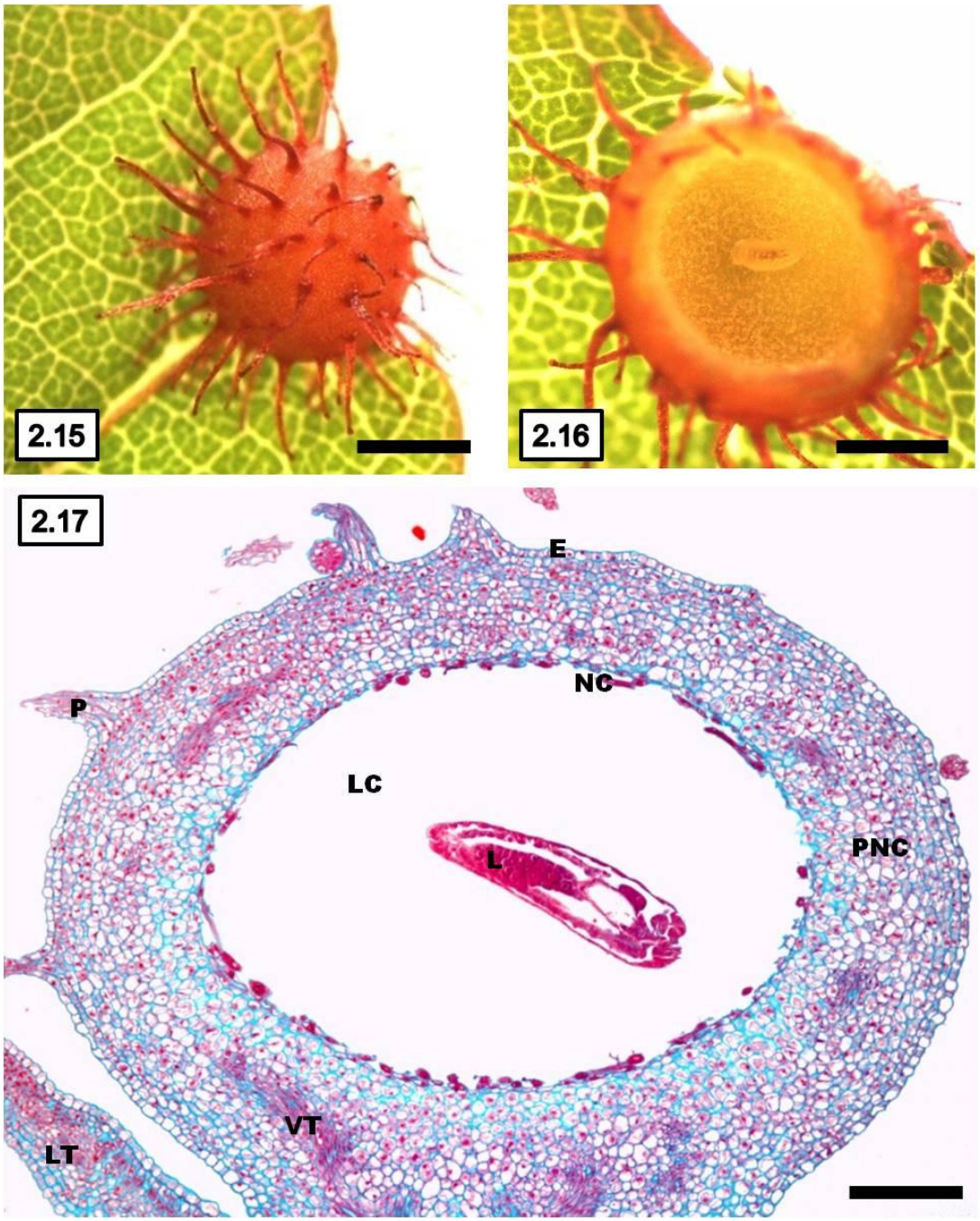




Figs. 2.11 – 2.13a: Galls of *Diplolepis polita* at the end of the initiation stage. **Fig. 2.11.** Cross section of proliferating gall cells forming the larval chamber. The egg shell is still present at the chamber opening. Note that the boundaries of gall tissues within the leaf are clearly defined (arrow) and the development of PNC and immature prickles. Scale bar = 60 µ. **Fig. 2.12.** Habitus of a cluster of red, immature galls developing immature prickles. Scale bar = 0.4 mm. **Fig. 2.13a.** Cross section of an immature gall at low power showing the size of the larva relative to the volume of the gall. Scale bar = 80 µ. E, epidermis; EgS, egg shell; L, larva; LC, larval chamber; PNC, parenchymatous nutritive cells; P, prickle; VT, vascular tissue.



Figs. 2.13b and 2.14: Sections of galls of *Diplolepis polita* at the end of the initiation phase. **Fig. 2.13b.** High magnification of galls at the end of the initiation phase showing the arrangement of NC, PNC, VT, and E surrounding the larval chamber. Note the thick vascular bundle entering the gall from adjacent leaf tissues and the elongated appearance of the NC. Scale bar = 40 µ. **Fig. 2.14.** Cross section of a gall showing the juncture (arrows) of the gall cells that complete of the larval chamber. Note the differentiation of nutritive cells and vascular tissue. Scale bar = 70 µ.E, epidermis; EgS, egg shell; L, larva; LC, larval chamber; NC, nutritive cells; PNC, parenchymatous nutritive cells; P, prickle; VT, vascular tissue.



Figs. 2.15 – 2.17: Galls of *Diplolepis polita* in the mid growth phase. **Fig. 2.15.** Habitus of a gall in the growth phase showing the elongated and hardening prickles. Scale bar = 1.0 mm. **Fig. 2.16.** Dissected gall in the growth phase showing the size of the small larva in relation to the increased volume of the larval chamber. Scale bar = 0.58 mm. **Fig. 2.17.** Cross section of a gall in the early-mid growth phase. Scale bar = 190 µ. E, epidermis; L, larva; LC, larval chamber; NC, nutritive cells; PNC, parenchymatous nutritive cells; P, prickle; VT, vascular tissue.

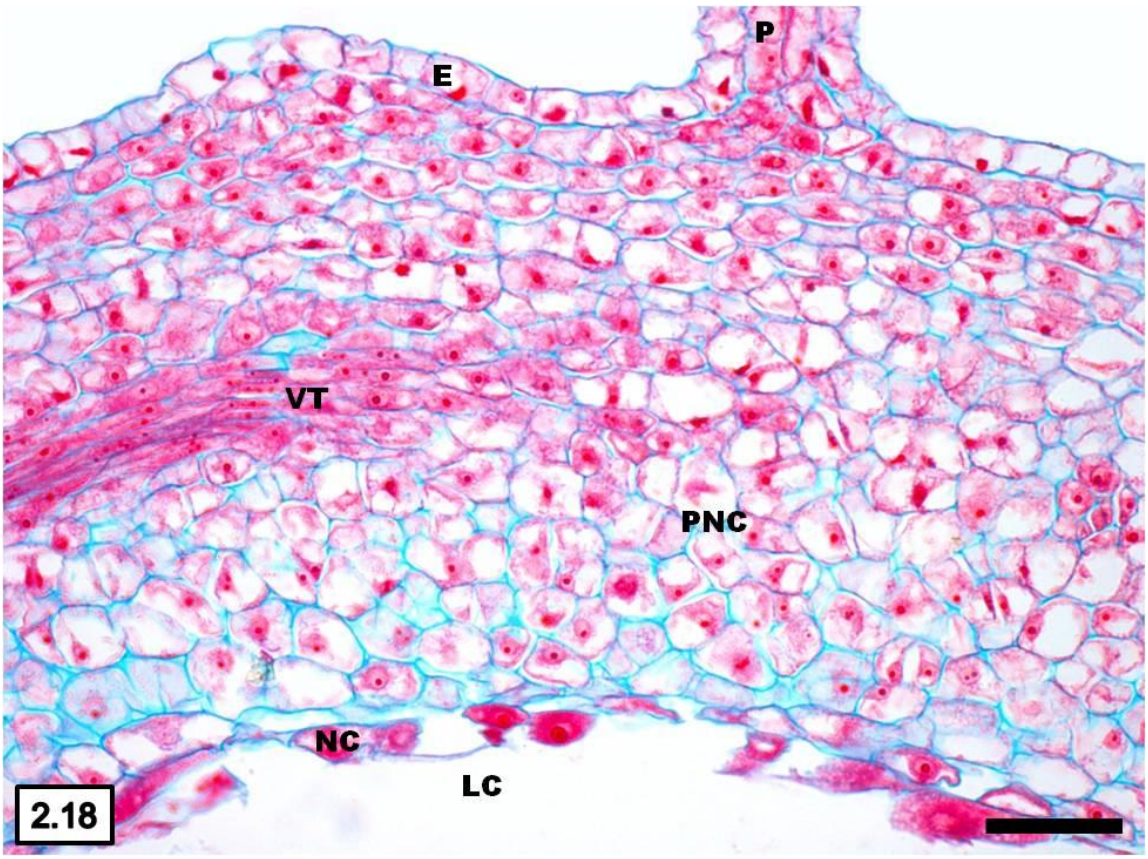


Fig. 2.18: Cross section of a portion of the wall of a gall of *Diplolepis polita* in the mid growth phase showing the patchy distribution of NC. Scale bar = 60 µ. E, epidermis; LC, larval chamber; NC, nutritive cells; PNC, parenchymatous nutritive cells; P, Prickle; VT, vascular tissue.

they were at the end of the initiation phase (Fig. 2.13b) and by the end of the growth phase, cells are vacuolated and do not proliferate.

d. MATURATION PHASE

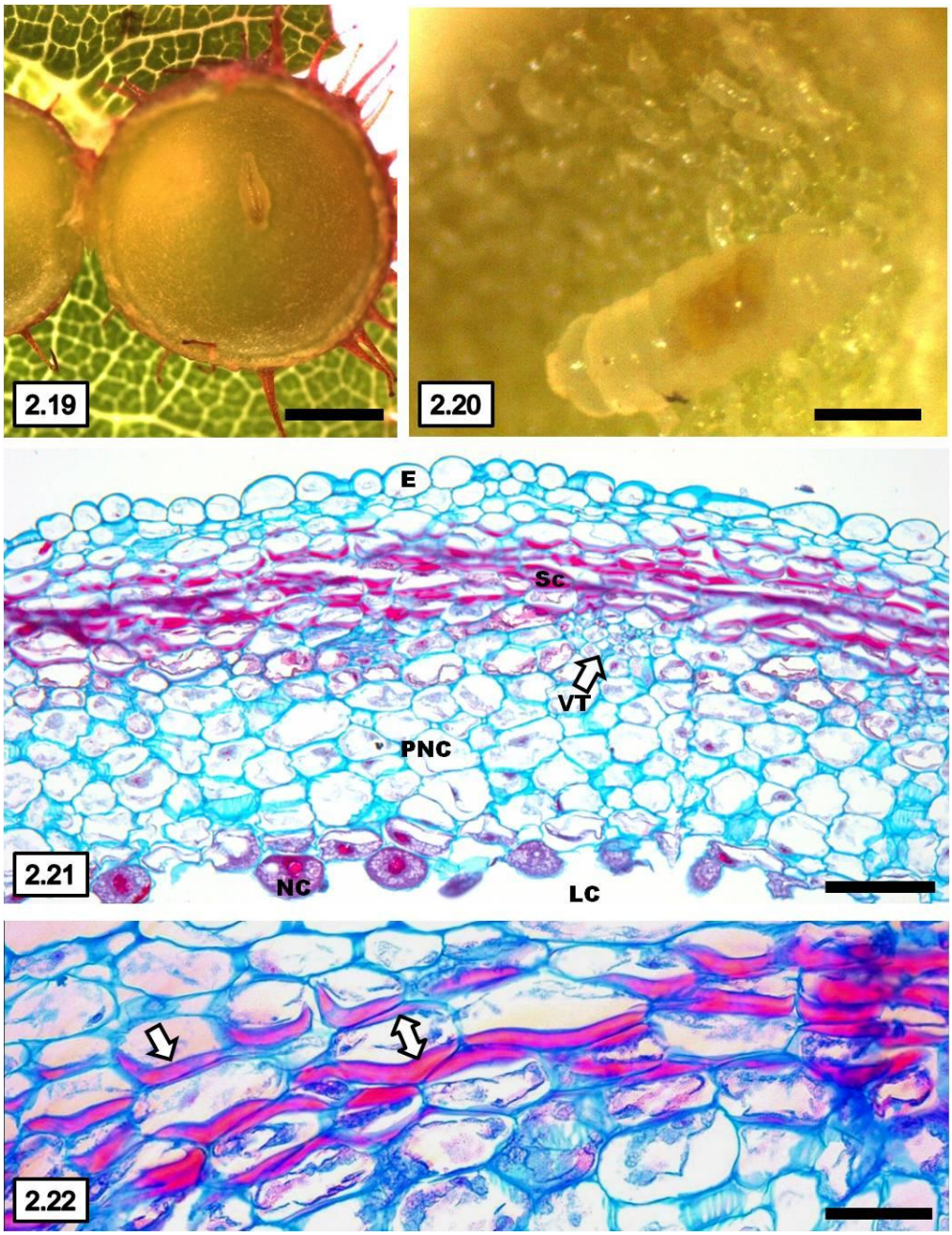
Galls mature from early to late June (Fig. 1.7) and turn reddish-green, while the prickles remain red (Figs 2.1 and 2.2). The onset of gall maturation is characterized by the cessation of gall growth and hardening of gall tissues through sclerification.

Larval chambers reach maximum volume early in the maturation phase; whereas, larvae do not appear to have increased in size since the growth phase (Fig. 2.19). Larvae actively feed on NC during this phase. NC retain their fabiform appearance (Fig. 2.20) and are significantly larger than those in the growth phase, with a mean area of 1986 μ^2 (Fig. 2.48); however, they are still sparsely distributed around the larval chamber (Fig. 2.21). PNC form a layer 4-8 cells in thickness and have also significantly increased in size from the growth phase and have a mean area of 1576 μ^2 (Fig. 2.48). The gradient in cell size observed at the end of the initiation phase also exists in galls in early maturation, where cell size decreases away from the larval chamber (Figs. 2.21and 2.48).

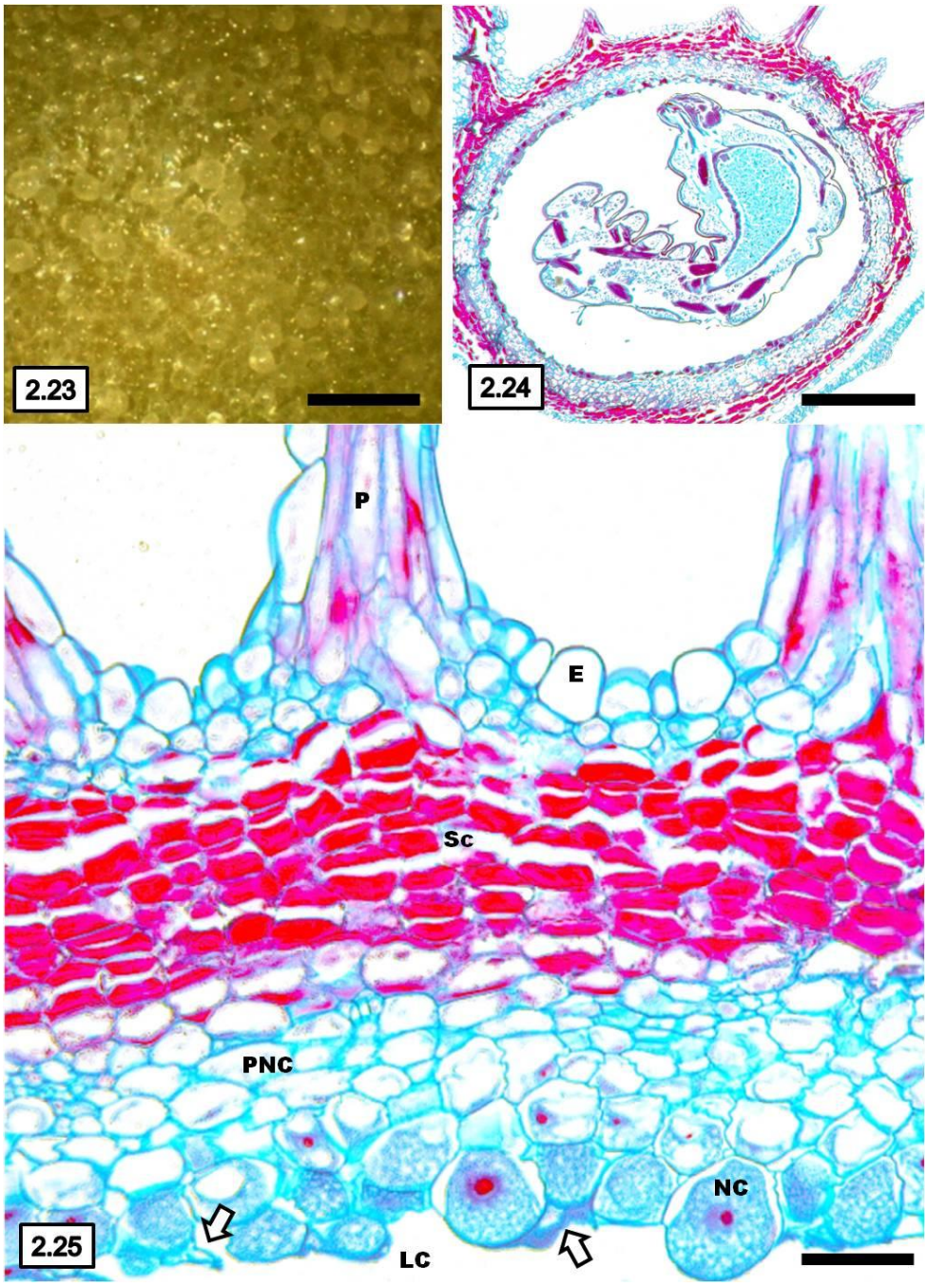
A thin layer of sclerenchyma (Sc) differentiates early in gall maturation and circumscribes the exterior portion of the chamber wall (Fig. 2.21). The layer of Sc is 3-4 cells thick (Figs. 2.21 and 2.22) and is composed of cells smaller than PNC that average 1212 μ^2 (Fig. 2.48). In addition, secondary walls of sclerenchyma cells are not uniformly thickened. As seen in cross section, the lateral walls of cells are never lignified, and some cells (mostly those on the border of the sclerenchyma layer) only have one thickened secondary cell wall (Figs. 2.21 and 2.22).

Galls become increasingly hard as maturation progresses and are difficult to open with a blade. Layers of Sc and NC can be seen with a dissecting microscope and sometimes the naked eye when galls have been cut open. Sc appears as a pale band of tissue with a brittle and flaky texture (Fig. 2.2) and NC appear as dark green, spherical cells lining the larval chamber (Fig 2.24). Larvae are mature and have increased in size, nearly filling their larval chambers (Figs. 2.2 and 2.23).

The most significant change to gall anatomy late in the maturation phase is the change in NC. NC were fabiform throughout earlier gall development; however, by late maturation they become globular (Fig. 2.24 and 2.25) and significantly enlarged, with a mean area of 2701 μ^2 (Fig. 2.48). They are the largest cells of the gall (Fig. 2.48) and form a dense layer (2-3 cells in thickness) that circumscribes the larval chamber (Fig. 2.25). Larvae actively feed on NC, as shown by the presence of collapsed NC surrounding the larval chamber (Fig. 2.25). As NC are consumed, PNC are converted into NC and fed upon by larvae. This process continues until few cell layers remain between the larval chamber and the Sc. Once larvae complete their development and discontinue feeding, remaining nutritive cells revert to parenchyma. PNC are not significantly larger than those at the beginning of gall maturation, with a mean area of 1593 μ^2 (Fig. 2.48). Although Sc cells are dead at maturity, there is a significant increase in cell size from those in the early maturation phase; having a mean diameter of 1501 μ^2 late in maturation (Fig. 2.48). The layer of Sc (Fig. 2.25) generally ranges from 4-7 cells in thickness.



Figs. 2.19 – 2.22: Galls of *Diplolepis polita* early in the maturation phase. **Fig. 2.19.** Dissection of a gall early in the maturation phase showing the thickness of the gall wall and an immature larva. Scale bar = 1 mm. **Fig. 2.20.** Dissection of a gall at high magnification showing the shape and abundance of NC lining the larval chamber. Scale bar = 0.2 mm. **Fig. 2.21.** Cross section of a gall early in the maturation phase that has developed a thin layer of Sc cells. Note that NC are sparsely distributed around the larval chamber. Scale bar = 120 µ. **Fig. 2.22.** Cross section of a gall early in the maturation phase showing a thin layer of Sc. Note the thickened secondary cell walls of the sclerenchyma may occur on only one (arrow) or two (double-arrow) surfaces of each cell in cross section. Scale bar = 60 µ. E, epidermis; LC, larval chamber; NC, nutritive cells; PNT, parenchymatous nutritive cells; Sc, sclerenchyma; VT, vascular tissue



Figs. 2.23 – 2.25: Mature galls of *D.* polita. **Fig. 2.23.** Dissection of a gall late in the maturation phase at high magnification showing the large, globular NC lining the larval chamber. Scale bar = 350 µ. **Fig. 2.24.** Dissection of a gall late in the maturation phase showing a full-grown larval that nearly fills the larval chamber. Scale bar = 0.75 mm. **Fig. 2.25.** Cross section of a gall late in the maturation phase showing a thick layer of Sc and highly enlarged NC that form a layer 2-3 cells in thickness around the larval chamber. Note the collapsed NC from larvae feeding (arrows). Scale bar = 110 µ. E, epidermis; LC, larval chamber; NC, nutritive cells; PNC, parenchymatous nutritive cells; Sc, sclerenchyma; P, Prickle; VT, vascular tissue.

ii. GALLS OF *Diplolepis nebulosa*

a. OVIPOSITION

Females of *D. nebulosa* exit from galls from mid May to mid June (Fig. 1.7). Females oviposit onto plant tissues within a narrow range of development when the compound leaf has elongated, but leaflets are still folded together (Fig. 2.26). This phase of leaf development occurs throughout the season as additional leaves are produced by the plant and thus, the window of opportunity for oviposition is lengthy (Fig. 1.7). Females tap immature leaflets with their antennae to assess the oviposition site and will often probe folded leaflets with their ovipositors. Females were observed stroking the lateral surface of the gaster with their hind legs prior to oviposition. Once a leaf is selected, they position themselves in an inverted position along the midribs of the folded leaflets, lower the hypopygium to a 90° angle (Fig. 2.26), and insert the ovipositor between the folded leaflets. Females of *D. nebulosa* have a small hypopygium in relation to body size (Fig. 1.3), and it is likely they have short ovipositors as well as eggs are not deposited deep within a folded bud, but rather are attached to unfolding and exposed tissues (Figs. 2.27). Based on the number of galls found on leaves, it is estimated that *D. nebulosa* lays between1 and 12, with a mean of three (n= 160 galled leaves). Eggs are typically laid in close association with the midrib or primary veins on each leaflet (Fig. 2.27). Following oviposition, females search for additional suitable oviposition sites.

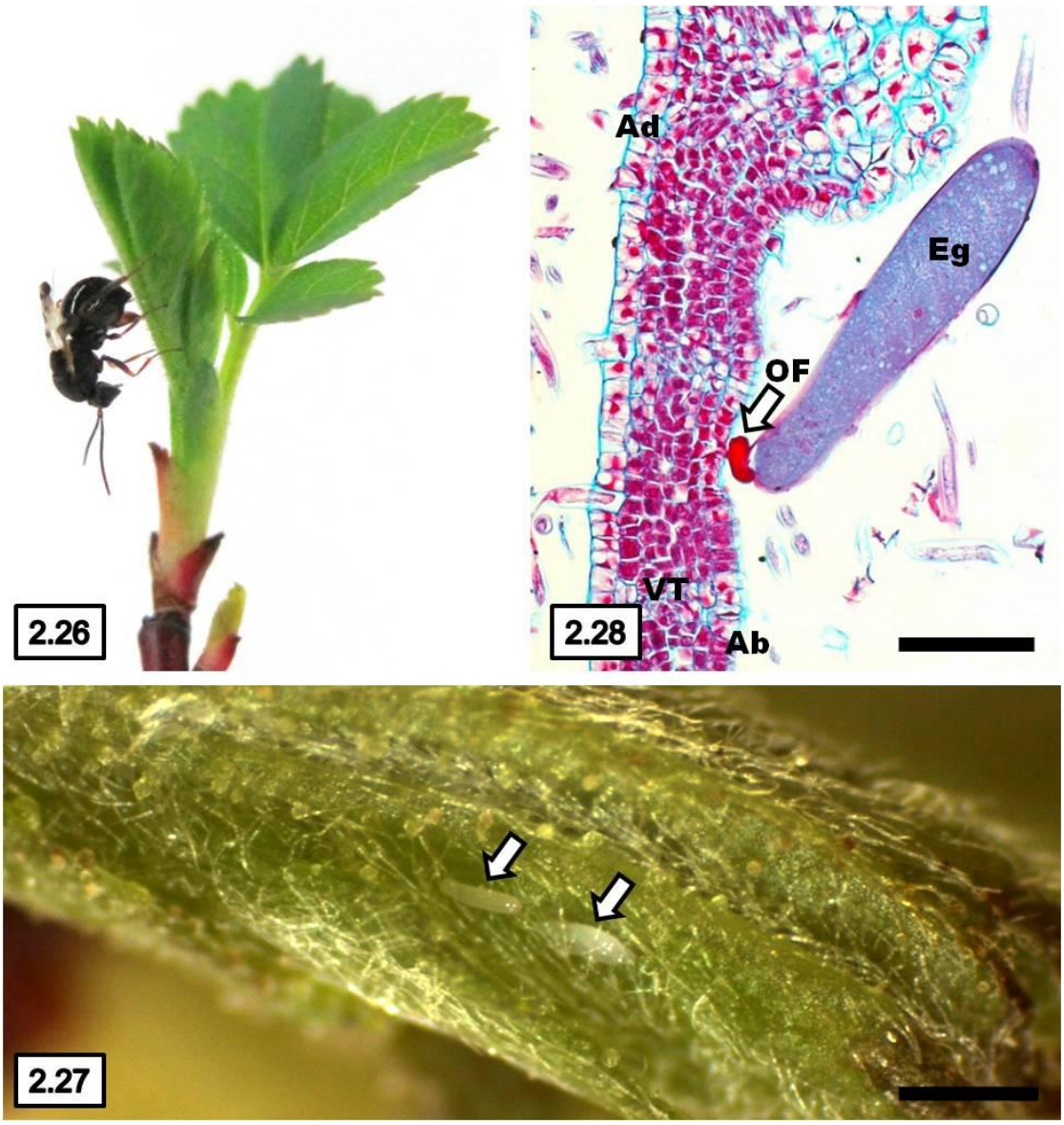
b. INITIATION PHASE

Eggs are deposited with their proximal pole anchored to the abaxial epidermis by a ‘plug’ of ovipositional fluids (Fig. 2.28). Leaf tissues are immature at the time of oviposition; epidermal cells and vascular tissues are weakly defined, but cells that will become

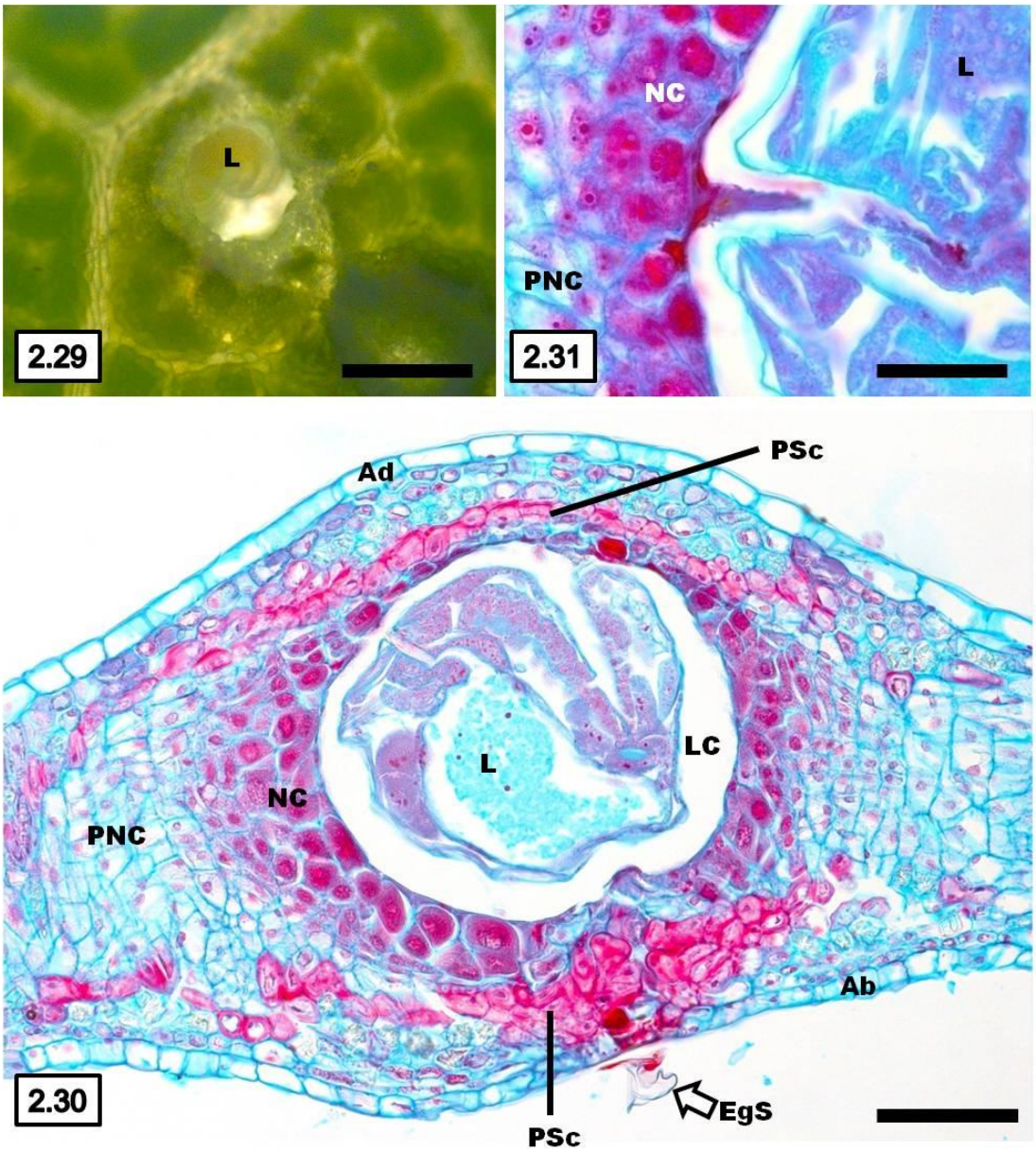
mesophyll have not differentiated (Fig. 2.28). Leaf cells that would normally differentiate into spongy mesophyll are those first affected by gall initiation and larvae are encapsulated in their chambers between the epidermal layers of the leaf upon hatching from eggs.

Although adults are present in the field until the third week of June, the most immature galls (those at the end of the initiation phase) are not found until mid–late July (Fig. 1.7). Leaf tissues adjacent to galls at the end of the initiation phase are differentiated when the first galls are found, and leaves are unfolded. Galls appear as small (<1 mm), light green to red spots on the adaxial surface of leaflets and larvae nearly fill their larval chambers (Fig. 2.29). The egg shell remains where opposing chamber walls have met to close the larval chamber and cells around this site appear crushed (Fig. 2.30). A layer of cytoplasmically dense, spherical, NC line larval chambers (Figs. 2.30 and 2.31). The layer of NC at the poles of the gall is either absent or one cell thick; however, is 3-5 cells thick at the equatorial region (Fig 2.30). Larvae in galls at the end of the initiation phase are likely still in the first instar and actively feed on the NC by slicing them with their mandibles and imbibing the cellular contents (Fig. 2.31). A layer of PNC is found adjacent to NC at the equatorial region of galls and is typically 5-10 cells in thickness (Fig. 2.30). PNC are arranged in columns between the epidermal layers of the leaf and are less cytoplasmically dense and have smaller nuclei than NC. NC and PNC at this phase are similarly sized; the mean area of NC is 157 μ^2 and 165 μ^2 for PNC (Fig. 2.48).

The larval chamber and layers of NC and PNC are enclosed between two sheaths of sclerenchyma that are 1-2 cells in thickness and located at the poles of the gall (Fig. 2.30). These sheaths are referred to as primary sclerenchyma (PSc) as it is the first layer



Figs. 2.26 – 2.28: Oviposition of *Diplolepis nebulosa* onto leaves of *Rosa blanda*. **Fig. 2.26.** Female ovipositing into folded leaflets. **Fig. 2.27.** Eggs (arrows) deposited in a row on the abaxial surface of closed leaflets. Note the placement of eggs near the midrib. Scale bar = 0.4 mm. **Fig. 2.28.** Longitudinal section of an egg deposited onto the abaxial surface of immature leaflets. Note the ovipositional fluid securing the egg to the surface of the leaf. Scale bar = 65 µ. Ab, abaxial epidermis; Ad, adaxial epidermis; Eg, Egg; OF, ovipositional fluid; VB, vascular bundle.

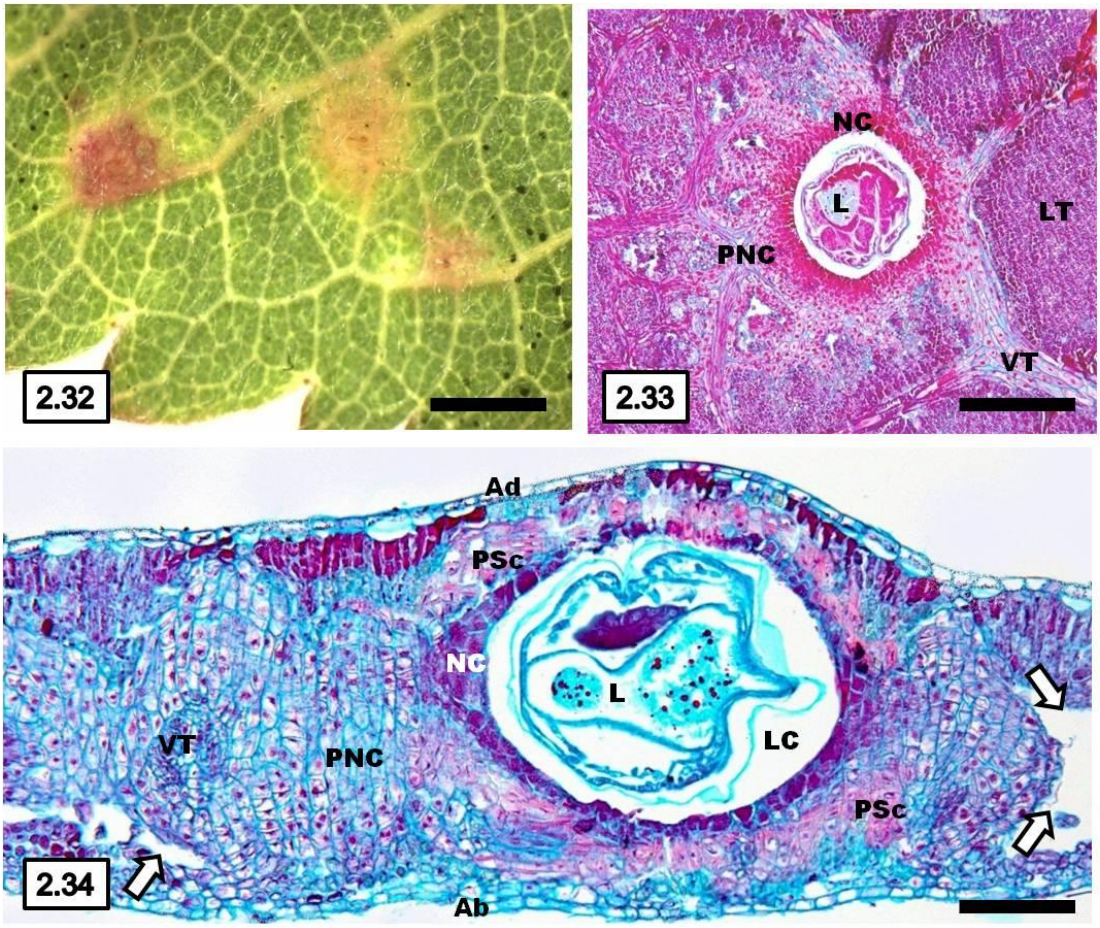


Figs. 2.29 – 2.31: Galls of *Diplolepis nebulosa* at the end of the initiation phase. **Fig. 2.29.** Dissected gall showing a freshly hatched larva inside. Scale bar = 0.2 mm. **Fig. 2.30.** Cross section of a gall at the end of the initiation phase showing three differentiated cell types surrounding the immature larva. Note the egg shell remaining where the chamber walls have enclosed the larva. Scale bar = 15 µ. **Fig. 2.31.** Cross section of a gall at high magnification showing the immature larva imbibing fluids from nutritive cells. Scale bar = 120 µ.Ab, abaxial epidermis; Ad, adaxial epidermis; EgS, egg shell; L, larva; LC, larval chamber; NC, nutritive cells; PNC, parenchymatous nutritive cells; PSc, primary sclerenchyma.

of sclerenchyma to develop within galls of *D. nebulosa.* PSc cells are spherical or cuboidal (Fig 2.30) and usually have such thickened secondary cell walls that the lumen (internal space) is nearly obliterated. The thickness of the layers of NC and PNC at the poles of the chamber, adjacent to the PSc sheaths, is highly reduced or absent (Fig. 2.30). Also, some differentiated leaf mesophyll cells remain beneath each epidermal layer of the host leaf exterior to the PSc and have not been modified by gall development (Fig. 2.30).

c. GROWTH PHASE

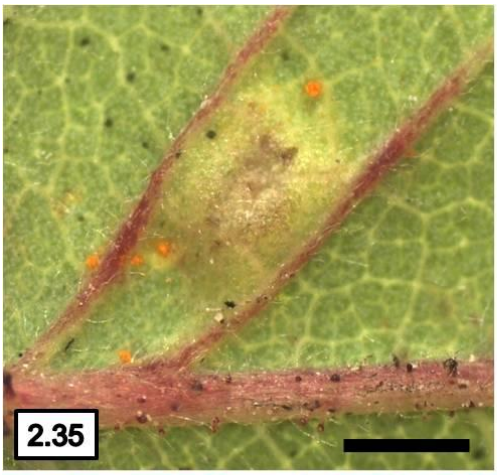
Galls in the early growth phase are visible on the abaxial surface of leaflets and appear as 1-2mm raised circular pads of tissue that are cream to red. Veins of the leaf adjacent to galls appear slightly thickened (Fig. 2.32). Larvae fill nearly the entire volume of their larval chambers (Figs. 2.33 and 2.34). When galls are transversely sectioned, PNC are shown to have proliferated radially into leaf tissues along the vascular bundles (Fig. 2.33). Transverse section implies that the leaf is sectioned parallel to the microtome blade such that only one layer of leaf tissue and enclosed gall can be observed in each section (eg. palisade mesophyll). Tissues are arranged in the same manner as those at the end of the initiation phase; however, the layer of PNC has increased to 10-35 cells in thickness and surrounds the vascular tissue of the leaf. The proliferation of PNC both laterally into the leaf and outward from the abaxial surface of the leaf caused the abaxial leaf tissues to rupture from the gall resulting in a small air space between the gall and leaf tissues (Fig. 2.34).

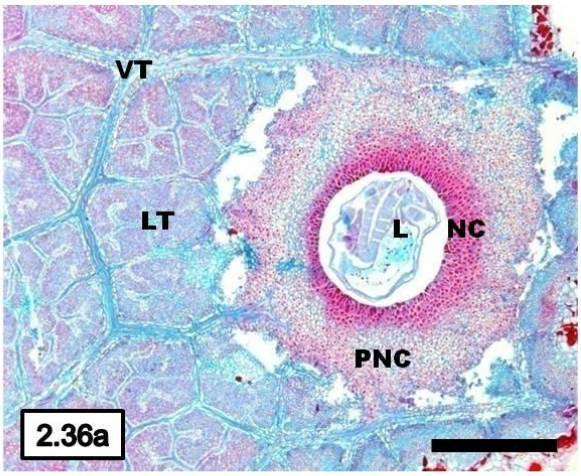


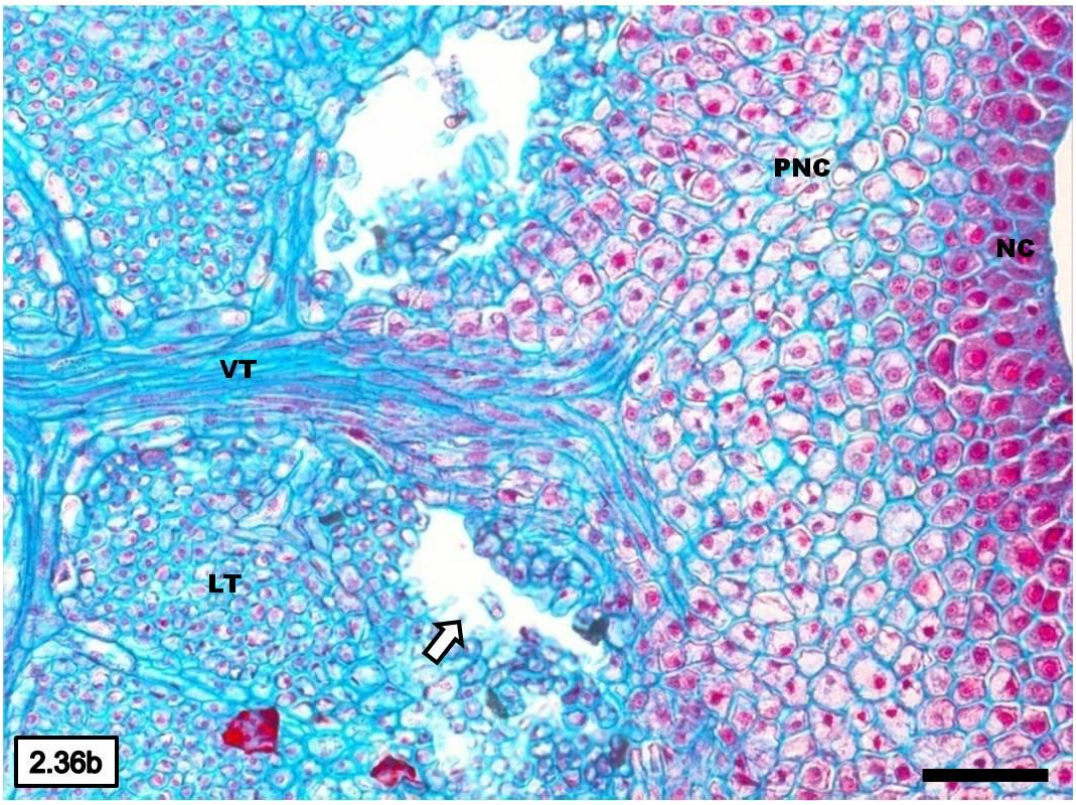
Figs. 2.32 – 2.34. Galls of *Diplolepis nebulosa* in the early growth phase showing lateral growth of the gall. **Fig. 2.32.** Habitus of galls in the early growth phase showing thickened vasculature of the leaf in proximity to galls. Note the lateral growth of the gall. Scale bar = 0.75 mm. **Fig. 2.33.** Transverse section of a gall early in the growth phase showing PNC proliferating laterally into the leaf and surrounding adjacent VT of the leaf. Note that transverse section implies sectioning the leaf through one tissue type at a time from the adaxial epidermis to the abaxial epidermis. Scale bar = 220 µ. **Fig. 2.34.** Cross section of a gall in the early growth phase showing differentiated VT. Note the lateral expansion of the gall has caused adjacent leaf tissues to rupture (arrows). Scale bar = 75 µ. Ab, abaxial epidermis; Ad, adaxial epidermis; L, larva; LT, leaf tissue; LC, larval chamber; NC, nutritive cells; PNC, parenchymatous nutritive cells; PSc, primary sclerenchyma; VT, vascular tissue.

Continual proliferation of PNC from the abaxial surface of the leaf causes galls to appear as small (1-2 mm) swellings (Fig. 2.35). In addition, adjacent vascular tissues appear thickened and may have a red pigment (Fig. 2.35). Galls transversely sectioned at this phase of development show PNC that have proliferated to their maximum radius and galls are completely surrounded by an air space within the leaf (Fig. 2.36a). The thickened veins (Fig. 2.35) are caused by PNC surrounding VT within the leaf (Figs. 2.36a and 2.36b); VT then proliferates and differentiates within gall tissues. Sectioning galls transversely also shows a thick layer of NC, 4-6 cells in thickness, around the equatorial region of galls (Fig. 2.36b).

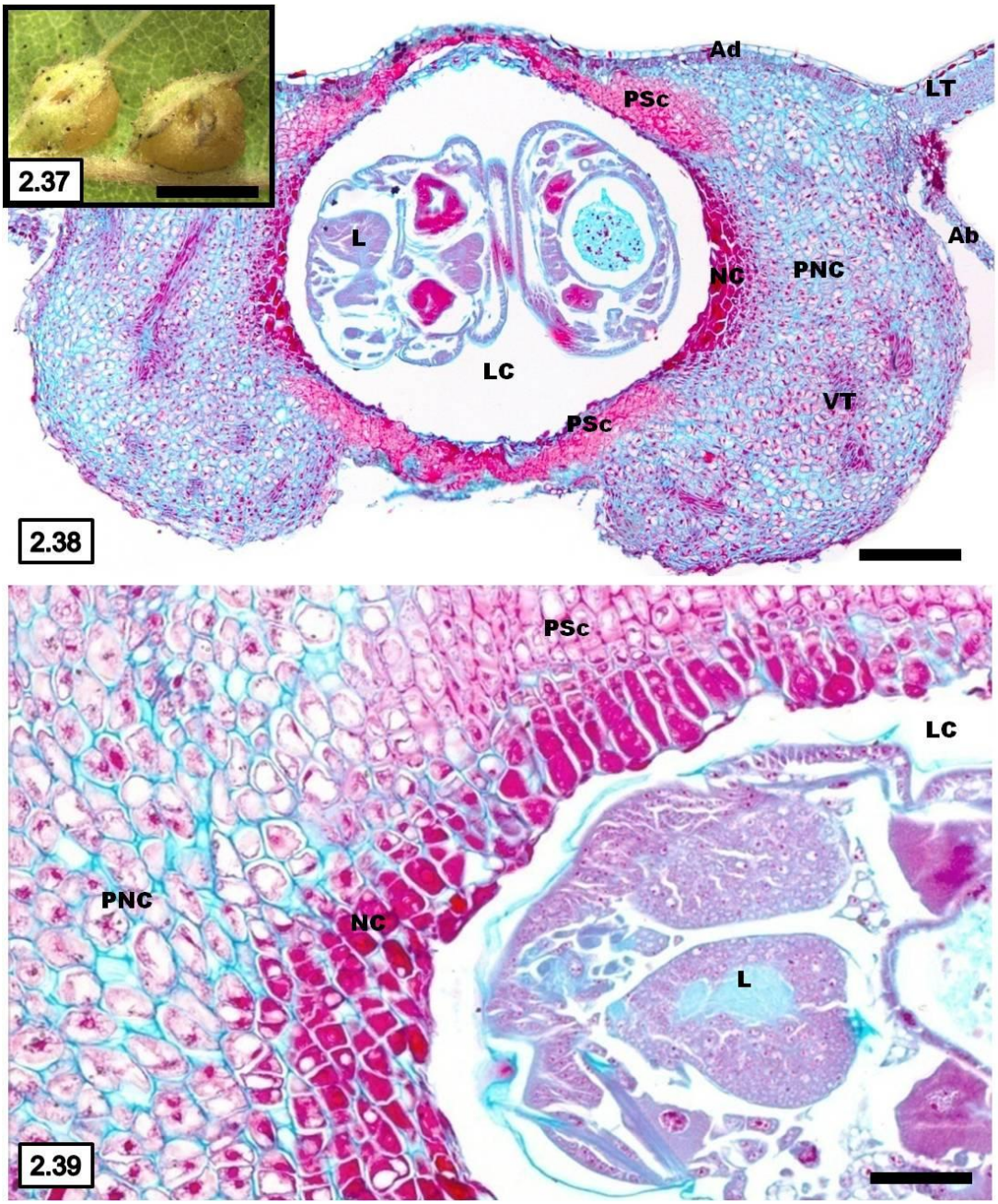
Galls in the mid-growth phase have broken through the abaxial epidermis of host leaves (Fig. 2.37) and any remaining pieces of leaf epidermis on galls detach from the PNC. Galls of *D. nebulosa* therefore lack an epidermal layer. Galls at this phase develop a small depression on the distal surface (Fig. 2.37). This depression is characteristic of galls of *D. nebulosa* and is created by the lack of PNC proliferation exterior of the distal PSc sheath (Fig. 2.38). When galls are dissected, tissues are easy to cut with a blade and are succulent. Larvae are large in relation to the volume of the larval chamber and are usually situated on their side (Figure 2.38) where a comma-shaped larva has been cross sectioned through a thoracic and posterior abdominal segment. The arrangement of cell types around larval chambers remains the same as in earlier development, with a thick band (4-7 cells) of NC arranged around the equatorial region of the chamber (Fig. 2.38). NC are elongated and proliferating, as shown by their columnar arrangement (Fig. 2.39), and have significantly increased in size compared to those at the end of initiation (Fig. 2.48).







Figs. 2.35 – 2.36. Galls of *Diplolepis nebulosa* in the mid-growth phase sectioned transversely. **Fig. 2.35.** Habitus of a gall in the mid-growth phase that is still encased within the abaxial epidermal layer of the leaf. Note the thickened veins in the leaf adjacent to the gall. Scale bar = 0.6 mm. **Fig. 2.36a.** Transverse section of a gall showing the separation of the gall tissues from the rest of the leaf with the exception of the vasculature of the leaf, which has been surrounded by PNC. Scale bar = 400 µ. **Fig. 2.36b.** Transverse section of the vascular tissue of the leaf joining the VT of the gall by extension of PNC. Note the air space created within the leaf from the outward growth of the gall rupturing the layers of tissue within the leaf (arrow). Scale bar = 60 µ. L, larva; LT, leaf tissue; NC, nutritive cells; PNC, parenchymatous nutritive cells; VT, vascular tissue.

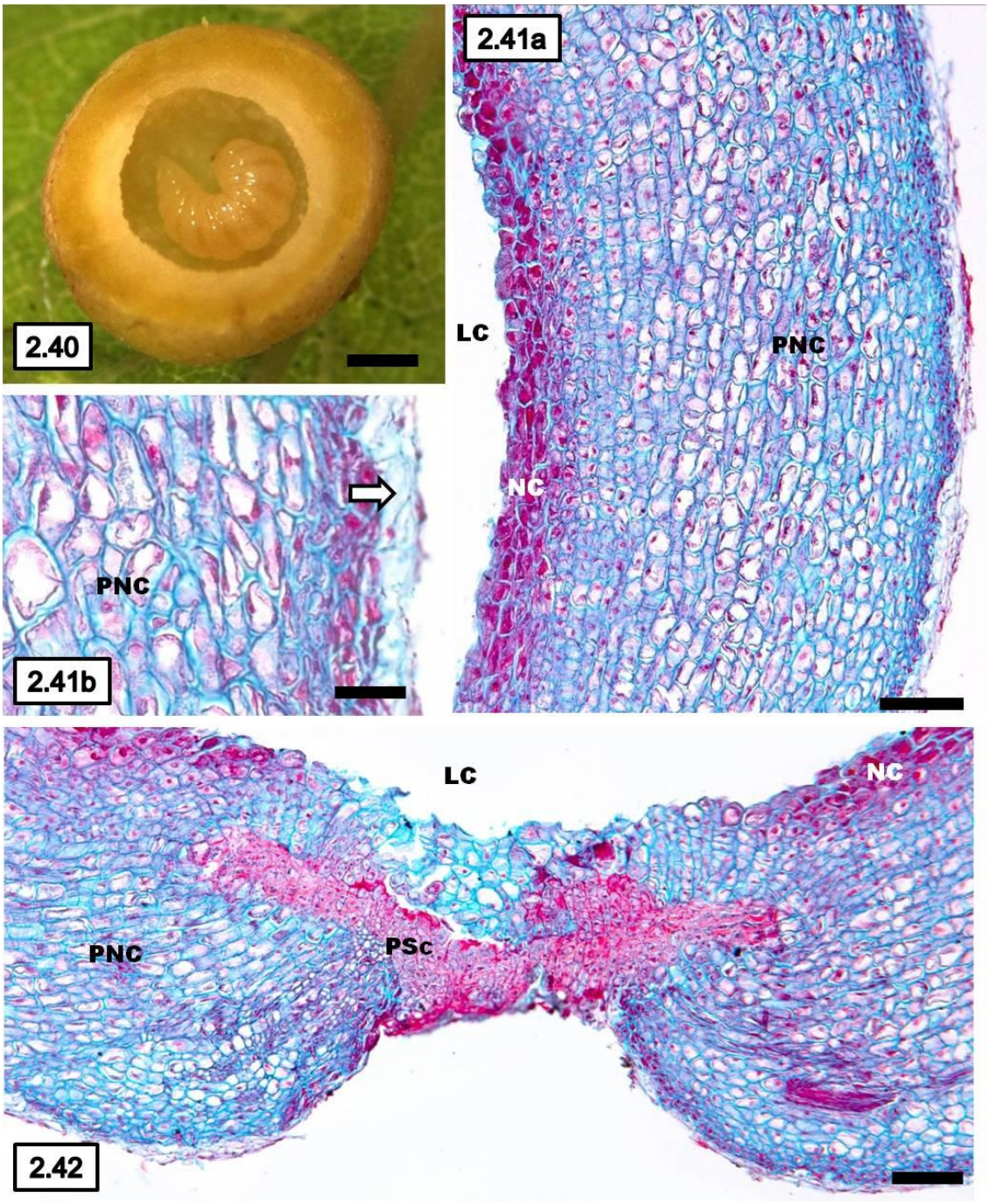


Figs. 2.37 – 2.39. Galls of *Diplolepis nebulosa* in mid-growth phase after the gall has ruptured through the abaxial epidermis. **Fig. 2.37.** Habitus of a gall in the mid-growth phase that has broken through the abaxial epidermis. Note the thickened veins in the leaf adjacent to the gall. Scale bar = 1.78 mm. **Fig. 2.38.** Cross section of a gall in the mid growth phase. Note the lobe-like appearance of the gall caused the rapid proliferation of PNC around the distal PSc sheath. Scale bar = 175 µ. **Fig. 2.39.** Cross section of a gall in the mid growth phase showing the layer of dense NC lining the larval chamber. Scale bar = 45 µ. Ab, abaxial epidermis; Ad, adaxial epidermis; L, larva; LT, leaf tissue; LC, larval chamber; NC, nutritive cells; PNT, parenchymatous nutritive cells; PSc, primary sclerenchyma; VT, vascular tissue.

The two PSc sheaths at the poles of each chamber are 3-5 cells in thickness and are spaced further apart due to the increase in size of the larval chamber as a result of PNC proliferation (Fig. 2.38). PNC are generally the same size as NC, and have also significantly increased in size compared to those at the end of the initiation phase (Fig. 2.48).

Galls late in the growth phase are cream to yellow, spherical, and are typically between three and four millimeters in diameter (Fig. 2.40). Dissected galls show a thick band of whitish NC that circumscribes larval chambers, surrounded by a succulent layer of yellow coloured PNC. Larvae are particularly active late in the growth phase and wriggle violently when probed during dissection.

Galls sectioned late in the growth phase show NC with a mean area of 525 μ^2 (Fig. 2.48) and circumscribe nearly the entire larval chamber in a layer 4-7 cells in thickness (Fig. 2.41a), except for regions of the larval chamber adjacent to the PSc (Fig. 2.42). The size of this region in relation to the size of the gall is smaller compared to earlier phases of development because PSc sheaths have not expanded since the mid-growth phase; whereas, all other gall cells have undergone hypertrophy and hyperplasia (Fig. 2.48). The majority of the chamber wall is composed of PNC of varying size which form a layer typically 20-30 cells in thickness (Fig. 2.41a). These cells have a mean area of 541 μ^2 (Fig. 2.48). Peripheral PNC (2-3 cell layers) that are exposed to the environment collapse likely due to desiccation, forming a ‘pseudo-epidermal’ layer (Fig. 2.41b) that can be peeled off with forceps. Beneath this ‘pseudo-epidermis’, PNC (a layer 3-6 cells thick) appear smaller than those comprising the rest of the chamber wall and are particularly dense with enlarged nuclei (Fig. 2.41b).



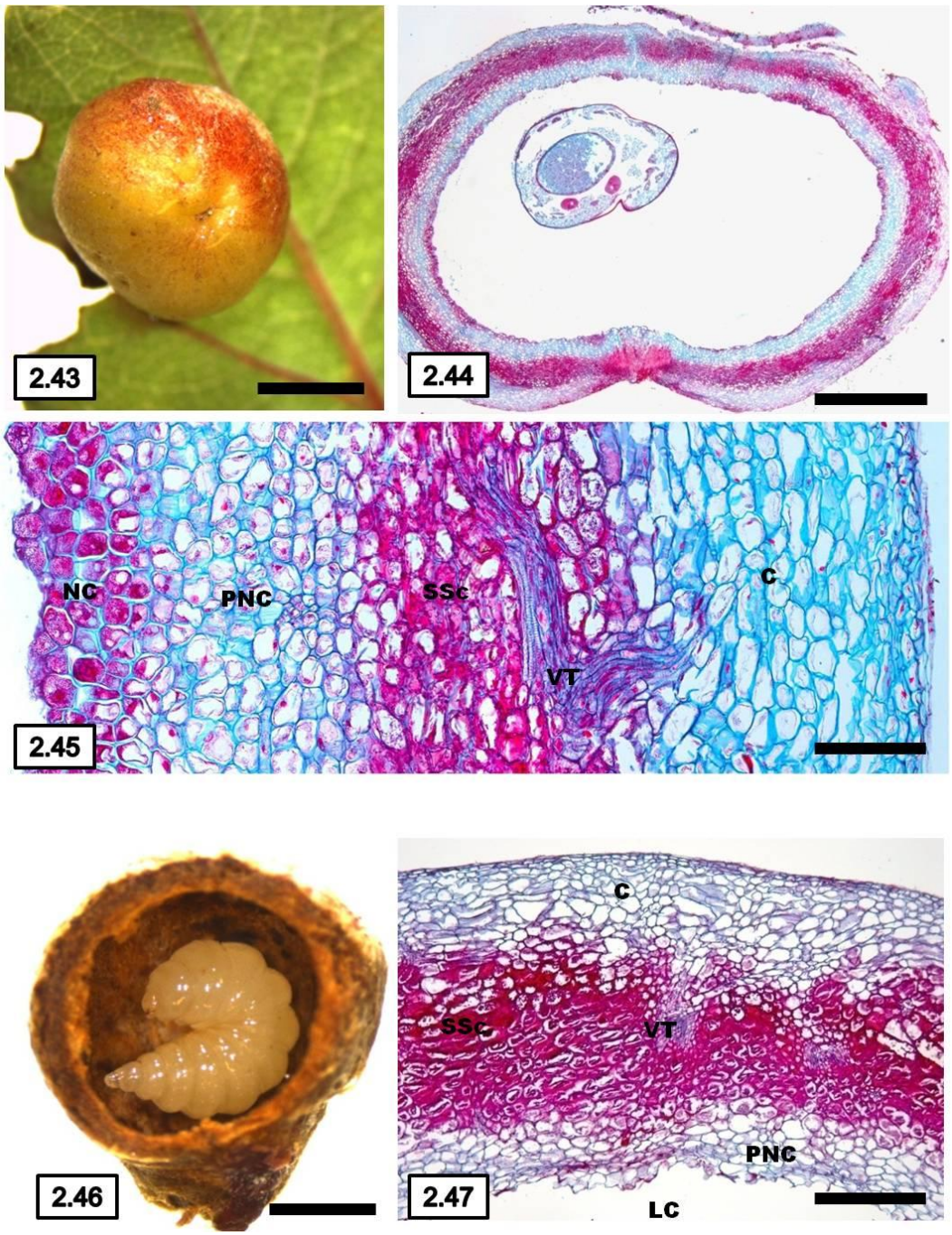
Figs. 2.40 – 2.42. Galls of *Diplolepis nebulosa* late in the growth phase. **Fig. 2.40.** Dissection of a gall late in the growth phase. Scale bar = 1.1 mm. **Fig. 2.41a.** Cross section of a portion of the wall of a gall showing the multi-cellular layer of NC surrounding the larval chamber and rapidly proliferating PNT. Scale bar = 90 µ. **Fig. 2.41b.** Cross section of a gal at high magnification showing the collapsed PNC (arrow) that make up the 'pseudo-epidermal' layer circumscribing the exterior of the gall. Scale bar = 60 µ. **Fig. 2.42.** Cross section of a portion of the wall of a gall showing the abaxial PSc influencing the proliferation of PNC and NC. Scale bar = 100 µ. LC, larval chamber; NC, nutritive cells; PNC, parenchymatous nutritive cells; PSc, primary sclerenchyma.

d. MATURATION PHASE

Mature galls of *D. nebulosa* are present in the field from early August to late September (Fig. 1.7), but are most abundant from the third week of August to the second week of September. Galls exposed to the sun develop a yellow to red pigment in the 'pseudo-epidermis' (Figs. 2.3 and 2.43) and may develop a white powdery substance on the exterior by late August.

The maturation phase begins with the differentiation of a thick layer (10-15 cells) of sclerenchyma, referred to as secondary sclerenchyma (SSc), in the medial portion of the chamber wall (Fig. 2.44). SSc cells are ovoid in cross section, have thin secondary cell walls, are filled with purple-staining ergastic substances (Fig 2.45), and average 649 μ^2 in area (Fig. 2.48). NC line the larval chambers with a semi-patchy distribution (Fig. 2.44) and form a layer 1-5 cells in thickness when present (Fig. 2.45). NC are the largest of the gall, and all other cell types are smaller and of similar size to one another (Fig. 2.48). Larvae feed on NC throughout the maturation phase; however, NC are not replaced by adjacent PNC, hence the patchy distribution. Considering many layers of PNC have lignified to form the SSc sheath, the remaining layer of PNC is only 5-15 cells thick, and a cortical layer differentiates to the exterior of the SSc. This layer is 5-15 cells in thickness and is composed of cells that vary in size and shape, and are vacuolated (Fig. 2.45).

Galls become brown and woody (Fig. 2.46) once larvae have completed development. Some galls detach from senescing leaves, whereas other leaves drop with galls attached. Sections of galls at this phase of development show the absence of nutritive cells – most



Figs. 2.43 – 2.47. Maturing and mature galls of *Diplolepis nebulosa*. **Fig. 2.43.** Habitus of a maturing gall that has a red pigmentation on the outer surface. Scale bar = 2 mm. **Fig. 2.44.** Cross section of a maturing gall showing the multi-cellular layer of SSc that circumscribes the larval chamber. Scale bar = 1.1 mm. **Fig. 2.45.** Cross section of a portion of the wall of a maturing gall showing all the cell types that circumscribe the larval chamber. Scale bar = 105 µ. **Fig. 2.46.** Dissection of a gall that has matured and is brown and woody. Scale bar = 1.7 mm. **Fig. 2.47.** Cross section of a mature gall after the larva has discontinued feeding. Note the absence of NT. Scale bar = 225 µ. C, cortex; LC, larval chamber; NC, nutritive cells; PNC, parenchymatous nutritive cells; SSc, secondary sclerenchyma.

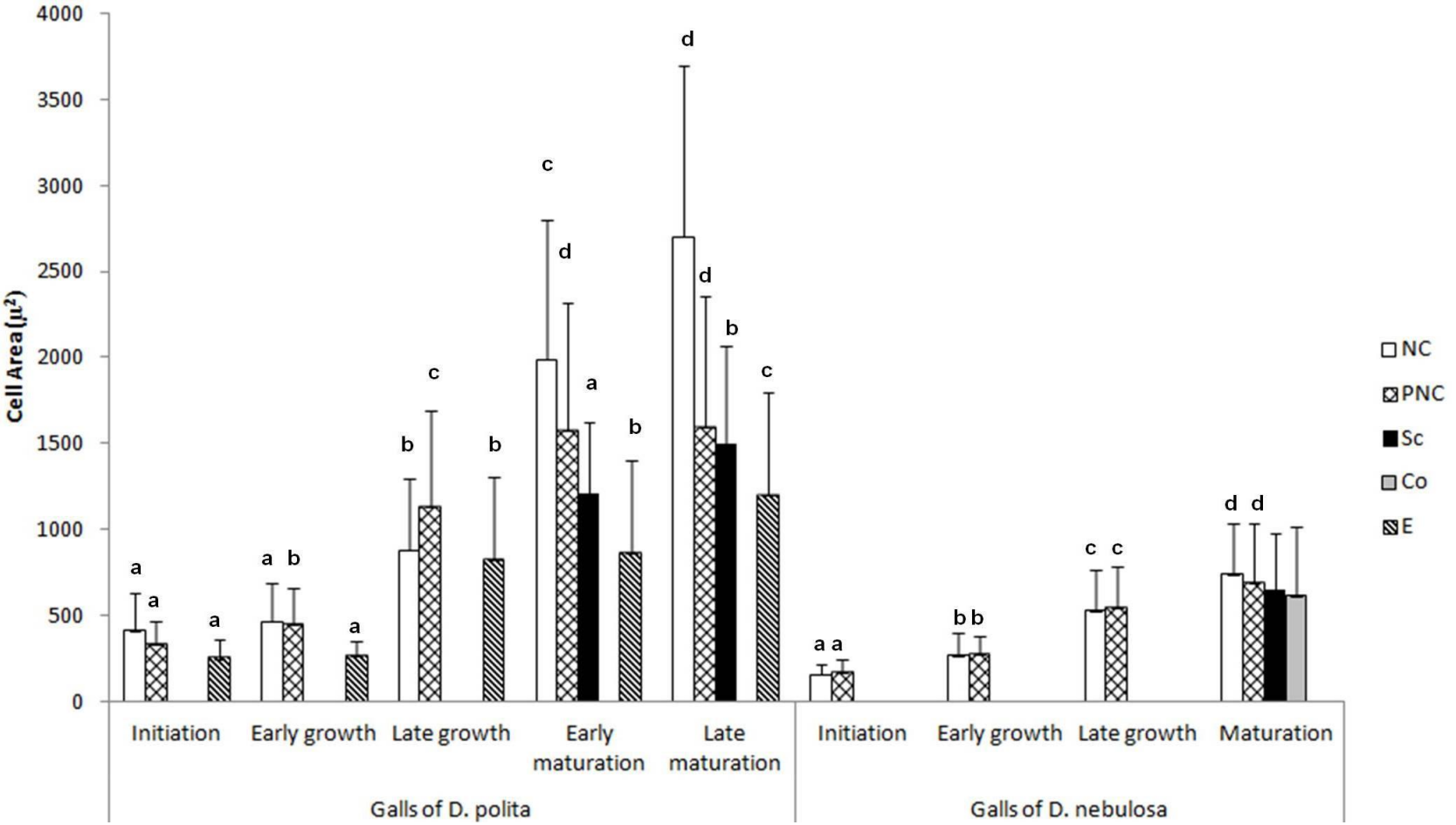


Fig. 2.48. Mean size of cells (μ^2) at each phase of development in galls of *D. polita* and *D. nebulosa.* Error bars represent standard deviations of the mean. Unlike lowercase letters represent significant differences ($p<0.05$) within each cell type across development within each gall based on student's t-test ($\alpha=0.05$) or ANOVA using Tukey's HSD for post-hoc comparisons ($\alpha=0.05$).

were consumed, and those that were not, revert to PNC (Fig. 2.47). In addition, SSc cells are more densely filled with the purple-staining ergastic substances (Fig. 2.47).

E. DISCUSSION

Ab, abaxial epidermis

Ad, adaxial epidermis

C, cortex

E, epidermis

Eg, egg

EgS, eggshell

HC, hypertrophied cells

L, larva

LC, larval chamber

LT, leaf tissue

NC, nutritive cells

OF, ovipositional fluid

P, prickle

PM, palisade mesophyll

PNC, parenchymatous nutritive cells

PSc, primary sclerenchyma

Sc, sclerenchyma

SM, spongy mesophyll

VB, vascular bundle

VT, vascular tissue.

This study represents the first detailed comparison of the anatomy and development of galls induced by two closely taxonomically related cynipid wasps of the same genus. Galls of *D. polita* and *D. nebulosa* are both common in northeastern Ontario and were thus ideal candidates for a comparative developmental study as they share features such as location on host plant, number of larval chambers, and similarity in size and shape. Examining galls at all stages of development in both field and laboratory settings revealed patterns that can further our understanding of strategies employed by cynipid gall-inducers as they gain control and redirect the developmental trajectory of their host organs on host plants. Furthermore, histological techniques used in this study demonstrate the abundance of anatomical characters within mature galls that vary even among taxonomically related species that can be used as characters for distinguishing species of inducers as well as phylogenetic analyses.

Diplolepis polita and *D. nebulosa* both have the ability to significantly alter the development of tissues within their host plant. Galls of *D. polita* are induced on the adaxial surface of leaflets of *R. acicularis*, while galls of *D. nebulosa* are induced on the abaxial surface of leaflets of *R. blanda*. Both galls undergo three phases of development as described in other cynipid galls (Meyer and Maresquelle 1983; Rohfritsch 1992), known as initiation, growth, and maturation. While the developmental events associated with each phase of development are similar across all cynipid galls, there are differences between galls induced by *D. polita* and *D. nebulosa* that reflect their respective galling strategies. Maturing chambers of both galls are encircled by concentric layers of gall cells. Typically, cynipid galls are composed of nutritive cells, parenchymatous nutritive cells, sclerenchyma cells, cortical parenchyma cells, and epidermal cells (Meyer and

Maresquelle 1983; Rohfritsch 1992). There are differences not only in the size of these cells between galls of *D. polita* and *D. nebulosa,* but also in their presence and arrangement. These differences reflect the species-specific nature of gall induction among cynipid wasps as well as the diversity of galling strategies.

i. OVIPOSITION

Adult gall wasps are short-lived and thus synchronization with the phenology of their host plant is critical because a time lag in synchronization could adversely affect the quantity and quality of resources available. Both species oviposit onto cells of immature leaflets of roses; however, there are a variety of differences in their oviposition strategies. Females of *D. polita* oviposit into folded leaflets within closed axillary leaf buds (Fig. 2.7) on their host plant from early to mid May (Fig. 1.7). They have a limited 'window of opportunity' for oviposition as suitable tissues are only available for a short period of time in early spring and thus adults exiting their galls must be synchronized with the development of the first leaf buds on rose stems. In contrast, females of *D. nebulosa* oviposit onto folded leaflets of leaves that have expanded from their respective buds (Fig. 2.26). Leaves remain at this phase of development for only a short period of time; however, leaves suitable for oviposition are continuously produced throughout the season. Thus, there is a wide 'window of opportunity' for oviposition by *D. nebulosa*, lasting from mid May to late June (Fig. 1.7).

A variety of synchronization patterns have evolved between phytophagous insects and their hosts such that insects are present when desirable host tissues are available. More specifically to gall-inducing insects, four synchronization patterns have been described by

Yukawa (2000): 1) insect emergence and host plant availability are both short; 2) the insect emergence period is short and host plant availability is lengthy; 3) the insect emergence is lengthy and host plant availability is short; or 4) insect emergence and host plant availability are both lengthy. Based on Yukawa's (2000) descriptions, the relationship between *D. polita* and its host plant is categorized as 'synchronization pattern 1' and *D. nebulosa* is categorized as 'synchronization pattern 4'.

Pattern 1 is most common in northern climates where bud burst occurs simultaneously on host plants (Yukawa 2000). This insect-host plant relationship can be risky; adult *D. polita* exiting their galls a few days prior to or later than the flush of host tissues at the appropriate phase of development would be unsuccessful in oviposition. To avoid such risks, it is likely that *D. polita* is intimately attuned to conditions of its external environment that are directly or indirectly related to host-plant phenology. This synchronization pattern could also be revealed in the high density of galls per leaf and leaflet considering all successful *D. polita* females exit within the same week in May (Fig. 1.7). The limited number of unforced buds available on each host plant results in females depositing many eggs per leaf bud. In addition, *D. polita* is a spring galler and is heavily attacked by inquilines (see next chapter) and parasitoids (Shorthouse 1973, 2010; Shorthouse *et al.* 2005). Inducing galls in high-density clusters is likely a strategy to compensate for high parasitism rates, assuming parasitoids would not oviposit into every gall within a cluster. This is supported by experiments of parasitoid host-patch usage, where parasitoids generally have 'leaving time rules' such as constant searching time or number of ovipositions (Godfray 1994).

Pattern 4 exhibited by *D. nebulosa* is less risky as adult emergence need not be synchronized with the appearance of leaves suitable for oviposition as they are present for at least six weeks (Fig. 1.7). *D. nebulosa* likely evolved this strategy to capitalize on the lengthy availability of host tissues by decreasing intra-specific competition for oviposition sites. In addition, galls of *D. nebulosa* have a lower parasitism rate than those of *D. polita* (Shorthouse *et al.* 2005; Shorthouse 2010), which could be attributed to the lengthy oviposition period. Galls of all stages are found in the field simultaneously and only a portion would be at the appropriate developmental phase for parasitoid oviposition. This could increase search time by the parasitoids, effectively decreasing their success.

Shorthouse *et al.* (2005) described the emergence period and oviposition strategies of six species of *Diplolepis,* including *D. polita* and found that there is extensive variation between species, not only in the first appearance of adults that have exited their galls, but also in the duration of emergence periods; which range from approximately ten days by species such as *D. polita* and *D. bicolor,* to a period greater than one month by species such as the stem gallers *D. triforma* and *D. spinosa. D. nebulosa* similarly has a lengthy emergence period, reported for the first time here (Fig. 1.7). Each species of *Diplolepis* is apparently specialized on a specific location or tissue type on their hosts (Shorthouse *et al.* 2005). Based on data presented in this thesis (Fig. 1.7) and by Shorthouse *et al.* (2005), it is suggested that the period of oviposition reflects the length of time each species' respective tissues are available in the field for successful oviposition.

ii. INITIATION PHASE

Egg hatch

Diplolepis polita and *D. nebulosa* both exhibit a remarkable ability to alter the developmental trajectories of cells within their host leaves to produce two anatomically and morphologically distinct galls. Both species deposit eggs onto immature rose leaflets without damaging nearby cells. Avoiding leaflet damage likely ensures that the host leaf will develop into a functional organ that can supply developing larvae with nutrients (Shorthouse *et al.* 2005).

Eggs of *D. polita* are deposited onto the adaxial surface of leaflets of *R. acicularis* and the first cells to react to the egg or fluids associated with the egg are those that would normally differentiate into palisade mesophyll (Fig. 2.8). Early gall cells stimulated by the egg proliferate outwards from the adaxial surface of the leaf, and surround the hatching larva, forming a small gall with immature prickles (Fig. 2.11). It is not until galled leaves expand from their respective buds and unfold that galls can be located in the field. Galls at this stage are at the end of initiation (Figs. 2.12 and 2.13a) and essentially resemble smaller versions of mature galls (compare Figs. 2.12 and 2.1); they are red, spherical, and are covered with immature prickles.

In contrast, eggs of *D. nebulosa* are deposited onto the abaxial surface of leaflets of *R. blanda* (Figs. 2.27 and 2.28). Eggs are laid in May and June, and despite careful weekly examination of study sites throughout the spring and summer, immature galls were not found until mid to late July (Fig. 1.7). This ‘gap’ in phenology is due to either a delay in egg hatch or a delay in gall growth and first instar larvae between the epidermal layers of

the leaves cannot be detected with the naked eye or with the use of a dissecting microscope. Considering all cynipids require meristematic or rapidly dividing tissues to induce their galls (LeBlanc and Lacroix 2001; Csóka *et al.* 2005), the latter is more likely as leaves would be nearly mature at the time of initiation if egg hatch was delayed until mid July or later. In addition, a delay in egg hatch would increase the risk of egg predation or desiccation. Leaf cells that normally develop into spongy mesophyll are redirected by *D. nebulosa* and become gall cells. Rather than gall cells proliferating outward from the leaf and surrounding larvae, as is the case in initiation of galls of *D. polita*, larvae of *D. nebulosa* hatch from their eggs and enter a depression within the host leaf and there is little outward proliferation of tissues. Thus, the initiation phase of galls of *D. nebulosa* occurs between the epidermal layers of host leaves (Figs. 2.29 and 2.30).

Late initiation phase galls

The developmental trajectories of galls induced by these two species are different by the end of the initiation phase. The surface of galls of *D. polita* has a red pigment and soft, immature prickles; whereas, the surface of galls of *D. nebulosa* is smooth. Differences in anatomy also occur in all cell types, but most notably in the arrangement of nutritive tissue and presence of sclerenchyma tissue (Figs. 2.13a and 2.30). Nutritive cells within immature galls of *D. polita* are small compared to other cells within galls at this phase of development and have a patchy distribution around the larval chamber (Fig. 2.13a). This finding is congruent with other cynipid galls (Brooks and Shorthouse 1998; Bronner 1992) and has been attributed to the absence of larval feeding in galls until later in gall development. This typical distribution and appearance of nutritive cells and absence of larval feeding is not observed in galls or larvae of *D. nebulosa*; nutritive cells are large,

and form a distinct, multi-cellular layer around the larval chamber (Fig. 2.30) and larvae actively feed on nutritive tissues in the first larval instar (Fig. 2.31).

The formation of a sclerenchyma sheath in cynipid galls is typically a defining feature of gall maturation (Csóka *et al.* 2005); however, galls of *D. nebulosa* develop two primary, cap-like sclerenchyma sheaths by the end of gall initiation. Two distinct primary sheaths that do not intersect form beneath each epidermal surface and encase the larval chamber (Fig. 2.30). Other galls induced by members of the genus *Diplolepis* also have primary sclerenchyma sheaths, including those induced by *D. rosaefolii* (LeBlanc and Lacroix 2001), *D. ignota, and D. gracilis* (Shorthouse 1975). One common characteristic between these four galls is they are induced late in the season (Fig. 1.7; LeBlanc and Lacroix 2001; Shorthouse 1975). They do not form a clade within *Diplolepis* phylogeny (Plantard *et al.* 1998), and do not have common larval, adult, or external gall morphologies (Shorthouse 2010). This primary sclerenchyma sheath likely protects against ovipositing inquilines and parasitoids when galls are small. Had galls of *D. nebulosa* undergone a lengthy initiation period there would be a prolonged period where larvae are easily reached by the ovipositors of enemies. In addition, sclerenchyma can restrict water loss inside the gall, creating a humid microenvironment which would prevent desiccation of the inducer (Formiga *et al.* 2011) and the same likely occurs with galls of *D. nebulosa*.

iii. GROWTH PHASE

Cynipid galls in the growth phase rapidly increase in size through proliferation and hypertrophy of gall parenchyma, and typically reach their maximum size by the beginning of gall maturation (Bronner 1992). Galls of *D. polita* do not significantly

change externally (Fig. 2.15) or internally (Figs. 2.17 and 2.18) from the late initiation phase to the growth phase. They increase in size, while larvae remain small (Fig 2.16) and galls are composed of small, sparse NC, PNC and a single E layer.

In contrast, galls of *D. nebulosa* change in morphology and internal tissue organization throughout the growth phase. Parenchyma first proliferates laterally into leaf tissues surrounding nearby vascular bundles (Figs. 2.32, 2.33, 2.36a and 2.36b), then proliferates outwards from the abaxial surface of the leaf, eventually resulting in a spherical gall (Fig. 2.35). The proliferation of gall parenchyma causes primary sclerenchyma plates to become spaced further apart (Fig. 2.38). This space leaves larvae vulnerable to attack by parasitoids and inquilines through the soft, spongy chamber wall around the equatorial regions of the gall (Fig. 2.38). Galls of *D. nebulosa* were not inhabited by inquiline or parasitoid eggs until galls had reached the early or mid-growth phase, thus supporting that the primary sclerenchyma plates provide protection against ovipositing enemies earlier in gall development.

'Pseudo-epidermis' in galls of D. nebulosa

As parenchyma within galls of *D. nebulosa* proliferates causing the gall to expand outward from the host leaf, the abaxial epidermis is not incorporated into the gall and ruptures, likely as a result of epidermal tissues being fully differentiated and mature when galls begin to grow (Fig. 2.37 and 2.38). The epidermal layer attached to the gall becomes dry and is sloughed off, thus galls lack an epidermal layer (Fig. 2.40 and 2.41b). The degree of control that gallers have over the development of host plant tissues has been shown to be affected by the degree of differentiation of host tissues at the time of gall

initiation (Raman 2011). For example, leaf galls of psyllids are induced when the epidermis is more differentiated than adjacent mesophyll, and thus mesophyll cells respond to the galling stimulus at a higher magnitude than cells of the epidermis. The function of the epidermis in cynipid galls is likely similar to normal leaves, primarily providing protection against water loss (Evert 2006). Late in the growth phase, galls of *D. nebulosa* develop a 'pseudo epidermis' on the exterior of galls that is composed of many layers of collapsed parenchyma cells. This layer becomes pigmented when exposed to the sun and can be peeled from the gall with forceps. It is likely the 'pseudo epidermis' replaces many of the functions of a true epidermis such as preventing water loss. Galls of *D. nebulosa* are most commonly found on stressed plants in arid habitats such as in grasslands and on sand dunes and the 'pseudo epidermis' could compensate for a true epidermal layer and would be beneficial under such environmental conditions.

iv. MATURATION PHASE

By the maturation phase, galls of both *D. polita* and *D. nebulosa* have reached their final shape and dimensions. The most prominent change to galls in the maturation phase is the differentiation of a sclerenchyma layer. Sclerenchyma cells form by the deposition of lignin in PNC that have completed their growth and are thought to protect larvae from ovipositing parasitoids (Stone and Cook 1998; Ronquist and Liljeblad 2001; Stone and Schönrogge 2003; Csóka *et al.* 2005; Bailey *et al.*2009). Sclerenchyma tissue causes galls to become hard and brittle. The sclerenchyma cells of several galls induced by *Diplolepis* have been examined including those within single and multi-chambered galls on leaves and stems (Brooks and Shorthouse 1998; Sliva and Shorthouse 2005; Leggo and Shorthouse 2006; LeBlanc and Lacroix 2001) and all, including those induced by *D.*

nebulosa, are circular (in cross section) and have an even layer of lignin that forms around the entire inner surface of the cell wall (Fig. 2.45). Sclerenchyma cells in galls of *D. polita* are different from those in galls induced by other *Diplolepis* (Shorthouse 1975; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001; Sliva and Shorthouse 2005; Leggo and Shorthouse 2006) as well as other cynipids (Meyer and Maresquelle 1983; Rohfritsch 1992). There is uneven lignin deposition within cells (Fig. 2.22), where only two, and sometimes only one, inner surface of cell walls, as seen in cross section, is lignified. It is unknown why *D. polita* induces sclerenchyma cells that are different from those induced by other cynipids; however, it does illustrate the control wasps in this genus have over their host plant. Further studies of sclerenchyma cell structure in galls of *Diplolepis* and other cynipids would provide insight into patterns in lignin deposition in galls and would help to determine if the structure of sclerenchyma in galls of *D. polita* is unique to all cynipids, or if there is diversity in sclerenchyma structure across galls induced by cynipids.

In addition to differences in lignin deposition in galls of *D. polita* and *D. nebulosa*, differences also exist between nutritive cells. Throughout earlier gall development, nutritive cells in galls of *D. polita* are small and arranged singly around the larval chamber (Figs. 2.13a and 2.17); however, once galls begin to undergo lignification, nutritive cells increase in size and density (Fig. 2.21). By mid maturation, nutritive cells can be seen with the naked eye in dissected galls and are the largest cells within the gall (Fig. 2.25; Fig. 2.48). In contrast, nutritive cells within immature galls of *D. nebulosa* occur in a dense, multi-cellular layer circumscribing the larval chamber; however, by the maturation phase, they have a patchy distribution around the larval chamber (Fig. 2.44).

Larvae actively feed throughout gall development, from the first larval instar (Fig.2.31) and is assumed that larvae reach maturity and discontinue feeding once galls begin to mature. This is also supported by the thick layer of parenchyma that lines the larval chamber in brown, woody galls at the end of the season (Fig. 2.47). In galls of other *Diplolepis* species, the parenchymatous nutritive cells convert to nutritive cells in the maturation phase and larvae consume all cells up to the sclerenchyma layer (Roth 1949; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001; Sliva and Shorthouse 2005; Leggo and Shorthouse 2006).

v. VASCULARIZATION

Vascular tissue differentiates within cynipid galls in the growth phase and joins with existing vascular tissue of the host plant to supply developing larvae with nutrients (Csóka *et al.* 2005). *D. polita* and *D. nebulosa* use different strategies to connect their galls to the vascular tissue of their host plant. Galls of *D. polita* are induced on immature leaflets that are rapidly growing and do not have well defined or differentiated vascular tissue (Fig. 2.8). Early in gall development, vascular tissue is thickest and most well defined at the position where galls connect to host leaves (Figs. 2.13b and 2.17) and is sparse near the apex of galls (Figs. 2.13a, 2.14, and 2.17). This suggests that as vascular tissue differentiates and proliferates within normal leaves as a part of the typical developmental pattern, vascular tissue also proliferates into nearby gall tissues. Vascular tissue within galls of *D. polita* at all phases of development is also immature and does not have well defined vascular bundles with xylem and phloem. In contrast, galls of *D. nebulosa* remain immature on leaflets for many weeks before they enter the growth phase (Fig. 1.7). Vascular tissue does not differentiate in galls during this developmental 'lag'.

Galls of *D. nebulosa* usually develop in close proximity to the midrib or first order veins and it is likely that water and nutrients are passed by diffusion and osmosis through the gall cells from nearby leaf vascular tissue. Once galls enter the growth phase, PNC proliferate and surround nearby veins (Figs. 2.33, 2.36a, and 2.36b), essentially reinforcing the connection the gall has with the vascular tissue of the leaf, and then vascular tissue proliferates and differentiates into the gall (Figs. 2.36b and 2.38). In addition, the vascular tissue within galls of *D. nebulosa* matures with gall development and by gall maturation vascular tissue is in the form of distinct bundles with xylem and phloem easily distinguished (Fig. 2.45). Thus, Galls of *D. nebulosa* seem to have more abundant and well defined vascular bundles than do galls of *D. polita.* This could be because galls of *D. polita* develop quickly (Fig. 1.7) on their host plants and undergo most of their growth while leaves are still developing and thus acting as physiological sinks. Galls of *D. polita* may not need to develop a robust pipeline to deliver nutrients seeing nutrients are already being transported to immature host leaves in abundance. In contrast, galls of *D. nebulosa* do not develop until leaves have matured and are also found in arid habitats, thus the differentiation of thick, well defined vascular bundles would be beneficial.

vi. SIZE OF GALL CELLS

This is the first study comparing the size of each cell type throughout gall development as well as the first to compare cells between galls induced by different species. All cell types in both galls increase in size throughout development (Fig. 2.48). Insect galls are known to rapidly increase in size throughout the growth phase (Bronner 1992; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001); however, all gall cells in these two

Diplolepis galls are smallest during the initiation and growth phase compared to those in the maturation phase, demonstrating that cells undergo hyperplasia while the galls as a whole increase in size most rapidly. Once gall growth slows and galls enter the maturation phase, cells increase in size. Of interest, all cells of galls induced by *D. polita* are significantly larger ($p<0.001$) than those in galls of *D. nebulosa* at all phases of development (Fig. 2.48, further statistical details are provided in the appendix). Considering size of gall cells is an aspect of gall studies not previously investigated, there has been little speculation as to the significance of this feature and the impact it has on the developing inducers. Studies of typical plant cells have shown that cell size can affect the water relations of plants and the ability to survive drought is inversely correlated with cell size (references within Cutler *et al.* 1977). This may explain why young leaves and meristematic tissue have some of the smallest cells within their respective plant and show the least susceptibility to damage from water stress (Cutler *et al.* 1977). Galls of *D. nebulosa* are most common on water stressed *R. woodsii* in the grasslands of southern Alberta and Saskatchewan (Shorthouse 2010), and likewise only on *R. blanda* in Ontario growing in arid habitats such as sand dunes of Manitoulin Island (Fig. 1.2). Roses on sand dunes of Manitoulin Island are heavily galled by *D. nebulosa* and exhibit signs of stress such as small, folded leaflets and overall small plant size, whereas plants in moist habitats are rarely galled. Shorthouse (2010) argued that *D. nebulosa* is adapted to grassland conditions and the current study showing that the induction of small cells within a thick-walled gall supports this hypothesis as both features would help to maintain a microclimate with high humidity.

In contrast to *R. blanda*, *R. acicularis* is a boreal plant most commonly found in moist, partially shaded habitats. The occurrence of galls on hosts growing in partially shaded, moist conditions suggest that galls of *D. polita* are not as susceptible to damage from water stress as are galls of *D. nebulosa* and thus there has not been a selection for smaller gall cells. While relative differences in the size of cells among the galls of various cynipids may be an unexplored aspect of gall biology, results from the current study reveal yet another anatomical feature of galls of *D. polita* and *D. nebulosa* that demonstrate the high degree of control both species have over the development of the cells comprising their galls.

vii. GENERAL GALLING STRATEGIES OF *D. polita* AND *D. nebulosa*

Although rates of gall growth were not measured in this study, I concluded there are two strategies of *Diplolepis* wasps based on dissections and histological results. Galls of *D. polita* undergo rapid exponential growth soon after gall initiation whereas, larvae remain small in early instars (Fig. 2.16) waiting to increase in size exponentially during gall maturation after the development of the sclerenchyma layer (Figs. 2.19 and 2.2). Similar growth patterns of galls and larvae of a tephritid gall fly were reported by Lalonde and Shorthouse (1985), where galls expand rapidly during the growth phase and then slow at maturation. Larvae in this gall remain in the second instar throughout the growth phase and then grow quickly, attaining 98% of their mature larval mass during gall maturation. According to Raman (2011), galls of psyllids grow quickly when the insect is in the first and second instar and slow in the later instars as the insect approaches the final developmental phase before becoming adults. Thus, gall and larval/nymphal development pattern is common among many gall insects, and is similar to that of many endoparasitic

hymenoptera which oviposit into immature hosts and then delay development until their hosts are mature (See references in Lalonde and Shorthouse 1985). One explanation for such a pattern is that increased gall size is correlated with lower parasitism rates in a variety of gall systems because ovipositors of enemies cannot reach the centre of the larval chamber of larger galls (Price and Clancy 1986; Weis *et al.* 1992; Fernandes *et al.* 1999; Stone *et al.* 2002; Bailey *et al.* 2009; Zargaran *et al.* 2011). Fernandes *et al.* (1999) showed that larval mortality from parasitoids and inquilines is typically high in the first month of gall development when galls are small and have not developed tough chamber walls and larvae that survive the first month of development are less likely to be attacked. Galls of *D. polita* rapidly increase in size and mature quickly (Fig. 1.7) likely as a strategy to avoid parasitism. Once larvae are protected within large chambers reinforced with sclerenchyma, they begin to feed and increase in size.

Galls and larvae of *D. nebulosa* do not follow the development pattern associated with other galls, including *D. polita.* Larvae nearly fill the entire larval chamber from gall initiation (2.30) to maturation (2.36a) and thus galls and larvae seem to develop at similar rates. Most other galls induced by *Diplolepis* in Ontario are initiated in the early spring and are heavily attacked by parasitoids when galls are small and chamber walls are soft and succulent (Shorthouse 2010). The galling strategy of *D. nebulosa* likely evolved to reduce attack by inquilines and parasitoids. While other immature *Diplolepis* galls are small, succulent, and easily penetrated by the ovipositors of enemies, galls of *D. nebulosa* remain nearly undetectable over a lengthy period of time (Fig. 1.7) and are protected from attack from enemies by two primary sclerenchyma plates (Fig. 2.30).

Price *et al.* (1987) proposed three hypotheses for the adaptive value of galls to the insects within: the microenvironment hypothesis, which suggests that galls provide insects with protection from harsh environmental conditions; the enemy hypothesis, which proposes that being enclosed within plant tissues provides gallers with protection from predation and parasitism; and the nutrition hypothesis which suggests that gall tissues are of superior nutritional quality than other plant tissues. It is widely accepted that external gall morphology is diverse among cynipid galls because of pressures of parasitism over evolutionary time (Stone and Schönrogge 2003; Bailey *et al.* 2009). Many of the characters that differ between galls and life history strategies of *D. polita* and *D. nebulosa* have likely been shaped by the selective pressures of parasitism over evolutionary time, and thus the observations for these two galls also supports the enemy hypothesis.

In conclusion, the results presented here support the hypothesis of Stone and Schönrogge (2003) that the modification of outer gall tissues (epidermis and cortex) is mostly responsible for the morphological diversity of cynipid galls. However, this study showed that external morphological diversity only accounts for a portion of gall diversity as many inner gall characters and developmental pathways have also undergone extensive diversification among cynipids and it is only by histological examination that the extent of the control cynipid gall wasps have over their host plant can be fully understood and appreciated. In addition, many useful characters are revealed from histological examination of galls that will prove useful when searching for gall strategy patterns in future phylogenetic analyses of the family Cynipidae.

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G. APPENDIX

Table. 2.1. Mean cell area with standard deviations in galls of *Diplolepis polita* (μ^2). Unlike lowercase letters represent significant differences ($p<0.05$) within each cell type based on student's t-test ($\alpha=0.05$) or ANOVA using Tukey's HSD for post-hoc comparisons ($\alpha=0.05$). n = 210 for each cell type.

Phase of Development	Cell Type			
	NC	PNC	Sc	E
End of Initiation	412±220 **a**	330±137 **a**	N/A	249±106 **a**
Early Growth	465±222 **a**	447±210 **b**	N/A	265±85 **a**
Late Growth	880±417 **b**	1129±564 **c**	N/A	824± 483 **b**
Early Maturation	1986±813 **c**	1576±746 **d**	1212±407 **a**	858± 542 **b**
Late Maturation	2701±993 **d**	1593±764 **d**	2266±565 **b**	1199±595 **c**

Table. 2.2. Mean cell area with standard deviations in galls of *D. nebulosa* (μ^2). Unlike lowercase letters represent significant differences ($p<0.05$) within each cell type based on analysis of variance using Tukey's HSD for post-hoc comparisons ($\alpha=0.05$). n = 210 for each cell type.

Phase of Development	Cell Type			
	NC	PNC	SSc	C
End of Initiation	157±58 **a**	165±74 **a**	N/A	N/A
Early Growth	265±130 **b**	269±105 **b**	N/A	N/A
Late Growth	525±237 **c**	541±244 **c**	N/A	N/A
Maturation	736±296 **d**	687±352 **d**	649± 324	611±409

Table 2.3. Summary of statistical analysis of the mean size of cells at each phase of development between galls of *D. polita* and *D. nebulosa*.

Phase of Development	Cell Type	Species of Inducer	Mean (μ^2)	*N*	T value	P
Late Initiation	NC	*D. polita*	412	210	10.818	<0.001
		D. nebulosa	157	210		
	PNC	*D. polita*	330	210	10.733	<0.001
		D. nebulosa	165	210		
Early Growth	NC	*D. polita*	222	210	11.233	<0.001
		D. nebulosa	265	210		
	PNC	*D. polita*	447	210	10.945	<0.001
		D. nebulosa	269	210		
Late Growth	NC	*D. polita*	880	210	10.711	<0.001
		D. nebulosa	525	210		
	PNC	*D. polita*	1129	210	13.860	<0.001
		D. nebulosa	541	210		
Maturation	NC	*D. polita*	2701	210	27.493	<0.001
		D. nebulosa	736	210		
	PNC	*D. polita*	1593	210	15.609	<0.001
		D. nebulosa	687	210		
	Sc	*D. polita*	1501	210	18.964	<0.001
		D. nebulosa	649	210		

CHAPTER III: DIFFERENCES IN THE MODIFICATION OF GALLS OF *Diplolepis polita* AND *Diplolepis nebulosa* BY *Periclistus* INQUILINES (HYMENOPTERA: CYNIPIDAE).

A. ABSTRACT

Leaf galls induced by the cynipid wasps *Diplolepis polita* and *D. nebulosa* on *Rosa acicularis* and *R. blanda*, respectively, are attacked and structurally modified by inquiline wasps of the genus *Periclistus*. Two undescribed species of *Periclistus* are gall-specific, and are referred to here as *Periclistus* 1, for those in galls of *D. polita*, and *Periclistus* 2 for those in galls of *D. nebulosa*. Galls induced by *Diplolepis* undergo distinct phases of development referred to as oviposition, initiation, growth, and maturation, and are composed of gall cells known as nutritive, parenchymatous nutritive, sclerenchyma, cortex and epidermis, along with vascular bundles, that appear in layers from the surface of the chambers to the outside. Galls inhabited by *Periclistus* do not undergo similar phases. Instead, each *Periclistus* sp. oviposits into immature galls, killing the inducer larvae with their ovipositors, and then the presence of *Periclistus* eggs along the inner chamber surface causes changes in gall structure. *Diplolepis*-induced nutritive cells degrade and *Diplolepis*-induced parenchymatous nutritive cells enlarge, and as a result, the galls increase in size. Feeding by first-instar *Periclistus* larvae stimulates the differentiation and proliferation of *Periclistus*-induced parenchymatous nutritive cells and nutritive cells. Vascular bundles differentiate between the *Diplolepis* and *Periclistus*-induced tissues and join those that were initiated in gall tissues prior to *Periclistus* modification. These vascular bundles join those of the host leaflet, allowing assimilates from the plant to reach each of the *Periclistus* feeding sites where they are imbibed by the larvae. *Periclistus* larvae then restrict their feeding to one spot on the inner chamber

surface and nutritive cells with dense cytoplasm begin to differentiate. Proliferating cells surround and encase each larva in its own chamber. While this is happening in galls of *D. polita*, a layer of sclerenchyma cells, known as the inquiline-induced primary sclerenchyma, also differentiates. This does not occur in galls of *D. nebulosa* until later in modification. Nutritive and parenchymatous nutritive cells induced by *Periclistus* in galls of both species are larger than those induced by *Diplolepis* and appear in dense clusters throughout the inside surface of their chambers. Once galls enter the maturation phase, inquiline-induced primary sclerenchyma differentiates in modified galls of *D. nebulosa,* circumscribing the outer gall. In modified galls of both *D. polita* and *D. nebulosa,* a second layer of inquiline-induced sclerenchyma, known as secondary sclerenchyma, differentiates between each of the inquiline chambers. Secondary sclerenchyma cells in the walls of *Periclistus* chambers are smaller than primary sclerenchyma cells circumscribing the entire gall. All cells in *Periclistus*-modified galls of *D. polita* are larger than cells in the same location in *Periclistus*-modified galls of *D. nebulosa*. Significance of the different phenologies of the two species of *Periclistus* and the manner by which they modify their respective host galls are discussed in light of perceived patterns in the strategies of *Periclistus* inquilines.

B. INTRODUCTION

Insect galls are atypical plant growths that provide enhanced nutrition and shelter to the immatures of inducers (Raman 2011). Galls also represent an attractive resource for other non-galling insects such as entomophagous parasitoids that secondarily inhabit galls and exploit immature inducers as a food source along with phytophagous insects that feed on gall tissues (Sanver and Hawkins 2000). These phytophagous insects are referred to as

inquilines (Sanver and Hawkins 2000), a term derived from the Latin *Inquilinus*, meaning tenant or guest (Yang *et al.* 2001). The impacts of inquilines on gall inducers can be diverse (Sanver and Hawkins 2000). Inquilines can have little or no impact on gall inducers (Miller 2004); however, most studies on inquiline inhabited galls report negative impacts on inducers including food deprivation, increased risk of fungal infection, limited physical space inside galls, and directly being killed by inquilines (Ronquist 1994; Heard and Buchanan 1998; Sanver and Hawkins 2000; Yang *et al.* 2001; Shorthouse 1973; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001).

Inquilines are found in galls induced by a variety of insect taxa including psyllids (Yang *et al.* 2001; Heard and Buchanan 1998), aphids (Miller 2004), gall midges, and sawflies, with the most diversity within the family Cynipidae (Sanver and Hawkins 2000). In many cases, inquilines evolved within the same lineage as their gall-inducing hosts (Yang *et al.* 2001). This relationship has been termed agastoparasitism by Ronquist (1994), where inquilines and the majority of their hosts are within the same family or even genus and form a monophyletic group. Perhaps the most striking example of this occurs within the subfamily Cynipinae, which is composed largely of gall inducers (approximately 1 300 described species), but also includes the large inquiline tribe Synergini (approximately 145 species) (Csóka *et al.* 2005), members of which primarily attack galls of other cynipids (Ronquist 1994; Csóka *et al.* 2005). There are seven genera within Synergini; *Ceroptres, Saphonercus, Synergus, Synophrus*, *Periclistus*, *Synophromorpha*, and *Rhoophilus*, each of which are generally associated with one genus of gall-inducing cynipid (Csóka *et al.* 2005). For example, *Synophromorpha* is only found in galls induced by *Diastrophus* on *Rubus* and *Periclistus* in galls induced by *Diplolepis* on *Rosa* (Csóka

et al. 2005). In addition, each species of cynipid inquiline tends to be restricted to galls induced by a single host species (Ronquist 1994).

It has been hypothesized that cynipid inquilines were once gall inducers that secondarily lost the ability to induce galls of their own (Ronquist 1994). Ronquist (1994) argued that this event was in response to competition for favorable sites of gall induction. However, cynipid inquilines of the genus *Periclistus*, and possibly those of other genera, retained the ability to control the development of plant cells and remodify the structures of the galls they inhabit. Indeed, *Periclistus* inquilines stimulate the tissues of their host galls resulting in each inquiline larva becoming enclosed within its own chamber (Shorthouse 1975, 1998; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001). Larval chambers of inquilines have been found either within the host larval chamber (in which case the gall inducer is always killed) or in the outer tissues of the host gall (Csóka *et al.* 2005). Inquiline-modification of galls can also have a significant impact on the overall size of both single-(Shorthouse 1973; Brooks and Shorthouse 1998) and multi-chambered galls (László and Tóthmérész 2006). For example, *D. nodulosa* on the stems of *R. blanda* are three times larger in diameter when occupied by the inquiline, *Periclistus pirata* (Brooks and Shorthouse 1998). Thus, the strategies employed by inquilines when they modify galls, the effect they have on the host gall inducer, and the extent of their modifications are diverse.

Most studies on cynipid inquilines have focused on their phylogeny and evolution (Ronquist 1994; Liljeblad and Ronquist 1998; Ronquist and Liljeblad 2001, Acs *et al.* 2010; Nylander *et al.* 2004; Van Noort *et al.* 2007), or the impact on gall communities (Brooks and Shorthouse 1997; Sanver and Hawkins 2000; László 2001; László and

Tóthmérész 2006; Bailey *et al.* 2009). Few botanical studies have been undertaken illustrating the manner by which cynipid inquilines modify their host galls, the most notable examples being Shorthouse (1975); Shorthouse (1980); Brooks and Shorthouse (1998); Shorthouse (1998); and LeBlanc and Lacroix (2001). Each of these studies compared the anatomy of *Diplolepis* galls modified by *Periclistus*, with normal or unmodified galls; however, there is still much to be learned about the manner by which inquilines gain control and modify host galls. For example, in galls induced by *Diplolepis*, one could ask if *Periclistus* that attack galls of closely related *Diplolepis* on the same host organ, modify host galls in a similar manner or does the degree of modification vary among species of *Periclistus*.

One approach to answering such questions is to compare the developmental events from oviposition to maturation of galls induced by two or more species of *Periclistus*. To do this, one should have a clear understanding of all events in the typical developmental trajectory of the host galls such that comparisons can be made between modified and normal galls. The galls of two species of inducers for which this information is known are found in central Ontario and are the subject of the present study. One of these species is *D. polita* which is found on the leaves of *R. acicularis* and the other is *D. nebulosa* which is found on the leaves of *R. blanda* (see Chapter II). Galls of both species are commonly attacked by *Periclistus* inquilines. As in Chapter II, the approach taken is histological which can be argued is the only way in which anatomical differences in cell types can be observed. Thus, the purpose of this study was to compare the developmental events of inquiline-modified galls of *D. polita* and *D. nebulosa* by their respective *Periclistus* inquilines to determine if inquilines exhibit species-specific patterns of gall modification.

In addition, the size of cells influenced by *Periclistus* within the various layers of tissues of the galls of both species of inducers are compared.

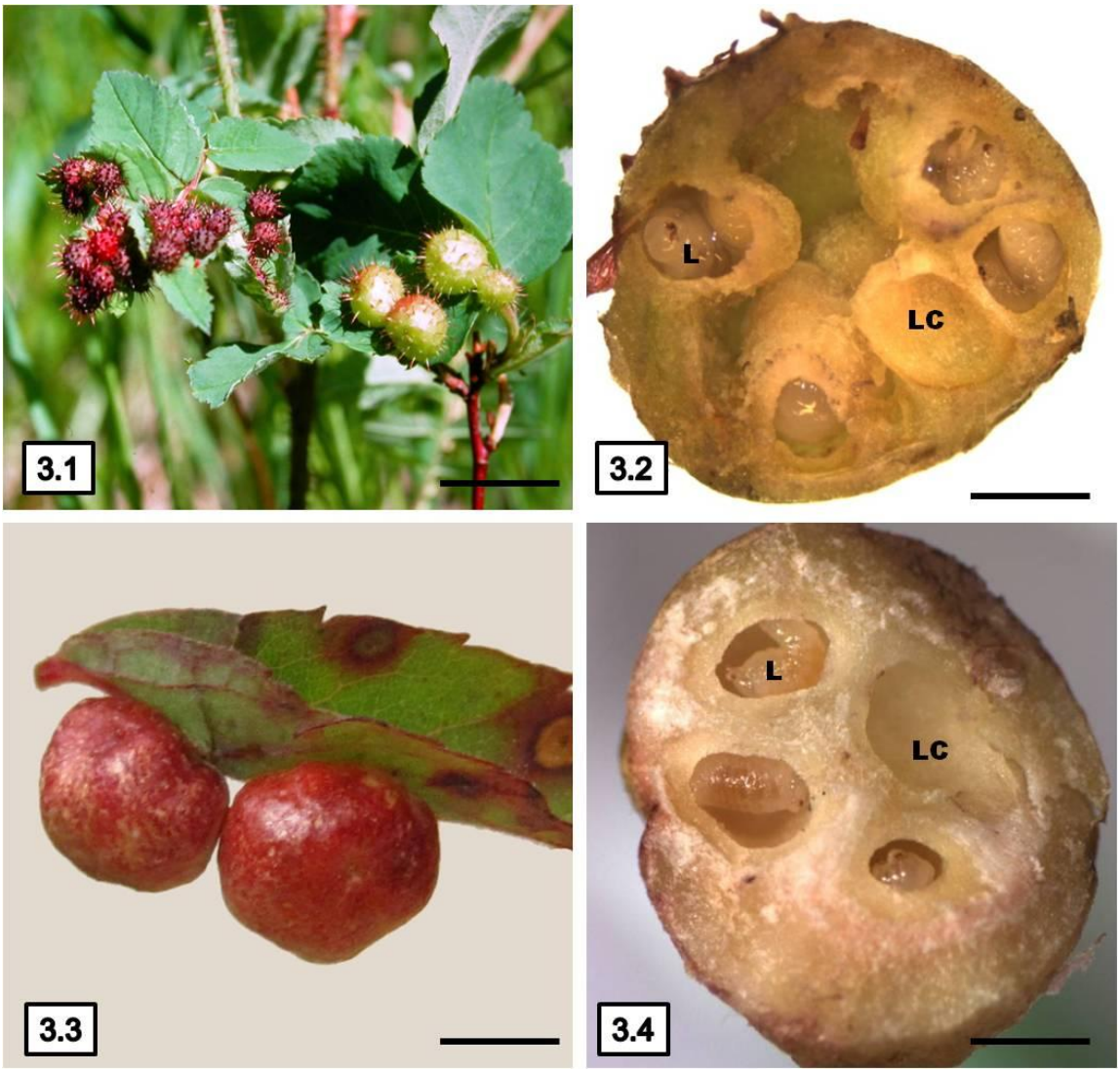
C. Materials and Methods

i. Inquiline modified galls used for study

The inquilines found within galls induced by *D. polita* will be referred to as *Periclistus* 1 and those found within galls induced by *D. nebulosa* will be referred to as *Periclistus* 2 for the rest of this chapter. *Periclistus* 1 inhabits galls induced by *D. polita* and causes extensive changes to the outer appearance of galls such that one can easily distinguish modified galls from normal galls in the field (Fig. 3.1). Inducer-inhabited galls are red and exhibit high radial symmetry (spherical), whereas those inhabited by *Periclistus* 1 are significantly enlarged, non symmetrical, and are not heavily pigmented (Fig. 3.1). Mature chambers inhabited by *Periclistus* 1 are distributed around the periphery of the inner gall and there is always a hollow space in the centre of the gall (Fig. 3.2). In contrast, the external appearance of galls of *D. nebulosa* inhabited by *Periclistus* 2 are similar to inducer-inhabited galls inhabits galls, there being only a slight irregularity to gall shape (Fig. 3.3). Mature chambers inhabited by *Periclistus* 2 are distributed throughout the inner gall with inquiline-modified tissues filling the entire chamber once inhabited by the larva of *D. nebulosa* (Fig. 3.4).

ii. Collection of botanical material

Inquiline modified galls at all phases of development were collected between May 2009 and October 2011 from sites previously identified (see Chapter II) as having large



Figs. 3.1 – 3.4: Habitus and dissections of galls of *Diplolepis polita* and *Diplolepis nebulosa* inhabited by full-grown larvae of *Periclistus.* **Fig 3.1.** Cluster of normal (left) and modified (right) galls of *D. polita.* Note the differences in size and colouration. Scale bar = 2 cm. **Fig. 3.2.** Dissection of a mature gall of *D. polita* inhabited by *Periclistus* 1 showing numerous chambers. Scale bar = 3 mm. **Fig. 3.3.** Mature modified galls of *D. nebulosa* modified by *Periclistus* 2. Note that modified galls are not spherical and have bulges around their circumference. Scale bar = 3 mm. **Fig. 3.4** Dissected gall of *D. nebulosa* showing multiple larvae of *Periclistus* 2 within individual chambers. Scale bar = 2 mm.

populations of host galls. More specifically, modified galls of *D. polita* were collected from roses growing along roadsides near Chelmsford and Timmins, Ontario, and La Sarre, Quebec. Modified galls of *D. nebulosa* were collected from sites near Sheguiandah and Providence Bay on Manitoulin Island, Ontario. Galls of both species were collected by walking haphazardly through rose patches and removing all galled leaves observed. Galled leaves were placed in Whirl pak® bags and transported to the laboratory in a cooler with ice. Galls were dissected to identify those inhabited by *Periclistus* and to estimate the phase of development prior to fixation. Gall diameter, phase of development, and number of inquiline chambers were recorded for each.

Ovipositions by *Periclistus* 1 were obtained by rearing adults from mature galls induced by *D. bicolor*, as galls of both *D. bicolor* and *D. polita* were shown by Ritchie (1994) to be inhabited by the same species of *Periclistus*. Females that had exited galls of *D. bicolor* were placed on branches of *R. acicularis* with clusters of immature galls of *D. polita.* Ovipositions were photographed and galls with eggs at various days post oviposition were fixed in FAA. In addition, many ovipositions by *Periclistus* 1 were observed in the field and galls of *D. polita* collected with eggs of *Periclistus* 1 were also used for this study. Unfortunately, no observations or photographs of *Periclistus* 2 ovipositing into immature galls of *D. nebulosa* were made in either of the collection seasons, even though the presence of numerous *Periclistus* 2 females was confirmed throughout the season by regularly sweeping galled roses with a sturdy net.

iii. PREPARATION OF HISTOLOGICAL SECTIONS

All specimens were fixed, sectioned, and stained as outlined in Chapter II.

iv. PHOTOGRAPHY AND ANALYSIS OF CELLS WITHIN INQUILINE-MODIFIED GALLS

Photographs of all sections were taken using a compound microscope fitted with a Leica camera. The areas, as observed in cross section, of 15 inquiline-induced nutritive, parenchymatous nutritive, primary sclerenchyma, secondary sclerenchyma; and *Diplolepis*-induced parenchyma, along with epidermal cells nearest the midpoint of the gall exterior were measured from 14 galls at each phase of development using ImageJ software. Analyses included: i) mean areas with standard deviations for cells at each stage of development; ii) analysis of variance (ANOVA) with a Tukey's Post Hoc test, or student's t-test of each cell type across development to determine whether or not the size of cells of each tissue type changes during throughout gall modification. For example, an attempt was made to determine if inquiline-induced nutritive cells within modified galls of *D. polita* in the egg, gall enlargement, chamber formation, and maturation phase were of the same size. Also, a student's t-test between cell types of modified galls of *D. polita* and *D. nebulosa* at similar developmental phases was performed to determine if cells of both galls are of similar size. For example, an attempt was made to determine if inquiline-induced primary sclerenchyma cells in the maturation phase of both species were the same. All statistical analyses were performed using IBM SPSS statistics 20.

v. ABBREVIATIONS

Throughout the remainder of the thesis when discussing cell types comprising the galls, abbreviations will be used both in the text and within figure captions. Each of the cell types are listed in alphabetical order as they appear in figure captions throughout the

results section of this chapter. For the convenience of the reader, each of the cell types with their respective abbreviations will also be listed at the beginning of the discussion.

D. RESULTS

i. MODIFICATION OF GALLS OF *Diplolepis polita*

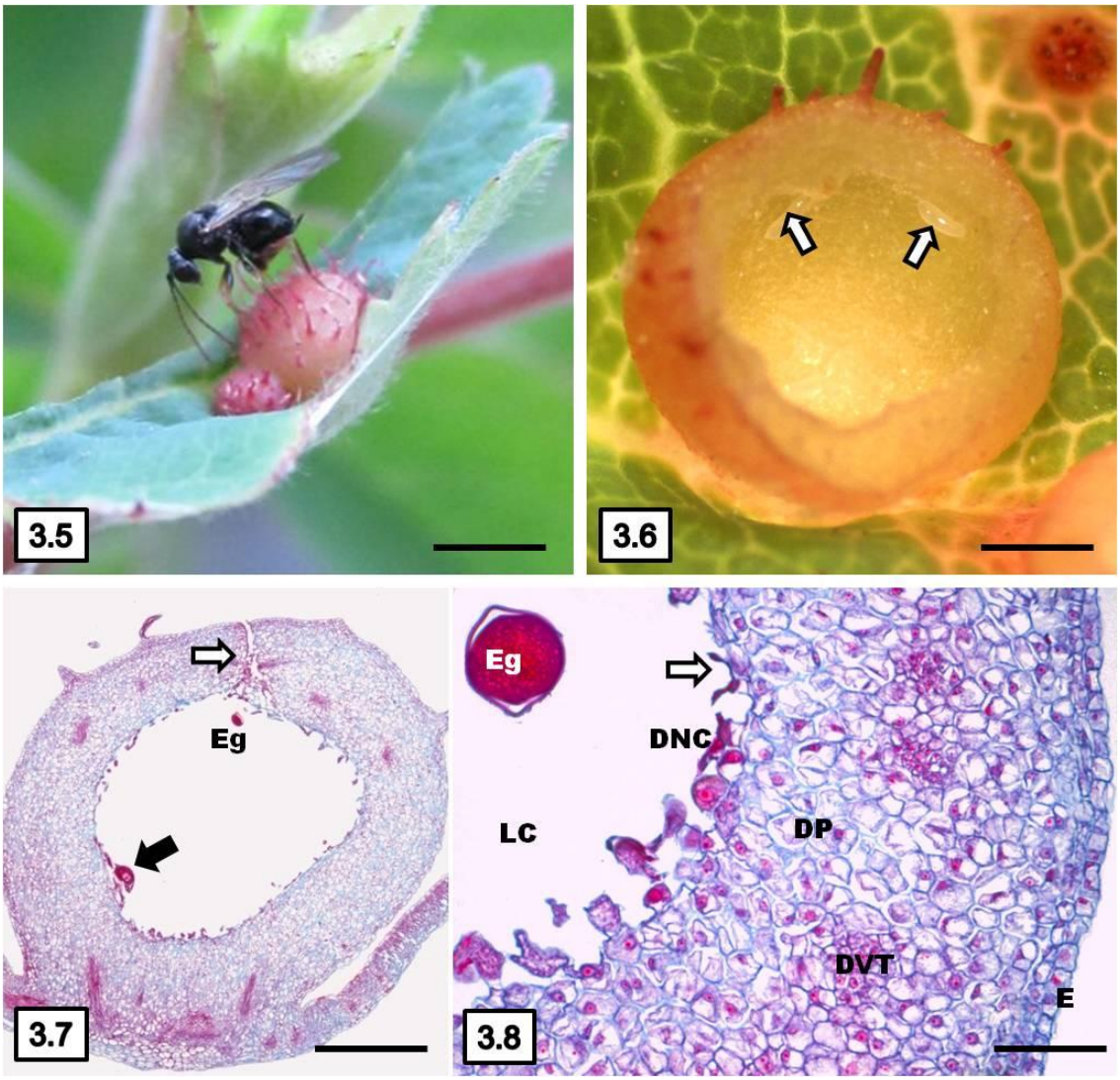
a. OVIPOSITION

Adult *Periclistus* 1 females exit host galls from mid to late May, approximately 7-14 days after the adults of *D. polita* exited galls and oviposited (Fig. 3.5). Females begin to search for freshly initiated host gall after chewing their way out of galls of *D. bicolor* and search for immature galls of *D. polita* in which to oviposit (Fig. 1.7) (note that figure numbers beginning with 1 appear in Chapter I of this thesis). Once a gall is located, females tap the exterior with their antennae and probe with their ovipositors. The ovipositor is then inserted (Fig. 3.5) typically through the chamber walls; however, some females were observed ovipositing into galls through the abaxial surface of the leaf beneath. It is important to note that *D. polita* larvae are killed by the *Periclistus* ovipositor during the course of oviposition. Several eggs are deposited on the inner surface of the chamber wall (Fig. 3.6) and it is likely that communal oviposition is possible as several females were observed ovipositing into galls that were already enlarged by *Periclistus* 1. Assuming galls collected for this study were the result of a single female oviposition, based on the number of inquiline chambers in mature galls collected later in the season, it was estimated that the number of eggs laid by a female in each gall ranges from 1-12 eggs with a mean of 5.49 (n= 55 galls). Once eggs are deposited, females search for additional suitable galls for oviposition. Galls of *D. polita* are typically found in dense clusters of

the same developmental phase (Figs. 1.2, 2.1, and 3.1) and thus females of *Periclistus* 1 typically oviposit into many or all of the galls within a cluster prior to searching for galls on other plants.

b. *Periclistus* EGGS

Oviposition channels are cut into the chamber wall by the ovipositor and appear as dark, lines when galls are dissected. Channels are lined with necrotic tissue that is densely stained (Fig. 3.7) and remains open for several days until closed by proliferating tissues. Dead inducer larvae appear shriveled and are present alongside *Periclistus* eggs in larval chambers (Fig. 3.7). Eggs with their elongated peduncle are loosely attached to the inner chamber and are easily removed with a fine dissecting probe. The layer of NC present in inducer-inhabited galls loses its characteristics soon after the inducer is killed, reverting to parenchyma or in some cases, they appear to disintegrate. *Diplolepis*-induced parenchyma cells (DP) lack nuclei, are not cytoplasmically dense (Fig. 3.8), and have a mean area of 1038 μ^2 (Fig. 3.43, additional statistical information can be found in the appendix). Galls containing eggs are larger than inducer-inhabited galls found in the field at the same time. Galls enlarge in size prior to larvae hatching which is demonstrated by the increase in the size of cells comprising the chamber wall and epidermis from the early phases of normal gall development to the egg phase of *Periclistus* modification (Compare Figs. 2.48 and 3.43) (note that any figures beginning with a 2 are found in Chapter II of this thesis).



Figs. 3.5 – 3.8: Oviposition and egg phases of inquiline-inhabited galls of *Diplolepis polita*. **Fig. 3.5.** Female of *Periclistus* 1 ovipositing into an immature gall. Scale bar = 1.5 mm. **Fig. 3.6.** Dissected gall showing eggs of *Periclistus* 1. Scale bar = 0.4 mm **Fig. 3.7.** Cross section of a gall after inquiline oviposition showing the oviposition channel (white arrow), dead *D. polita* larva (black arrow), and inquiline eggs. Scale bar = 117 µ. **Fig. 3.8.** Cross section of a gall at high magnification after inquiline oviposition showing eggs and DNC (arrow) that has disintegrated. Scale bar = 82 µ. LC, larval chamber; DNC, *Diplolepis*-induced nutritive cells; DP, *Diplolepis*-induced parenchyma cells; DVT, *Diplolepis*-induced vascular tissue. E, epidermis; Eg, inquiline egg.

C. GALL ENLARGEMENT

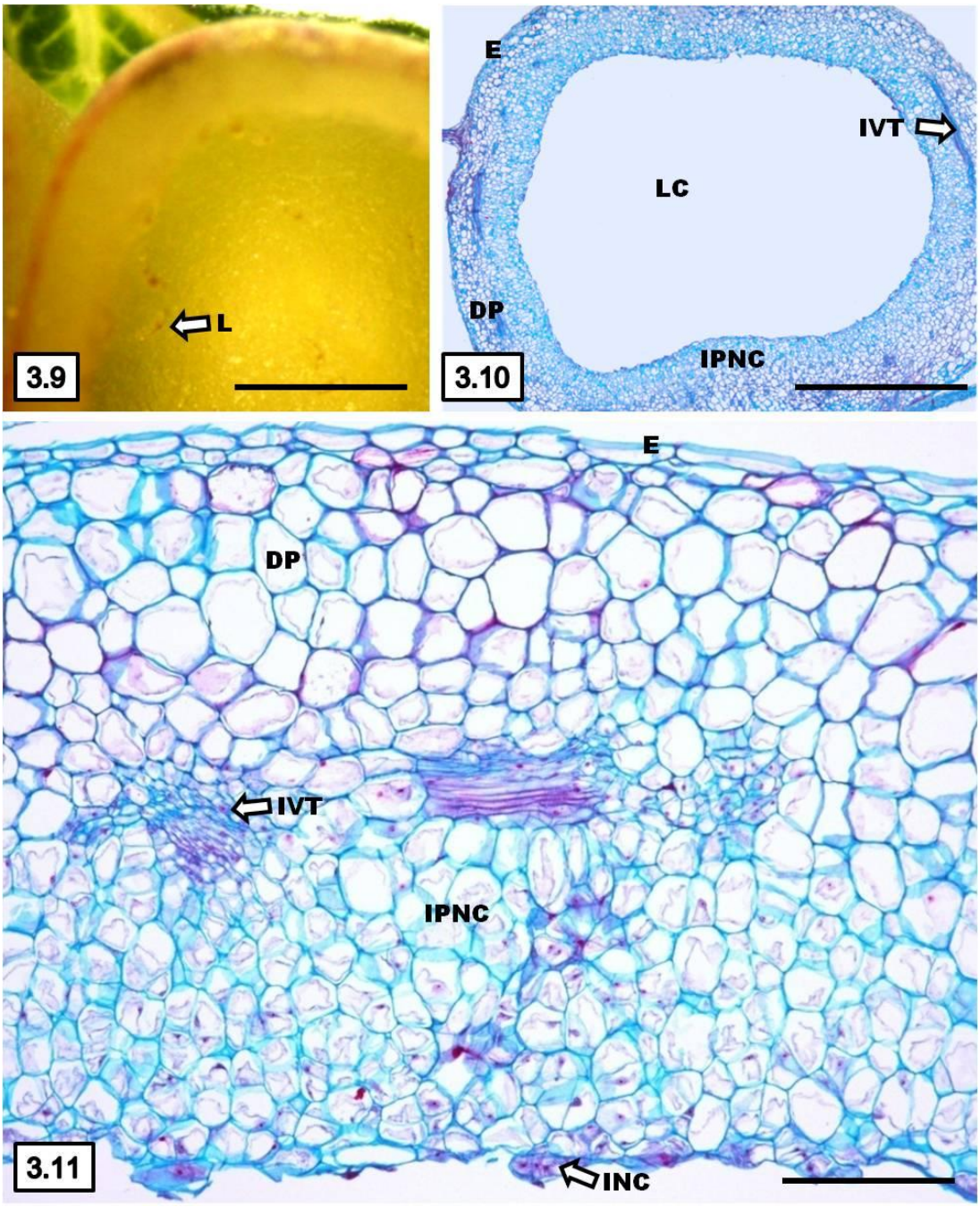
Larvae hatch approximately seven days after being deposited (Fig. 1.7). Galls at this phase in development appear as large, hollow spheres (Figs. 3.9 and 3.10) and freshly-hatched larvae can be seen with the naked eye even though they are small (< 1 mm in length) and have translucent integument (Fig. 3.9). Larvae move about grazing on gall tissues on the surface of the gall chamber (Fig. 3.9), which is much larger in diameter than galls in the inducer initiation phase.

The size of galls increases as a result of hypertrophy of DP (Fig. 3.43). These cells are larger than all other cells within the gall with a mean area of 3172 μ^2, and are significantly larger than those within galls containing *Periclistus* eggs (Fig. 3.43). These polygonal cells lack nuclei and are not cytoplasmically dense (Fig. 3.11). Exterior to the DP is the epidermis, which is comprised of a single layer of elongated or stretched, vacuolated cells that average 1236 μ^2 in area, and are significantly larger than epidermal cells of galls containing *Periclistus* eggs (Fig. 3.43). In addition to modifying pre-existing *Diplolepis*-induced tissues (DP and epidermis), *Periclistus* also induce their own tissues that are located adjacent to the larval chamber and are first observed in galls at this phase of development (Figs. 3.10 and 3.11). This includes a thick layer (5-10 cells) of inquiline-induced parenchymatous nutritive cells (IPNC) that are thin-walled, polygonal to spherical, and have a mean area of 2010 μ^2 (Figs. 3.11 and 3.43). This also includes inquiline-induced nutritive cells (INC) which are small, cytoplasmically dense, have a mean area of 1182 μ^2 (Fig. 3.43), and are sparsely distributed around the larval chamber (Fig. 3.11). There is a cytoplasmic gradient in galls at this phase of development, where cell types closest to the larval chamber are most cytoplasmically dense and have enlarged

nuclei (Figs. 3.10 and 3.11). Lastly, a network of inquiline-induced vascular tissue (IVT) develops between the modified and inquiline-induced tissues and consists of large, poorly defined vascular bundles (Fig. 3.11).

d. Formation of Periclistus 1 chambers

Individual chambers begin to form in galls near the end of May (Fig. 1.7). Larvae at the beginning of the chamber formation phase are larger than those in the initiation phase and discontinue feeding over the entire inside surface of the enlarged chamber that was once inhabited by the inducer and become sedentary (Fig. 3.12). The inside surface of the gall has an uneven appearance due to proliferation of cells around larval feeding sites (Figs. 3.12 and 3.13). Feeding sites appear as depressions along the inner chamber wall (Fig. 3.13) and are lined with a layer of INC that is 2-5 cells in thickness (Fig. 3.14). These polygonal (usually triangular or rectangular in cross section) cells are significantly larger (Fig. 3.43) and more cytoplasmically dense than those in the initiation phase (Figs. 3.11 and 3.13), and rapidly proliferate (cells in the final stage of mitosis were commonly observed). Larvae feed on INC, as shown by some collapsed cells lining the larval chamber (Fig. 3.14). Adjacent to the INC is a layer of less cytoplasmically dense, polygonal IPNC which appear in columns showing the direction of tissue proliferation (Fig. 3.14). DP cells are the largest of the gall (Fig. 3.43) and undergo sclerification over the course of chamber formation, forming a layer of inquiline-induced primary

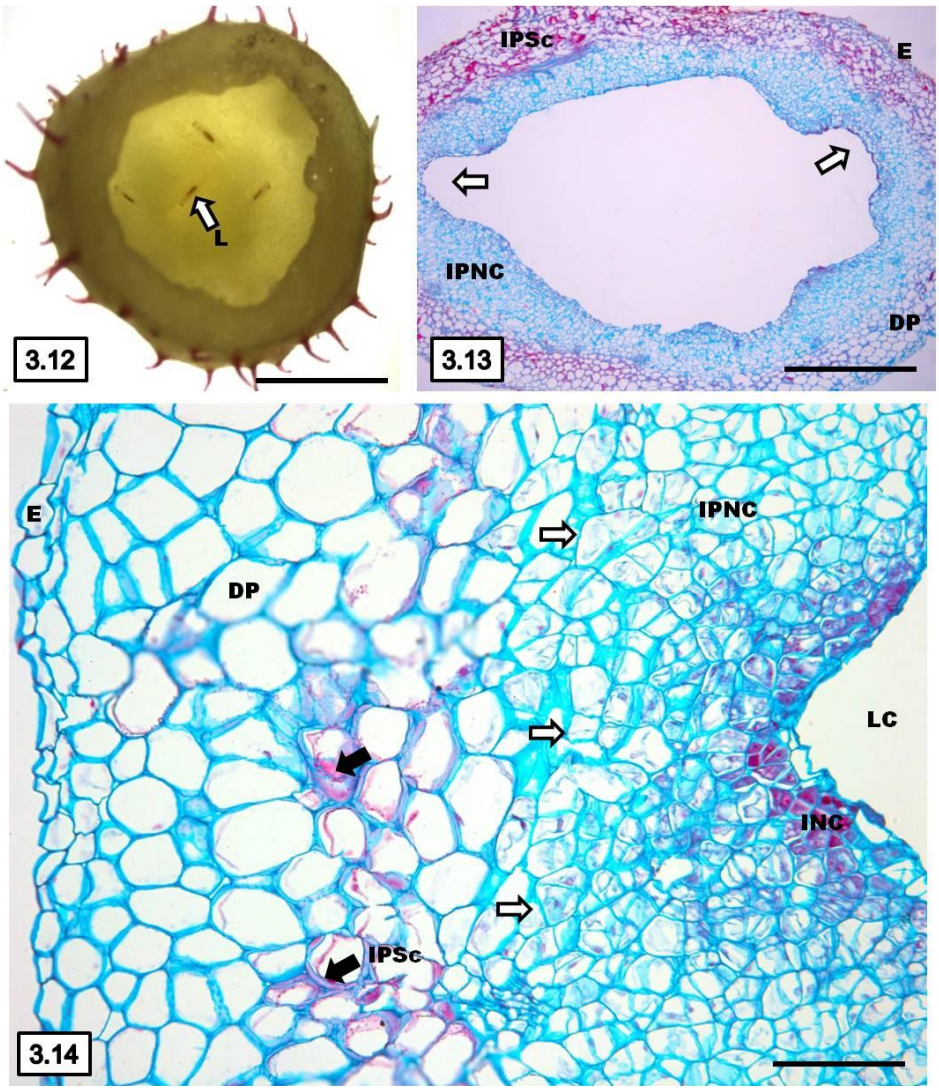


Figs. 3.9 – 3.11: Gall enlargement phase of inquiline-modified galls of *Diplolepis polita* with freshly-hatched *Periclistus*1 larvae and proliferation of inquiline-induced gall tissues. **Fig. 3.9.** Dissected gall showing freshly hatched larvae. Scale bar = 1.5 mm. **Fig. 3.10.** Cross section of proliferated inquiline-induced tissues soon after larvae have hatched. Galls are much larger at this phase than when inhabited by inducers. Scale bar = 1900 µ. **Fig. 3.11.** Cross section of a gall after larvae have hatched showing enlarged *Diplolepis*-induced cells making up the DP and proliferating *Periclistus*-induced cells. Scale bar = 300µ. DP; *Diplolepis*-induced parenchyma; E, epidermis; INC, inquiline-induced nutritive cells; IPNC, inquiline-induced parenchymatous nutritive cells; IVT, inquiline-induced vascular tissue; L, larva.

sclerenchyma (IPSc) that has a patchy distribution among the layer of DP (Figs. 3.13 and 3.14).

Larvae of *Periclistus* 1 are completely enclosed in their own chambers by late June (Fig. 1.7). The surface of each *Periclistus* 1 chamber is lined with proliferating gall tissues (Figs. 3.15 and 3.16). The whitish-yellow inquiline-induced cells (INC and IPNC) can be distinguished from the dull-green inquiline-modified cells (IPSc and E) in dissected galls (Figs. 3.15 and 3.16).

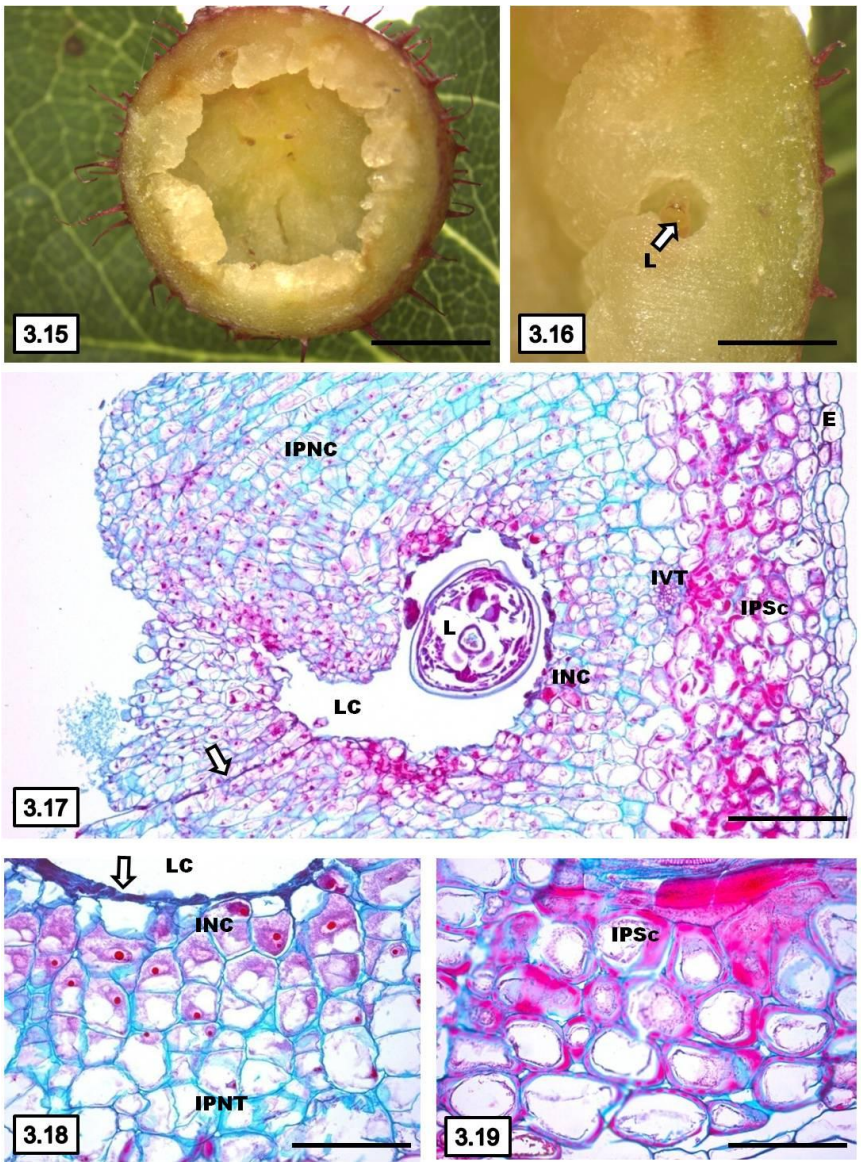
Larvae nearly fill their larval chambers (Figs. 3.16 and 3.17), which are lined with a layer of INC that is 2-5 layers in thickness. Larval feeding is indicated by a thick layer of collapsed cells on the chamber surface (Figs. 3.17 and 3.18). INC average 912 μ^2 and have enlarged nuclei (Figs. 3.18 and 3.43). Cuboidal IPNC are arranged in columns of proliferating cells around each larva (Fig. 3.17), which can be seen in fresh galls under a dissecting microscope (Fig. 3.16). IPNC have a mean area of 1400 μ^2 (Fig 3.43). All of the DP cells are lignified by the end of chamber formation which forms a thick layer of IPSc that circumscribes the gall and is comprised of large cells, averaging 3634 μ^2 (Fig 3.43), with thickened secondary cell walls (Fig. 3.17 and 3.19).



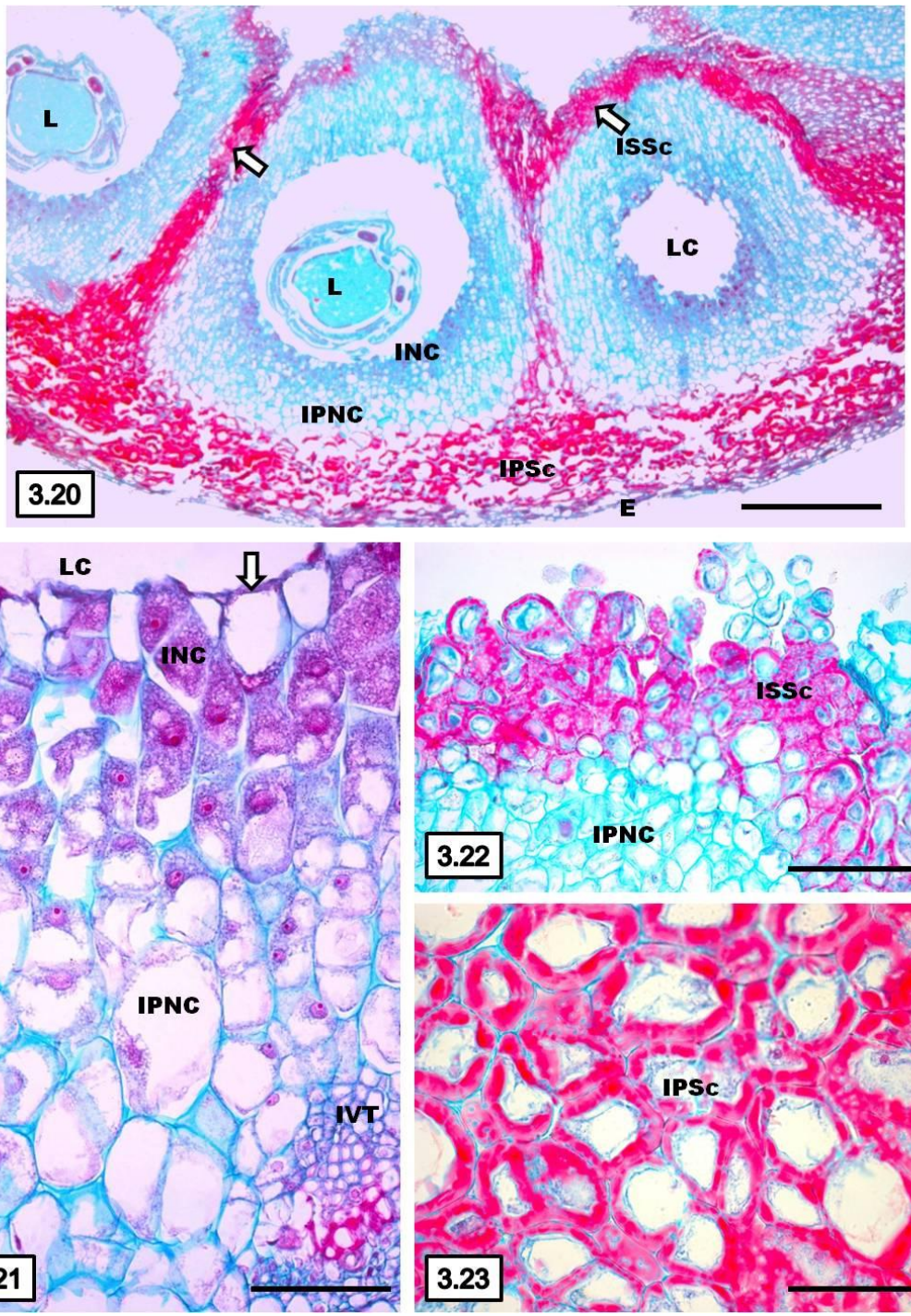
Figs. 3.12 – 3.14: Early chamber formation of inquiline-modified galls of *Diplolepis polita*. **Fig. 3.12.** Dissected gall showing proliferation of inquiline-induced parenchyma around larval feeding sites. Note that tissue proliferation has caused the inner surface of the gall chamber to become uneven. Scale bar = 1.5 mm. **Fig. 3.13.** Cross section of a gall showing uneven appearance of inquiline-induced cells. Arrow shows areas of larval feeding where parenchyma has proliferated starting to form the inquiline larval chamber. Scale bar = 1220 µ. **Fig. 3.14.** Cross section of a portion of a gall wall showing proliferation of INC near larval feeding sites. INC is well defined at each feeding site and some cells of the DP have lignified and are referred to as IPSc (black arrows). Note the white arrows indicate the division between tissues that were induced by the inducer larva and were present at the time of inquiline oviposition and tissues that have differentiated and proliferated under the influence of inquiline larvae. Scale bar = 175 µ. DP; *Diplolepis*-induced parenchyma; E, epidermis; L, larva; LC, larval chamber; INC, inquiline-induced nutritive cells; IPNC, inquiline-induced parenchymatous nutritive cells; IPSc, inquiline-induced primary sclerenchyma.

e. MATURATION

Inquiline-modified galls begin to mature in mid June (Fig. 1.7) and are so firm they are difficult to cut with a blade. Larvae are enclosed within hardened, individual chambers that are positioned around the periphery of the inner gall and a hollow space remains in the centre of each gall (Fig. 3.2). In galls of *D. polita* modified by *Periclistus* 1, a layer of sclerenchyma (IPSc) circumscribes the entire gall when the chambers begin to develop. A secondary sclerenchyma sheath (ISSc) forms in the maturation phase around each inquiline chamber as a result of lignification of IPNC (Figs 3.20 and 3.22). Layers of IPNC around each chamber are typically 3-10 cells in thickness, and cells are the same size as INC, but much smaller than IPSc (Figs. 3.22, 3.23, and 3.43). Larvae increase in size during the maturation phase (Figs. 3.20 and 3.21) and actively feed on nutritive cells. As INC are consumed, adjacent IPNC develop cytological features of INC until INC completely surrounds larval chambers up to the layer of ISSc.



Figs. 3.15 – 3.19: Late chamber formation of inquiline-modified galls of *Diplolepis polita*. **Fig. 3.15.** Dissected gall showing larvae enclosed in individual chambers. Note that the large central chamber remains that was once inhabited by the inducer larva remains. Scale bar = 2.5 mm. **Fig. 3.16.** Cross section of a portion of the wall of a gall showing a larva enclosed within its chamber. Scale bar = 0.7 mm. **Fig. 3.17.** Cross section of a portion of the wall of a gall showing a larva completely enclosed within its chamber. Note the region of crushed cells between opposing chamber walls (arrow) and the thick layer of IPSc that now circumscribes the entire gall. Scale bar = 250 µ. **Fig. 3.18.** Cross section of inquiline-induced nutritive tissue lining the completed larval chamber. Note the layer of collapsed cells (arrow) resulting from larvae imbibing the contents of INC. Scale bar = 130 µ. **Fig. 3.19.** Cross section of inquiline-induced primary sclerenchyma cells. Scale bar = 140 µ. E, epidermis; INC, inquiline-induced nutritive cells; IPNC, inquiline-induced parenchymatous nutritive cells; IPSc, inquiline-induced primary sclerenchyma; IVT, inquiline-induced vascular tissue; L, larva; LC, larval chamber.



Figs. 3.20 – 3.23: Modified galls of *D. polita* in the maturation phase with mature larvae of *Periclistus* 1. **Fig. 3.20.** Cross section of a portion of the wall of a gall showing mature inquiline chambers. Note the ISSc that surrounds each larval chamber (arrows). Scale bar = 900 µ. **Fig. 3.21.** Cross section of INC and IPNC. Note the collapsed cells from larval feeding (arrow). Scale bar = 150 µ. **3.22.** Cross section of ISSc. Scale bar = 91 µ. **Fig. 3.23.** Cross section of IPSc Scale bar = 110 µ. INC, inquiline-induced nutritive cells; IPNC, inquiline-induced parenchymatous nutritive cells; IPSc, inquiline-induced primary sclerenchyma.

ii. MODIFICATION OF GALLS OF *Diplolepis nebulosa*

a. OVIPOSITION

The first females of *Periclistus* 2 exit from galls in late July and early August, approximately two months after the first *D. nebulosa* females appeared and oviposited. The emergence period of *Periclistus* 2 is lengthy, lasting several weeks (Fig. 1.7). Females search for immature host galls to lay their eggs, which is from the inducer early to mid growth phase (Figs. 1.7 and 3.24). When a suitable gall is selected, the ovipositor is inserted through the lateral regions of the gall rather than at the poles of the gall. Galls of *D. nebulosa* are spherical and are described (as in Chapter II) as having poles, one at the point of attachment to the host leaf and the other opposite in the larval chamber, and an 'equatorial' or lateral region describing the widest portion of the chamber, central to either poles. *D. nebulosa* larvae are killed by the *Periclistus* 2 ovipositor during oviposition and several eggs are deposited along the chamber wall (Fig. 3.24). Assuming all eggs per gall were deposited by one female, based on the number of inquiline chambers in mature galls collected later in the season, an estimated 1-18 eggs with a mean of 4.85 (n= 40 galls) are deposited by each female.

b. *Periclistus* EGGS

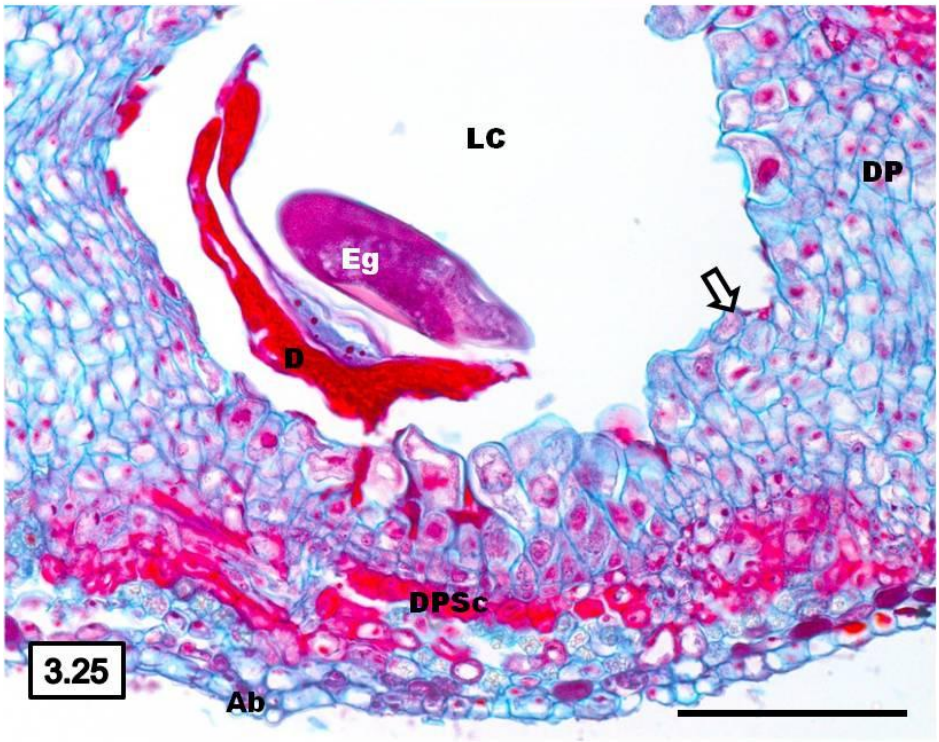
Externally, galls containing eggs of *Periclistus* 2 are indistinguishable from those containing live inducer larvae. Black channels made by the ovipositors are clearly seen in dissected chamber walls. In addition to the *Periclistus* eggs (Fig. 3.24), remains of the dead inducer larvae are also observed in dissected and sectioned galls (Fig. 3.25). Eggs are loosely attached to chamber walls and are easily dislodged with a fine dissecting probe. *Diplolepis*-induced NC that lined the larval chamber when the inducer larva was

alive loses its characteristics (dense cytoplasm and enlarged nuclei) and reverts to parenchyma or disintegrates (Fig. 3.25). In addition, *Diplolepis*-induced parenchyma cells (DP) become less cytoplasmically dense and some lack nuclei (Fig. 3.25). These cells average 402 μ^2 in area (Fig. 3.43). Lastly, the *Diplolepis*-induced primary sclerenchyma (DPSc) sheaths are not altered during inquiline modification (Fig. 3.25).

C. GALL ENLARGEMENT

Larvae hatch from their eggs approximately 7 days after oviposition (Fig. 1.7). While galls at this phase of development are larger than those inhabited by inducer larvae that were induced at the same time, it is not possible to distinguish galls from those inhabited by inducer larvae based on diameter because of the wide range of overlapping developmental phases simultaneously present in the field (Fig. 1.7). Galls show thicker chamber walls than those inhabited by inducer larvae and have a spongy appearance with dark patches of vascular tissue dispersed throughout the chamber walls (Fig. 3.26). Galls also have an enlarged spherical chamber in which the inquiline larvae move about as they feed on gall tissues (Fig. 3.26).

Galls increase in size as a result of proliferating of IPNC and hypertrophy of DP. The proliferation of IPNC causes the chamber walls to increase in thickness, and contributes to the overall size increase in galls (Figs 3.27 and 3.38). The DPSc seems to be the source of IPNC, especially the adaxial sheath, where IPNC are arranged in columns perpendicular to the larval chamber and are cuboidal with large nuclei (Figs. 3.27 and 3.28). IPNC closer in proximity to the larval chamber are more polygonal to spherical in

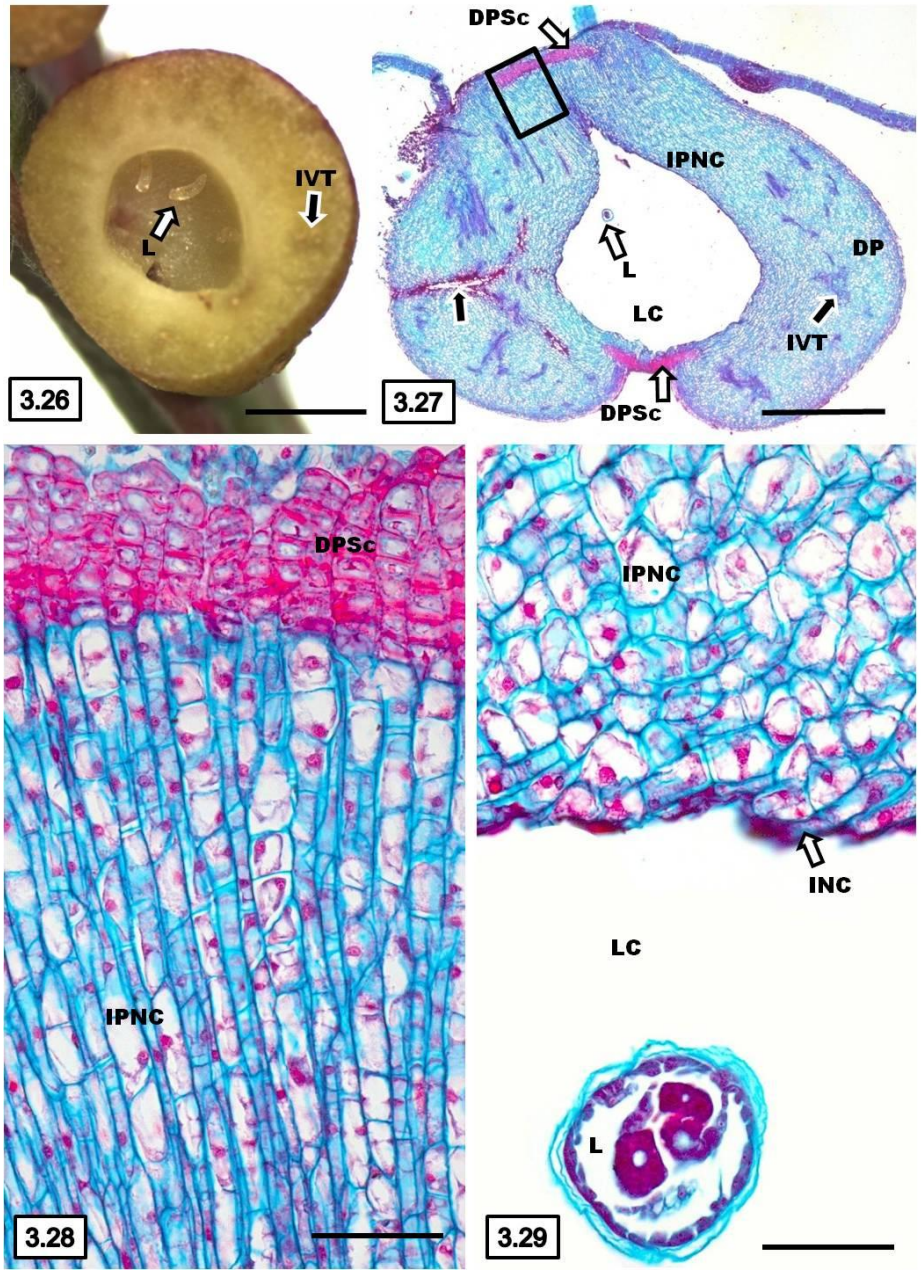


Figs. 3.24 – 3.25: Oviposition and egg phase of inquiline-inhabited galls of *Diplolepis nebulosa*. **Fig. 3.24.** Dissected gall showing inquiline eggs (arrow). Scale bar = 1.5 mm. **Fig. 3.25.** Cross section of a gall after inquiline oviposition showing the dead *D. nebulosa* larva and an inquiline egg. Note the disintegrating DNC (arrow). Scale bar = 136 µ. Ab, abaxial epidermis; D, dead *Diplolepis* larva; DP, *Diplolepis*-induced parenchyma cells; DPSc, *Diplolepis*-induced primary sclerenchyma; Eg, egg; LC, larval chamber.

shape (Fig. 3.29) than those further away from the larval chamber (Fig. 3.28) and have a mean area of 573.50 μ^2. INC are not well defined, with cytoplasm that is only slightly more dense than the adjacent IPNC. At this phase of development, inquiline-induced vascular tissue is found throughout the chamber wall, particularly in the medial portion (Figs. 3.26 and 3.27). When dissected gall tissues oxidize, it is easy to distinguish the IVT as it appears as dark strands in the chamber wall (Fig. 3.26). A gradient in cell size exists at this phase of development, with cells size increasing away from the larval chamber. The smallest cells within the gall are the INC with a mean area of 388 μ^2, whereas the largest cells are DP which are located around the periphery of the gall and are hypertrophied with a mean area of 931 μ^2 (Fig. 3.43). Cytologically, DP cells do not appear different from IPNC, but are spherical and are not arranged in columns.

d. CHAMBER FORMATION

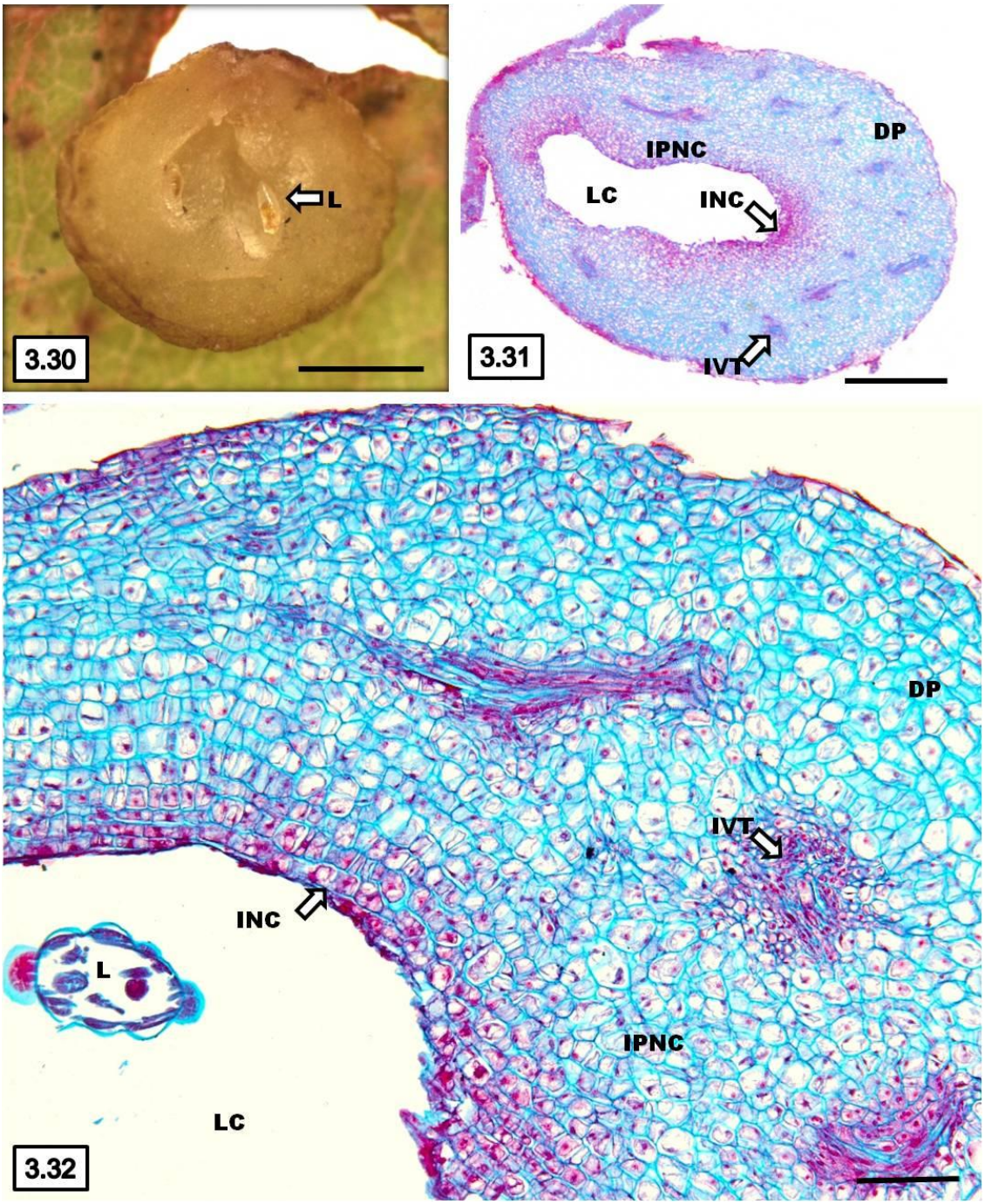
Galls with developing inquiline chambers are found from late July to early September (Fig. 1.7). Dissected galls, and those sectioned and photographed at low magnification, show inner chamber walls with an uneven appearance resulting from proliferation of IPNC around larval feeding sites (Figs 3.30 and 3.31). IPNC are cuboidal and arranged in columns parallel to the larval chamber (Fig. 3.32). INC form a distinct layer around the entire chamber (Fig, 3.31), but is thickest (3-5 cells) at larval feeding sites (Fig. 3.32). INC are the most cytoplasmically dense of modified galls in the phase of chamber formation and a gradient exists where cytoplasmic density decreases away from the larval chamber. IVT is in the form of thick bundles found in the median portion of the chamber wall between the modified (DP) and inquiline-induced (IPNC and INC) layers (Fig. 3.31



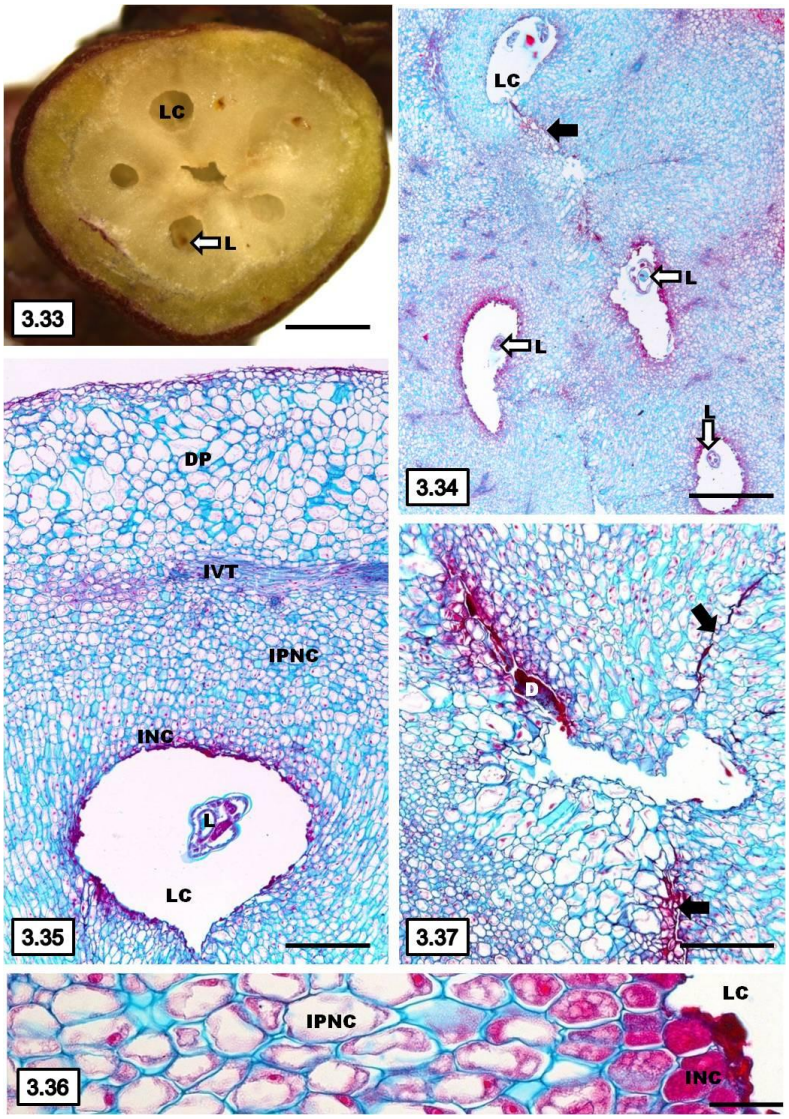
Figures 3.26 – 3.29: Modified galls of *Diplolepis nebulosa* showing hatching inquiline larvae and proliferating gall tissues. **Fig. 3.26.** Dissected gall showing recently hatched larvae. Scale bar = 1.6 mm. **Fig. 3.27.** Cross section of a gall that has increased in size after larvae have hatched. The gall wall is thicker than those of galls inhabited by the inducer due to proliferation of IPNC. Note the oviposition channel (black arrow) and IVT throughout the in the chamber wall and the black box which represents the general location within galls where the cells in figure 3.28 are found. Scale bar = 1 mm. **Fig. 3.28.** Cross section of IPNC proliferation. Note the elongated appearance of the cells and their arrangement in rows from proliferation. Scale bar = 120 µ. **Fig. 3.29.** Cross section of cells lining the larval chamber after the larvae have hatched. Scale bar = 160 µ. DPSc, *Diplolepis*-induced primary sclerenchyma; IPNC, inquiline-induced parenchymatous nutritive cells; L, larva; LC, larval chamber.

and 3.32). These vascular bundles are well-defined and have differentiated xylem and phloem. DP circumscribes the outermost portion of the chamber wall (Fig. 3.32).

Larvae of *Periclistus* 2 are completely enclosed by gall tissues in their individual chambers at the end of the chamber formation phase (Fig. 3.33). They are not active; displaying little reaction when stimulated with a fine dissecting probe. Larval chambers are found throughout the central portion of galls with all of the inner gall space filled with IPNC (Figs. 3.33 and 3.34). It is easy to distinguish the whitish-yellow inquiline-induced cells (INC and IPNC), from the dull-green modified cells (DP) of dissected galls (Figs. 3.15 and 3.16). DP cells vary in size and shape, are vacuolate (3.35), and significantly larger than in previous developmental phases (Fig. 3.43). Thick bundles of IVT occur between the layers of DP and IPNC (Fig. 3.35); whereas, many thin bundles are found throughout the layer of IPNC that surrounds all larval chambers (Fig. 3.34). IPNC are arranged in columns at sites of extensive proliferation and there are many sites within the layer of IPNC where proliferating cells associated with the completion of different larval chambers intersect. Cells appear crushed at these sites (Fig. 3.37). The central site where IPNC associated with multiple chamber walls have intersected is a small hollow space that contains the corpse of the inducer larva and is lined with enlarged, vacuolated cells (Fig. 3.37). Larval chambers are surrounded by a layer of cytoplasmically dense, spherical INC (2-5 cells in thickness) that are significantly larger than those at the gall enlargement phase (Figs. 3.36 and 3.43). These cells are the smallest of the gall with a mean area of 563.60 μ^2. Cellular size and cytological gradients exist in galls at this phase; cell size increases and cytoplasmic density decreases away from the larval chamber (Figs. 3.35, 3.36, and 3.43).



Figures 3.30 – 3.32: Early chamber formation of inquiline-modified galls of *Diplolepis nebulosa*. **Fig. 3.30.** Dissection of a modified gall showing proliferation of IPNC around larval feeding sites. Note the tissue proliferation has caused the inner surface of the gall chamber to become uneven. Scale bar = 1.3 mm. **Fig. 3.31.** Cross section of a gall showing uneven appearance of inquiline-induced tissues. Scale bar = 900 µ. **Fig. 3.32.** Cross section of a portion of the wall of a gall showing differentiating INC and IVT. Scale bar = 122 µ. DP, *Diplolepis*-induced parenchyma; INC, inquiline-induced nutritive cells; IPNC, inquiline-induced parenchymatous nutritive cells; IVT, inquiline-induced vascular tissue; L, larva ; LC, larval chamber.



Figures 3.33 – 3.37: Late chamber formation of inquiline-modified galls of *Diplolepis nebulosa* showing completed *Periclistus* 2 larval chambers. **Fig. 3.33.** Dissection of a modified gall showing larvae enclosed in chambers. Note the clear distinction between inducer tissues around the outside of the gall and those induced by the inquiline in the central region of the gall. Scale bar = 1.4 mm. **Fig. 3.34.** Cross section showing larvae enclosed in chambers. Scale bar = 700 µ. Black arrows show the regions where opposing chamber walls intersect. **Fig. 3.35.** Cross section of a portion of the wall of a gall showing a larva enclosed within its chamber. Note enlarged DP cells around the most distal region of the gall. Scale bar = 330 µ. **Fig. 3.36.** Cross section of the INC layer lining the complete larval chamber. Note the collapsed cells which result from larvae imbibing the contents of INC. Scale bar = 50 µ. **Fig. 3.37.** Cross section of the small central chamber occupied by the dead inducer larva. Note the regions of crushed cells (black arrows) between opposing chamber walls. Scale bar = 370 µ. D, Dead *Diplolepis* larva; INC, inquiline-induced nutritive cells; DP, *Diplolepis*-induced parenchyma; IPNC, inquiline-induced parenchymatous nutritive cells; IVT, inquiline-induced vascular tissue; L, larva; LC, larval chamber.

e. MATURATION

Inquiline-modified galls begin to mature in mid August and are found in the field until leaves abscise in late September and early October (Fig. 1.7). Galls are difficult to cut with a blade and larvae are enclosed within individual, hardened chambers (Fig. 3.38). Larvae are full grown, occupying nearly the entire volume of their larval chambers (Fig. 3.38) and wriggle when disturbed during dissection or touched with a fine dissecting probe.

The maturation phase in *Periclistus* 2-modified galls of *D. nebulosa* is characterized by the formation of sclerenchyma that circumscribes the gall and each of the larval chambers (Fig. 3.39). Sclerenchyma is easy to distinguish within dissected galls and appears as whitish, flaky tissue (Fig. 3.38). There are two types of sclerenchyma cells within mature, modified galls. The first, inquiline-induced primary sclerenchyma cells (IPSc), form a sheath that surrounds the larval chambers collectively at the interface of the DP and IPNC layers (Figs. 3.39 and 3.40). IPSc have thick secondary walls and the lumen is sometimes completely occluded (Fig. 3.41). This layer is comprised of circular or ovoid cells (in cross section) that are compactly arranged and vary in size (Fig. 3.40). The second type is inquiline-induced secondary sclerenchyma cells (ISSc) which differentiate slightly later than IPSc. The ISSc layer which is 5-15 cells thick forms between larval chambers (Fig. 3.39) and is comprised of significantly smaller cells with thinner secondary walls than the IPSc; IPSc cells have a mean area of 609 μ^2 and ISSc cells have a mean area of 470 μ^2. A thick layer of thin-walled, vacuolated DP cells circumscribes the outermost portion of the gall. These cells have a mean area of 1293 μ^2 and are significantly larger than those in the chamber formation phase, and remain the largest gall cells (Figs. 3.40 and 3.43).

Chambers are surrounded by a thick layer of INC as nearly all IPNC are converted into INC (Fig. 3.40). Larvae consume all INC until chambers are surrounded only by sclerenchyma. Both INC and IPNC are not significantly larger than they were in the chamber formation phase and average 569 μ^2 and 670 μ^2 respectively (Fig. 3.43).

E. DISCUSSION

DP, *Diplolepis*-induced parenchyma

DPSc, *Diplolepis*-induced primary sclerenchyma (found in modified galls of *D. nebulosa* only)

DVT, *Diplolepis*-induced vascular tissue

DNC, *Diplolepis*-induced nutritive cells

E, Epidermis (found in modified galls of *D. polita* only)

INC, Inquiline-induced nutritive cells

IPNC, Inquiline-induced parenchymatous nutritive cells

IPSc, Inquiline-induced primary sclerenchyma

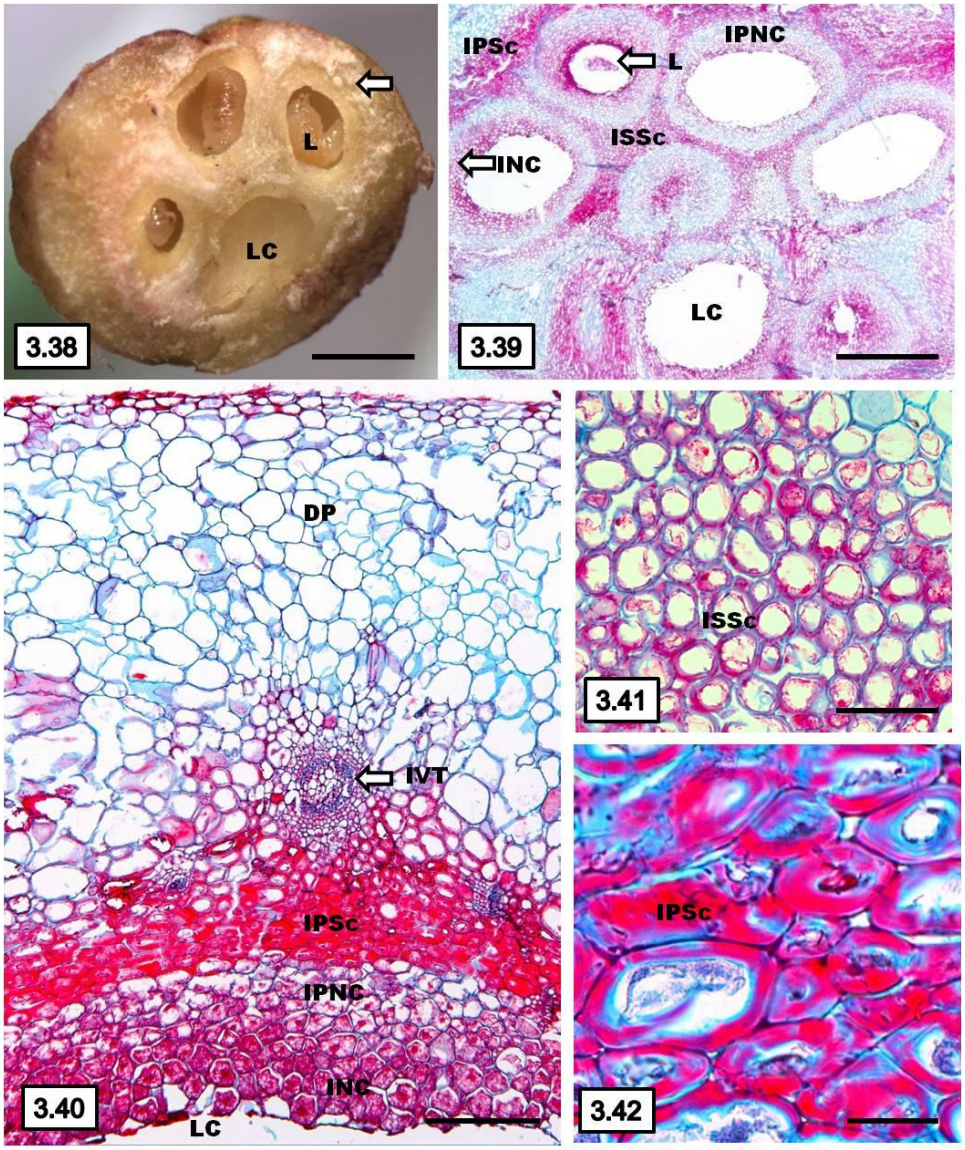
ISSc, Inquiline-induced secondary sclerenchyma

IVT, inquiline-induced vascular tissue

LC, Larval chamber

L, Larva.

Small wasps in the family Cynipidae induce some of the most structurally and anatomically complex galls (Csóka *et al.* 2005), all of which undergo three developmental phases known as; intiation, growth, and maturation (Meyer and



Figures 3.38 – 3.42: Maturation of *Periclistus* 2-modified galls of *Diplolepis nebulosa*. **Fig. 3.38.** Dissection of a gall showing maturing larvae. Note the dried, white appearance of sclerenchyma surrounding each chamber (arrow). Scale bar = 1.6 mm. **Fig. 3.39.** Cross section of a gall showing mature inquiline chambers. Note the sclerenchyma layer that surrounds each larval chamber (arrow). Scale bar = 1107 µ. **3.40.** Cross section of tissues surrounding each larval chamber. Note the development of IPSc proximal to the larval chamber and the thick layer of DP in the outer gall. Scale bar = 144 µ. **Fig. 3.41.** Cross section of ISSc that forms between the chamber walls. Scale bar = 83 µ. **Fig. 3.42.** Cross section of IPSc that circumscribes the entire gall. Scale bar = 58 µ. DP, *Diplolepis*-induced parenchyma; INC, inquiline-induced nutritive cells; IPNC, inquiline-induced parenchymatous nutritive cells; IPSc, inquiline-induced primary sclerenchyma; ISSc, inquiline-induced secondary sclerenchyma; IVT, inquiline-induced vascular tissue; L, larva ; LC, larval chamber.

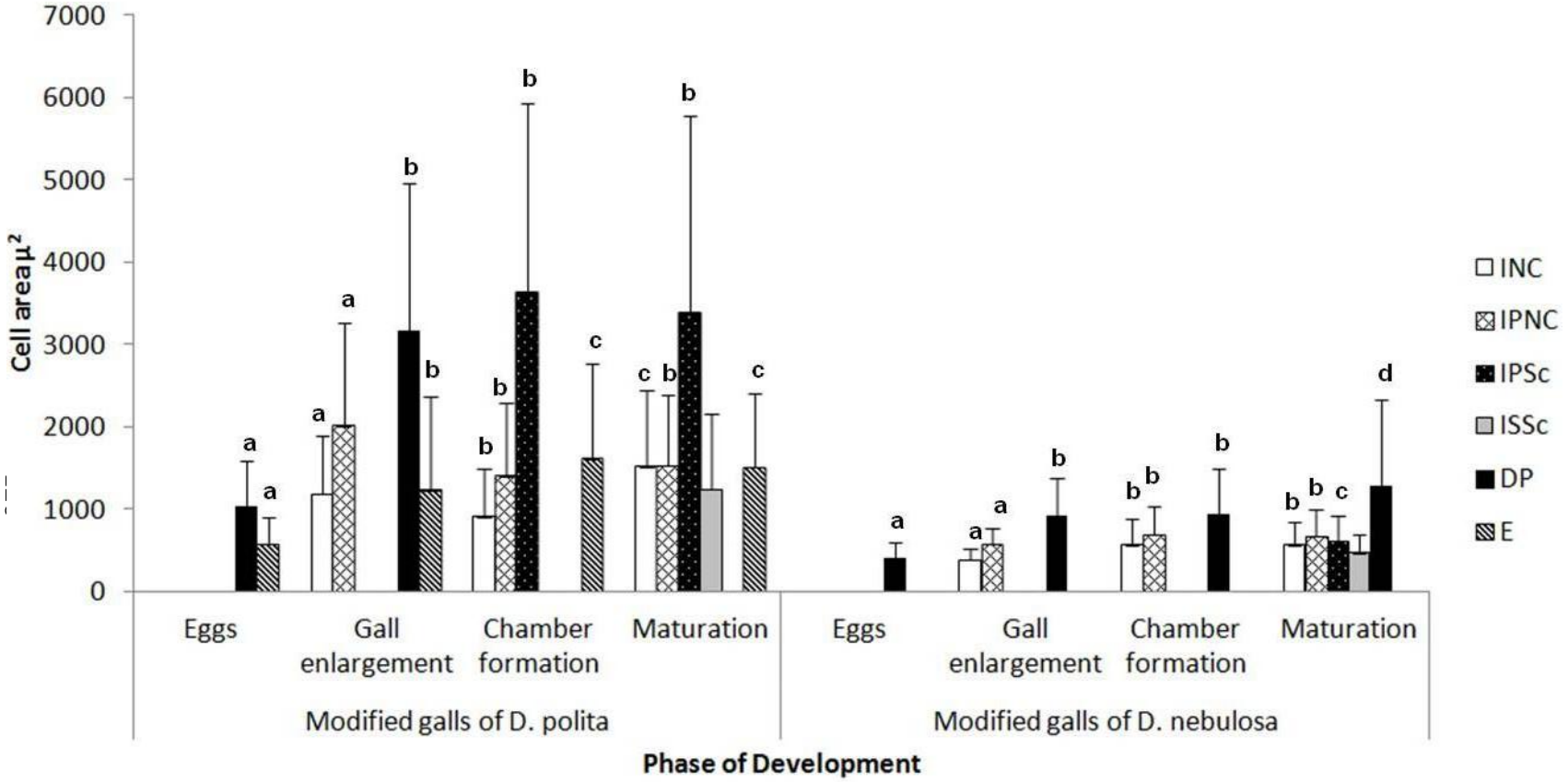


Fig. 3.43. Mean size of cells at each phase of development in *Periclistus* modified galls of *Diplolepis polita* and *Diplolepis nebulosa.* Error bars represent standard deviations of the mean. Unlike lowercase letters represent significant differences ($p<0.05$) within each cell type across development within each gall based on an ANOVA using Tukey's HSD for post-hoc comparisons ($\alpha=0.05$). Note that IPSc arises from DP in both galls and is included in the DP statistical analyses.

Maresquelle 1983; Rohfritsch 1992). The results from Chapter II, as well as other developmental studies (e.g. Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001; Sliva and Shorthouse 2005; Leggo and Shorthouse 2006) have shown that mature cynipid galls are all generally composed of the same types of gall cells. Larval chambers are lined with concentrically layered rows of nutritive cells, parenchymatous nutritive cells that are converted into nutritive cells as they are depleted by the feeding inducer larva, vascular tissue that supplies galls with nutrients, a protective layer that is typically in the form of hard sclerenchyma cells, and an epidermal layer on the exterior that may or may not be covered with structures such as hairs or prickles depending on the inducer species (Meyer and Maresquelle 1983; Rohfritsch 1992; Csóka *et al.*2005).

Not all phytophagous cynipids are capable of inducing galls. Of note, the tribe Synergini is composed of inquiline–cynipids that have lost the ability to induce their own galls (Ronquist 1994). They not only inhabit galls of other cynipids, but have maintained the ability to manipulate plant tissues within their host galls (Ronquist 1994). There are seven genera of cynipid inquilines (*Ceroptres, Saphonercus, Synergus, Synophrus, Periclistus, Rhoophilus*), each of which is generally associated with one genus of gall-inducing cynipid (Csóka *et al.* 2005). *Periclistus* is the only genus that has received attention concerning their ability to modify gall tissues. Detailed anatomical studies of *Periclistus*-inhabited galls of *D. nodulosa* (Brooks and Shorthouse 1998) and *D. rosaefolii* (LeBlanc and Lacroix 2001) have been published; however, no studies have compared the developmental events between multiple *Periclistus*-inhabited galls. Thus, this study provides the first detailed comparison of the development of inquiline-modified galls. Based on the results of this study and studies by Brooks and Shorthouse (1998),

Shorthouse (1998), and LeBlanc and Lacroix (2001), some generalized trends in inquiline modification of *Diplolepis* galls can be made.

Periclistus females kill *Diplolepis* larvae with their ovipositors at the time of oviposition. Periclistus-inhabited galls enlarge due to hypertrophy of *Diplolepis*-induced gall cells and the proliferation of *Periclistus*-induced parenchyma. Larvae of *Periclistus* are enclosed within individual chambers composed of parenchyma and nutritive cells, and undergo maturation with the differentiation of a sclerenchyma sheath surrounding each inquiline chamber. The following discussion compares the developmental events associated with of the modification of galls of *D. polita* and *D. nebulosa* by two species of *Periclistus* are discussed.

i. OVIPOSITION

Emergence period

The adults of both species of *Periclistus* are synchronized with their host galls, *Periclistus* 1 with immature galls of *D. polita*, and *Periclistus* 2 with immature galls of *D. nebulosa*, but both species of *Periclistus* undergo a similar life-history. All *Periclistus* are univoltine, with the emergence of both males and females occurring in late spring or early summer (depending on the species). Larvae of *Periclistus* develop in galls through the summer, and overwinter in the larval stage until emerging as adults the following year (Fig. 1.7) (Brooks and Shorthouse 1998; Leblanc and Lacroix 2001). Females oviposit into their respective inducer-inhabited galls in the early to mid-growth phase (Figs. 3.7 and 3.25) and thus the appearance of adults is closely synchronized with the presence of galls at the appropriate stage of development (Fig. 1.7). This suggests, especially in the case of *Periclistus* 2, that inquilines use different environmental cues than their inducer

hosts to exit from galls. In addition, this demonstrates that the emergence period of *Periclistus* inquilines (as was suggested in Chapter II for the synchronization pattern of *Diplolepis* with their host tissues) is closely associated with the availability of host tissues. Each species of *Periclistus* has lengthened or shortened their emergence period to exploit their resource. That is, immature galls of *D. polita* are only available for a short time, and thus the emergence period of the inquiline is also brief.

Lethal inquilines

All previous studies of modification by *Periclistus* report that they are lethal inquilines, as females puncture inducer larvae with their ovipositors while probing or depositing their eggs in galls of *Diplolepis* (Shorthouse 1998; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001). This is also true for *Periclistus* 1 in galls of *D. polita* and *Periclistus* 2 in galls of *D. nebulosa.* An explanation for this behaviour is lacking; however, based on the observations and results from this study, it is suggested that gall inducers must be killed in order for *Periclistus* larvae to gain control of the development of the host gall. It is likely that conflicting stimuli from both the *Diplolepis* and *Periclistus* larvae inhabiting the same chamber would result in the proliferation of gall tissues that are not optimal for the nutritional and physical (overwintering) requirements of *Periclistus* larvae.

ii. *Periclistus* EGGS

Galls of both *D. polita* and *D. nebulosa* containing *Periclistus* eggs are composed of *Diplolepis*-induced parenchyma cells (DP) as the *Diplolepis*-induced nutritive cells degrade or revert to parenchyma (Figs. 3.7, 3.8, and 3.25). In addition, the DP is less cytoplasmically dense (Figs. 3.7, 3.8, and 3.25) than in inducer-inhabited galls. This

pattern of nutritive cell degradation and DP cells becoming less cytoplasmically dense has been shown in other studies where inducer larvae were killed using systemic insecticide (Lalonde 1985), parasitoids (Leggo and Shorthouse 2006), and other inquilines (Bronner 1981; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001). Thus, a live inducer larva is required not only to stimulate further development and differentiation of gall tissues, but to also maintain the cytological aspects of the gall cells and maintain the flow of nutrients directed to the gall from other parts of the host plant (St. John and Shorthouse 2000). In essence, galls of all species begin to 'shut down' once inducer larvae are killed (Rohfritsch 1992); however, in the case of cynipid inquilines, *Diplolepis* galls are 'restarted' and follow a new developmental trajectory under the control of *Periclistus* larvae.

Cynipid gallers typically deposit their eggs into meristematic or rapidly dividing tissue (Rohfritsch 1992; Raman 2007, 2011). When both species of *Periclistus* deposit their eggs, host galls are in the 'early growth phase' (see Chapter II) and are composed of cytoplasmically dense, rapidly dividing *Diplolepis*-induced PNC. While *Periclistus* larvae do not manipulate meristematic plant tissues, they do manipulate gall tissues that are similar to meristematic tissues in that they are cytoplasmically dense and rapidly dividing. Although cynipid inquilines are not gall-inducers, this finding suggests that *Periclistus* inquilines are under similar developmental constraints as cynipid gallers when gaining control of plant tissues. LeBlanc and Lacroix (2001) reported that *Periclistus* in galls of *D. rosaefolii* oviposit into host galls at all phases of development, other than the earliest. This wide developmental window results in variability of the extent of inquiline modification, where the most substantial changes are observed when *Periclistus* eggs are

deposited into immature galls. In contrast, *Periclistus* 1 in galls of *D. polita* and *Periclistus* 2 in galls of *D. nebulosa* consistently oviposit into host galls in the early growth phase and the extent of modification does not vary. This suggests that gall tissues quickly lose their plasticity making it imperative that galls at an immature phase of development are used. LeBlanc and Lacroix (2001) are the only authors to report such variability in the phase of gall development in which *Periclistus* inquilines will deposit their eggs. This study and that by Brooks and Shorthouse (1998) show that the *Periclistus* associated with galls of *D. polita, D. nebulosa* and *D. nodulosa* deposit eggs in immature host galls. Interestingly, the extent of *Periclistus* modification in these three galls is much greater than in galls of *D. rosaefolii,* supporting the trend that the potential for modification in galls by cynipid inquilines is highest in immature galls composed of rapidly dividing tissues.

iii. GALL ENLARGEMENT

Once inquiline eggs hatch, significant changes are observed in the size and organization of cells comprising the chamber walls of galls of both *D. polita* and *D. nebulosa*. *Periclistus* 1-modified galls of *D. polita* are much enlarged (Figs. 3.9 and 3.10) compared to those inhabited by inducer larvae found in the field at the same time. The DP and E cells, which were present in inducer-inhabited galls, become modified by *Periclistus* 1 are significantly enlarged resulting in their becoming the largest cells within the gall (by area) (Figs. 3.11 and 3.43). In addition, there is an even layer of inquiline-induced parenchymatous nutritive cells (IPNC) surrounding the larval chamber (Fig. 3.11). The INC are not well defined and only slightly more cytoplasmically dense than the adjacent IPNC (3.11). Larvae move about the larval chamber feeding on *Periclistus* 1-induced

tissues. In contrast, galls of *D. nebulosa* inhabited by *Periclistus* 2 larvae are not significantly enlarged compared to inducer-inhabited galls found in the field at the same time. Walls of the chambers in modified galls of *D. nebulosa* are thicker than those of inducer-inhabited galls (Figs. 3.26 and 3.27). However, distinguishing *Diplolepis*-induced cells from *Periclistus* 2-induced cells at this phase is difficult. That is, the DP is not cytologically distinct from *Periclistus* 2-induced IPNC and INC and is not easily identified based on size. However, as with nutritive cells induced by inducers in galls of *D. polita*, *Periclistus* 1 and 2-induced nutritive cells are difficult to distinguish from adjacent IPNC, except for a slightly higher cytoplasmic density (Fig. 3.29). Brooks and Shorthouse (1998) reported similar results in inquiline-modified galls of *D. nodulosa* and suggested that the growth of inquiline larvae is initially slow, explaining the lack of nutritive cells early in modification. This is congruent with the growth and development of galls of *D. polita* in Chapter II of this thesis as well as for galls of non-cynipids (Lalonde and Shorthouse 1985; Raman 2011) and those induced by *Diplolepis* (Brooks and Shorthouse 1998).

Gall enlargement and proliferation of *Periclistus*-induced tissues has been attributed to stimuli from eggs (Shorthouse 1975; Brooks and Shorthouse 1998) and larvae (LeBlanc and Lacroix). Immature galls of both *D. polita* and *D. nebulosa* examined for this study that were enlarged and had differentiated inquiline-induced tissues were always inhabited by hatched larvae and not eggs. In addition, galls containing eggs in this study never showed signs of differentiating *Periclistus*-induced cells. Other works have suggest that cynipid gall initiation is stimulated (at least in part) by the egg or fluids associated with oviposition (Rohfritsch 1992; LeBlanc and Lacroix 2001; Shorthouse *et al.* 2005);

however, Roth (1949) suggested that plant cells do not show any signs of proliferation or differentiation as a result of cynipid gall-inducer eggs until they have hatched. Assuming *Periclistus* and other cynipid inquilines stimulate gall tissues in a similar manner as their gall-inducing relatives, it is likely that, changes to gall tissues in this phase of modification are the result of recently hatched larvae grazing on gall tissues and not their eggs. In addition, it is possibly advantageous to the *Periclistus* to not stimulate gall tissues in the egg phase in order to allow a period of time after the stimulus of the dead inducer larva has dissipated before imparting a new stimulus and directing gall tissues into a new developmental trajectory.

Increases in size of *Periclistus*-inhabited galls of both *D. polita* and *D. nebulosa* reported here, and in other studies occurs when larvae of *Periclistus* are small and in early instars (Brooks and Shorthouse 1998; Shorthouse 1998; Shorthouse 1973). Enlargement of galls of *D. polita* by *Periclistus* 1 and galls of *D. nebulosa* by *Periclistus* 2 by hypertrophy and proliferation of DP cells is a key event in *Periclistus* modification as gall enlargement expands the gall chamber such that many individual *Periclistus* chambers may form within one gall (Brooks and Shorthouse). Multi-chambered cynipid galls inhabited by several inducer larvae have lower rates of parasitism than do galls containing one or few larvae (Stone and Schönrogge 2003; Tabuchi and Amano 2004) and the same likely occurs in *Periclistus*-inhabited galls that become multi-chambered.

iv. CHAMBER FORMATION

The chamber formation phase for *Periclistus*-inhabited galls of both *D. polita* and *D. nebulosa* is characterized by the proliferation of IPNC around each *Periclistus* larva,

enclosing them within their own chamber. In modified galls of *D. polita,* IPNC proliferation essentially forms an enclosure around each larva and these enclosures occur around the inside surface of the enlarged chamber once inhabited by the *Diplolepis* larva (Figs. 3.15, 3.16, and 3.17). Cells appear crushed at sites where each *Periclistus* 1 chamber has been completed by opposing chamber walls (Fig. 3.17). Early in chamber formation, when feeding sites of *Periclistus* 1 are more bowl-like, INC are small, and are only present at larval feeding sites (Fig. 3.14). Once chambers are fully formed, INC circumscribes each of the *Periclistus* 1 chambers (Fig. 3.17). Interestingly, when chambers are first starting to form, IPSc begins to differentiate from DP circumscribing the outer gall (Fig. 3.14) and by the time chambers are fully formed, all DP are lignified (Figs. 3.17 and 3.19). In contrast, modified galls of *D. nebulosa* early in the chamber formation phase have uneven proliferation of IPNC and individual *Periclistus* 2 chambers are difficult to distinguish (Fig. 3.31). The most distinct difference between *Periclistus* 2-modified galls of *D. nebulosa* and *Periclistus*-modified 1 galls of *D. polita* at this phase of development is the differentiation of sclerenchyma as modified galls of *D. nebulosa* do not contain any sclerenchyma until the maturation phase.

Collective stimuli by larvae of Periclistus

Many gall inducing cynipids initiate their galls by each individual larva stimulating a few meristematic cells of their host organ (Roth 1949; Rohfritsch 1992: LeBlanc and Lacroix 2001). In contrast, hatching *Periclistus* larvae are surrounded by a large (in relation to the size of hatching larvae), pre-existing gall structure that they must gain control of to modify. Galls `shut down` soon after oviposition and the cells that comprise chamber walls at time of *Periclistus* egg hatch have dedifferentiated, and are vacuolated. Thus,

unlike their gall-inducing relatives, *Periclistus* inquilines do not alter the developmental trajectory of a few rapidly dividing meristematic plant tissues, but must alter the developmental trajectory of an entire gall induced by *Diplolepis* that has ceased development because the inducer has died. The oviposition of numerous eggs of *Periclistus* in each gall is likely due to the ease several larvae gaining control of a gall compared to the prospect of a single larva attempting to control gall development. A single larva of Periclistus would like have difficulty in gaining control of a large mass of plant tissues that would soon lose their susceptibility to redevelopment if the stimulation was weak. Thus, it is important for galls of Diplolepis to be inhabited by numerous larvae of *Periclistus* for they must collectively stimulate gall development at least during the gall enlargement phase.

Once galls enter the chamber formation phase, *Periclistus* larvae in galls of *D. polita* become sedentary and feed in one location within the chamber (Fig. 3.12). The stimulus from each larva then becomes localized and inward proliferation of INC surrounds each larva enclosing them in their own chamber (Figs. 3.12 and 3.13) suggesting that inquiline larvae no longer collectively stimulate gall tissues in the chamber formation phase. It is possible that a change in larval instar could be associated with the behavioral change as well as the change in the way in which gall tissues respond to larval stimuli. In contrast, inquiline larvae within modified galls of *D. nebulosa* continue to act collectively on gall tissues in the chamber formation phase. Gall parenchyma does not uniformly surround each larval chamber (Fig. 3.30) as it does in modified galls of *D. polita.* Instead, parenchyma tissue completely fills the inner space of the gall around each larva (Fig. 3.33 and 3.34) and it is difficult to distinguish which larva is in control of each portion of gall

tissue. Thus, it is presumed that there is some collective influence on the gall tissues into the chamber formation phase.

The most distinct difference between dissected galls of *D. polita* modified by *Periclistus* 1 and galls of *D. nebulosa* modified by *Periclistus* 2 is the arrangement of inquiline chambers in the gall. Chambers of *Periclistus* 1 are arranged strictly around the periphery of the gall, leaving a large central internal space (Figs. 3.2 and 3.15), whereas chambers of *Periclistus* 2 fill the space once occupied by *D. nebulosa* and there is no space between *Periclistus* 2 chambers as they are all coalesced (Figs. 3.33 and 3.38). This is a result of proliferation of IPNC, where in modified galls of *D. polita,* by each *Periclistus* larva is localized at the larval feeding sites as larvae are sedentary by this phase and thus areas of the gall wall that do not receive stimulation from feeding larvae do not increase in thickness from IPNC proliferation. Proliferation continues throughout chamber development filling in the central space within the gall around each larva, ultimately enclosing each within a larval chamber. *Periclistus* 2 larvae do not become sedentary until they are restricted to one area within the gall from IPNC. Again, the differences between actions of *Periclistus* 1 and 2 could be the result of 'ghost of competition past', where arrangement of larval chambers influenced rates of parasitism endured by *Periclistus* larvae. Perhaps having inquiline chambers around the periphery of enlarged modified galls of *D. polita* increased the search and oviposition effort by parasitoids.

v. MATURATION

Sclerenchyma

The maturation of cynipid galls is typically characterized by the differentiation of a hard, protective layer of sclerenchyma (Rohfritsch 1992; LeBlanc and Lacroix 2001; Leggo and Shorthouse 2006). Previous studies of inquiline-modified galls have shown that cells modified by inquilines undergo sclerification near the end of larval development (Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001). Sclerenchyma also differentiates within the tissues of *Periclistus* 1 and 2 chambers and has been distinguished into two types based on size, location, and time of differentiation. Inquiline-induced primary sclerenchyma (IPSc) in modified galls of both *D. polita* (Fig. 3.23) and *D. nebulosa* (Fig. 3.42) circumscribes the entire outer gall in the area once under the influence of the *Diplolepis* and the cells here are much larger compared to other gall cells (Figs. 3.20 and 3.39). Inquiline-induced secondary sclerenchyma (ISSc) circumscribes each of the *Periclistus* chambers (Figs. 3.20 and 3.39) and is composed of smaller lignified cells (Figs. 3.22 and 3.41). The size differences in the *Periclistus*-induced sclerenchyma cells (Fig. 3.43) of both galls is attributed to the size of the cells in which sclerenchyma is derived. Sclerenchyma forms once cells have reached maturity and then the secondary cell walls become thickened**.** IPSc cells are lignified DP cells, which became enlarged under the influence of *Periclistus* in the gall enlargement phase; whereas, the ISSc cells in the walls of the chambers are lignified IPNC. The IPSc and ISSc of both modified galls are circular or ovoid in cross-section and the thickness of the lignified secondary cell walls is even in each cell.

In modified galls of *D. polita,* the IPSc differentiates early in the chamber formation phase from the lignification of DP cells that circumscribe the entire outer periphery of the gall (Fig. 3.14) and are significantly enlarged under the influence of *Periclistus* 1 (Fig. 3.43). Apparently the differentiation of a protective layer in modified galls of *D. polita* so early in development serves to protect larvae of *Periclistus* 1 from ovipositing enemies. Dissecting galls throughout the season revealed that galls with *Periclistus* 1 are attacked by chalcidoid ectoparasitoids of the genus *Eurytoma* typically early in the chamber formation phase. Once *Periclistus* 1 chambers have formed, other parasitoids of the genus *Torymus* lay their eggs on maturing *Periclistus* 1 larvae. The ISSc layer does not form until later in gall development when larvae of *Periclistus* 1 are full grown (Figs. 3.20 and 3.22) and likely have a different function than the IPSc. In contrast, both IPSc and ISSc layers in modified galls of *D. nebulosa* do not differentiate until the last phase of gall development (Figs. 3.39, 3.41. and 3.42). Despite not having a protective layer of sclerenchyma earlier in development, *Periclistus* 2 larvae in galls of *D. nebulosa* suffer from lower rates of parasitism than larvae of *Periclistus* 1 in galls of *D. polita*.

Sclerenchyma causes galls to become hard and brittle (Roth 1949; Rohfritsch 1992) and has also been suggested to reduce or have once reduced the success of ovipositing inquilines and parasitoids in galls in the maturation phase (Ronquist and Liljeblad 2001; Stone and Schönrogge 2003; Zargaran *et al.* 2011). Gall hardness through sclerification likely represents a 'ghost of predation past' (Stone and Schönrogge 2003) as this protective layer could in past evolutionary history provided protection to inducer larvae from ovipositing inquilines and parasitoids. But then in time, various species of inquilines

changed their phenologies to be closely associated with immature stages of their respective host galls prior to gall hardening.

Nutritive cells

Nutritive cells are one of the defining features of cynipid galls (Bronner 1992; Rohfritsch 1992; Raman 2007). Even though cynipid inquilines are not gall inducers, their ability to induce nutritive cells shows that they have retained the ability to manipulate plant tissues as they diverged from the inducers. The differentiation patterns of nutritive cells induced by *Periclistus* 1 and 2 follow a generalized pattern where few, small nutritive cells are sparsely distributed around the enlarged chamber at the gall enlargement phase (Figs. 3.11 and 3.29). During the chamber formation phase, nutritive cells appear larger, more cytoplasmically dense, and become restricted to the regions of larval feeding (Figs. 3.14, 3.17, 3.18, 3.32 and 3.36). Collapsed *Periclistus*-induced nutritive cells about the surface of completed larval chambers (Figs. 3.18 and 3.36), suggests that larvae begin to actively feed during this phase of development. By the maturation phase, a thick layer of nutritive cells (IPNC) circumscribes each larval chamber (Figs. 3.21 and 3.40). This is interesting because at the maturation phase, larvae nearly fill their chambers (Figs. 3.20 and 3.38) and it is unlikely that they are able to turn about and feed on cells throughout the chamber surface.

This pattern of nutritive cell differentiation and proliferation is also supported by the study of modification of galls of *D. nodulosa* by *P. pirata.* However, the development of nutritive cells associated with *Periclistus* inquiline chambers is quite different from those induced by *Diplolepis.* Nutritive cells surround *Diplolepis* chambers soon after egg hatch

and are of the largest cells of galls by maturation (see results in Chapter II); however *Periclistus* nutritive cells are not densely distributed until inquiline chambers are closed.

The developmental pattern of inquiline-induced nutritive cells (INC) in *Periclistus* 1-modified galls of *D. polita* and *Periclistus* 2-modified galls of *D. nebulosa* was also demonstrated in galls of *D. nodulosa* modified by *P. pirata* (Brooks and Shorthouse 1998). Inquiline-induced nutritive cell differentiation is different than *Diplolepis*. Inducer nutritive cells differentiate and surround *Diplolepis* chambers soon after egg hatch and are of the largest cells of galls by maturation (see results in Chapter II); however *Periclistus* 1 and 2-induced nutritive cells are not densely distributed until late in gall development at the end of chamber formation or early maturation.

vi. SIZE OF GALL CELLS

This is the first study comparing the sizes of cells associated with inquiline modified galls throughout gall development and between galls modified by two different species. After eggs of *Periclistus* 1 and 2 are deposited into galls, *Diplolepis*-induced nutritive cells degrade. Galls of *D. polita* become composed of parenchyma and an epidermal layer, whereas galls of *D. nebulosa* with *Periclistus* 2 become composed solely of parenchyma. Parenchyma cells increase in size once eggs hatch and hypertrophy of these cells contributes to the overall increase in size of galls inhabited by *Periclistus,* particularly in galls of *D. polita*.

The major difference between the cells induced by the two species is the size; where gall cells of *Periclistus* 1 modified galls of *D. polita* are significantly larger (two to three or more times) in area than those of *Periclistus*-2 modified galls of *D. nebulosa* (Fig. 3.43).

This is similar to results of the size of cells in inducer-inhabited galls presented in Chapter II, where cells within galls of *D. polita* are significantly larger than those within galls *D. nebulosa* (Fig. 2.48). It is likely that *Periclistus* evolved under similar environmental and evolutionary pressures as their hosts and thus evolved to induce cells of a similar size to their hosts.

vii. VASCULARIZATION

Vascular tissue develops during the gall enlargement phase of galls of both *D. polita* and *D. nebulosa* modified by inquilines at the interface of the inquiline-modified (DP) and induced (IPNC) tissues (Figs. 3.11 and 3.27). Vascularization in modified galls is more extensive and well-defined than in inducer-inhabited galls and vascular bundles have distinct phloem and xylem (Figs. 3.21, 3.32, and 3.40). The same had been found in other galls modified by *Periclistus* (Bronner 1981; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001). Each of these galls are single-chambered and become enlarged and multi-chambered when inhabited by their respective inquilines. The increase in vascular tissue is likely required by the increased mass of gall tissue (from gall enlargement and proliferation of inquiline-induced cells) (Bronner 1981; LeBlanc and Lacroix). The most remarkable example of *Periclistus* increasing the vascularization within their host gall is in modified galls of *D. nodulosa,* where the inducer-inhabited gall has no new vascular tissue as the larva is supplied with nutrients from adjacent vascular tissues of the stem. Galls modified by *P. pirata* are three times larger than galls with a single inducer and contain many, well-defined inquiline-induced vascular bundles that supply galls with an increased nutrient supply (Brooks and Shorthouse 1998). It is likely that a number of factors are involved when vascular bundles are differentiating in *Diplolepis* galls

modified by *Periclistus* including the amount of nutrients required to sustain the larvae and the time of year in which galls are induced or modified (it was suggested in Chapter II that normal galls of *D. polita* have poorly differentiated vascular bundles because they are initiated on immature leaflets early in the season that are well supplied by nutrients).

viii. CANNIBALISM AMONG INQUILINE LARVAE

Cynipid gallers are derived from entomophagous parasitoids of concealed hosts (Ronquist 1995). Although galling larvae are phytophagous, Roth (1949) and Shorthouse (1993) reported that larvae will consume each other when a cluster is placed in a petri dish. It was noticed throughout the three seasons of dissecting galls of *D. polita* and *D. nebulosa* inhabited by *Periclistus* that the number of inquiline eggs was greater than freshly hatched larvae in older galls. Then as galls matured, the number of *Periclistus* per gall decreased even further. It is suggested that larvae of *Periclistus* 1 and 2 also practice cannibalism when in close proximity to each other, thus explaining the decreasing population of *Periclistus* per gall as galls mature. It has been suggested that sclerenchyma formation around each individual larval chamber in multi-chambered galls evolved to prevent larvae from chewing into the next larval chamber and consuming the inhabitant (Shorthouse 1993; Leggo and Shorthouse 2006). Mature chambers in galls modified by *Periclistus* 1 and 2 are surrounded by sclerenchyma (IPSSc) (Figs. 3.20 and 3.39) in addition to a thick layer of sclerenchyma circumscribing the entire gall (IPSc) (Figs. 3.20 and 3.39). The differentiation of IPSc in galls modified by both *Periclistus* 1 and 2 could prevent larvae of *Periclistus* from chewing into the next larval chamber and consuming its inhabitant.

ix. SPECIES-SPECIFIC GALL MODIFICATION BY *Periclistus*

Comparing the developmental events associated with two species of *Periclistus* in galls induced by *Diplolepis* histologically allows one to distinguish which events are common to *Periclistus* and which, if any, are species-specific. This study along with others (Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001) suggests a common pattern of modification across *Periclistus* .It is likely that all species of *Periclistus* kill the larvae of the inducers when they oviposit which causes the inducer-inhabited gall cells to lose their characteristics. Once larvae of *Periclistus* hatch, their host galls enlarge and inquiline-induced tissues proliferate, enclosing each larva in a chamber. Galls maturation includes the differentiation of a layer sclerenchyma that surrounds each inquiline chamber (ISSc) and the entire gall (IPSc). The results presented here suggest that each species of *Periclistus* inquiline manipulates their host galls in a species-specific manner, particularly pertaining to cell size, extent of gall enlargement, parenchyma proliferation, and time of primary sclerenchyma differentiation. Shorthouse (1998) also supports species-specific modification by *Periclistus.* He compared six mature *Diplolepis* galls modified by *Periclistus*, including *D. polita* and *D. nebulosa*, and highlighted the distribution of sclerenchyma and nutritive cells within each of the mature galls, and each were species-specific.

It is unknown what developmental constraints are faced by cynipid inquilines based on the anatomy of the host gall at time of oviposition. Inquilines do not 'take control' of the development of the plant tissues until the developmental trajectory of the host plant has been significantly altered by the gall inducer. It would be interesting to compare the development of several galls of *Diplolepis* modified by the same species of *Periclistus*.

For example, Ritchie (1984) showed that galls of *D. polita* and *D. bicolor* are both inhabited by *Periclistus* 1, and galls of *D. nebulosa, D. variabilis,* and *D. ignota* are inhabited by *Periclistus* 2. A detailed histological comparison of the developmental events of the same species of inquiline in different galls would provide a better understanding as to the species-specificity of inquiline modification and what constraints, if any, the original host gall anatomy has on modification.

Number and arrangement of inquiline chambers

The number of inquiline chambers in single-chambered cynipid galls has been reported by several authors (Shorthouse 1973; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001; Shorthouse 2010). The range is typically from one to two *Periclistus* chambers in galls induced by *D. rosaefolii* (LeBlanc and Lacroix 2001; Shorthouse 2010) to an average of 17 inquiline chambers in galls of *D. nebulosa* modified by *P. pirata* (Brooks and Shorthouse 1998). Galls of *D. polita* and *D. nebulosa* used in this thesis were both inhabited by an average of five inquiline chambers. Shorthouse (2010) reported the number of inquiline chambers in *Diplolepis* leaf galls across the grasslands of Canada and his findings for galls of *D. polita* and *D. nebulosa* were the same as reported in this thesis. In most cases, each species of *Periclistus* is associated with one species of *Diplolepis* in North America (Ritchie 1984), which would suggest that the number of chambers induced by *Periclistus* is a reflection on the species of *Periclistus.* However, a few closely taxonomically related species of *Diplolepis,* such as *D. polita* and *D. bicolor* induce galls that are attacked by the same species of *Periclistus* (Ritchie 1984). Interestingly, galls of *D. bicolor* contain nearly twice as many inquiline chambers (9.3) than galls of *D. polita* (5.4) (Shorthouse 2010). Therefore, it seems that the number of

larval chambers per gall is not dependent on the species of *Periclistus*, but perhaps a reflection of the host gall. Modified galls of *D. rosaefolii* contain few inquiline chambers and are of the smallest *Diplolepis* galls, whereas those containing many inquiline chambers per gall, such as those induced by *D. bicolor* are large galls.

x. COMPARISON OF INQUILINE-MODIFIED GALLS WITH TYPICAL CYNIPID GALL DEVELOPMENT PATTERNS

Cynipid galls undergo three phases of development (Meyer and Maresquelle 1981; Rohfritsch 1992; LeBlanc and Lacroix 2001); however, this thesis classifies the development of inquiline-modified galls as having four phases. These are *Periclistus* eggs, enlargement, chamber formation, and maturation as the events that typically characterize each developmental phase in induced-inhabited galls occur at different times during *Periclistus* modification with some of these events spanning multiple phases of development. For example, initiation in galls of other cynipids is considered from the period when eggs have hatched until larvae are enclosed in their larval chambers (Rohfritsch 1992; Bronner 1992; LeBlanc and Lacroix 2001); however, in *Periclistus*-modified galls of *D. polita* and *D. nebulosa*, these events occur over a lengthy period of time and encompass three inquiline developmental phases (eggs, gall enlargement and chamber formation) (Figs. 3.8, 3.11, 3.17, 3.25, 3.27, 3.35). In addition, gall maturation in inducer-inhabited galls is characterized by the differentiation of a layer of hard sclerenchyma. In *Periclistus*-modified galls of *D. polita*, sclerenchyma differentiation occurs early in development in the chamber formation phase (Fig. 3.14) and continues to differentiate until the maturation phase (Figs. 3.22 and 3.23). Thus, the events used to characterize each phase of development in inquiline galls are not congruent with normal gall development.

The placement of inquilines within cynipid phylogeny has been examined by several authors (Ronquist 1994, 1995; Ronquist and Liljeblad 2001; Nylander 2004). Some consider inquilines to be a polyphyletic group where inquilines are more taxonomically related to the gall inducers they attack than other inquilines (Nylander 2004) and accordingly, *Periclistus* would be more closely taxonomically related to *Diplolepis* than to other inquiline genera (Nylander 2004). However, there is also evidence suggesting that inquilines are monophyletic and most closely related to the gallers in the primitive tribe, Aylacini (Ronquist 1994; Ronquist and Liljeblad 2001; Csóka *et al.* 2005). Sliva and Shorthouse (2005) studied the development of galls of *Aulacidea hieracii* Bouché, a species that belong to the tribe Aylacini. The development of galls of *A. heiracii* is similar to *Periclistus*-inhabited galls of *D. polita* and *D. nebulosa.* The most distinct similarity is that many larvae hatch within a central chamber and proliferation of gall tissues then surrounds each larva within its own chamber. This is different from the developmental events of many other multi-chambered cynipid galls, where each larva is enclosed within its own chamber immediately after eggs hatch (Sliva and Shorthouse 2005; Leggo and Shorthouse 2006). It is accepted that cynipid inquilines were once gall-inducers and lost the ability to initiate galls of their own (Ronquist 1994). It appears that the modification of galls of *Diplolepis* by *Periclistus* is similar to the developmental patterns of galls induced by ancestral cynipids. Thus, the developmental similarities between galls induced by members of the Aylacini and *Periclistus* inquilines support the monophyletic inquiline phylogeny, suggesting that inquilines are most closely taxonomically related to the primitive gall-inducing tribe Aylacini.

In conclusion, the results presented here demonstrate that gall modification by *Periclistus* is species-specific with some of the key differences between *Periclistus* 1 and 2 being phenology, size of gall cells, timing of key gall developmental events such as differentiation of primary sclerenchyma, and the arrangement of larval chambers. Many useful characters in inquiline modification were revealed from histological examination will be useful when considering the placement of inquilines within cynipid phylogeny. In addition, this study showed that the developmental events associated with the modification of galls of *Periclistus* is as complex as the development of galls inhabited by inducers.

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G. APPENDIX

Table. 3.1. Mean cell area with standard deviations in galls of *D. polita* modified by *Periclistus* 1 (μ^2). Unlike lowercase letters represent significant differences ($p<0.05$) within each cell type based on analysis of variance using Tukey's HSD for post-hoc comparisons ($\alpha=0.05$).

Phase of Development	Cell Type					
	DP	INC	PNC	ISSc	IPSc	E
Eggs	1038 **a** ± 559	N/A	N/A	N/A	N/A	573 **a** ±331
Gall Enlargement	3172 **b** ±1798	1182 **a** ±719	2010 **a** ±1253	N/A	N/A	1236 **b** ±1140
Chamber Formation	N/A	912 **b** ±583	1400 **b** ±901	N/A	3634 **a** ±2302	1616 **c** ±1158
Maturation	N.A	1522 **c** ±922	1524 **b** ±857	1238 ±919	3396 **b** ±2378	1505 **c** ±899

Table. 3.2. Mean cell area with standard deviations in galls of *D. nebulosa* modified by *Periclistus* 2 (μ^2). Unlike lowercase letters represent significant differences ($p<0.05$) within each cell type based on analysis of variance using Tukey's HSD for post-hoc comparisons ($\alpha=0.05$).

Phase of Development	Cell Type				
	DP	INC	PNC	ISSc	IPSc
Eggs	402 **a** ± 187	N/A	N/A	N/A	N/A
Gall Enlargement	930 **b** ±457	387 **a** ±140	573 **a** ±195	N/A	N/A
Chamber Formation	943 **b** ±554	564 **b** ±326	686 **b** ±349	N/A	N/A
Maturation	1293 **c** ±1043	569 **b** ±267	670 **b** ±321	470 ±218	609 ±320

Table 3.3. Summary of statistical analysis of the mean size of cells at each phase of development between *Periclistus* 1-modified galls of *D. polita* and *Periclistus* 2-modified galls of *D. nebulosa*

Phase of Development	Cell Type	Species of Inquiline	Mean (μ^2)	*n*	P
Eggs	DP	*Periclistus* 1	1038	210	<0.001
		Periclistus 2	402	150	
Gall enlargement	DP	*Periclistus* 1	3172	210	<0.001
		Periclistus 2	931	210	
	INC	*Periclistus* 1	1182	210	<0.001
		Periclistus 2	388	210	
	IPNC	*Periclistus* 1	2010	210	<0.001
		Periclistus 2	573	210	
Chamber Formation	INC	*Periclistus* 1	912	210	<0.001
		Periclistus 2	563	210	
	IPNC	*Periclistus* 1	1400	210	<0.001
		Periclistus 2	686	210	
Maturation	INC	*Periclistus* 1	1522	210	<0.001
		Periclistus 2	569	210	
	IPNC	*Periclistus* 1	1524	210	<0.001
		Periclistus 2	670	210	
	IPSc	*Periclistus* 1	3396	210	<0.001
		Periclistus 2	609	210	
	ISSc	*Periclistus* 1	1238	210	<0.001
		Periclistus 2	470	210	

CHAPTER IV: GENERAL DISCUSSION

Galls induced by cynipid wasps have attracted the attention of naturalists since ancient times and there are numerous references in old literature as to the mysterious events that allow these small insects to gain control of plant tissues and direct the development of what can be considered new plant organs. Early naturalists fascinated by plant galls had no idea as to what stimulus was the source of the unusual plant growths and it was not until the 17th century when Marcello Malpighi (1630-1694), Anthony van Leeuwenhoeck 1632-1723) and Jan Schwammerdam (1630-1680) attributed the development of galls with insects (Raman *et al.* 2005). Since then, much has been learned about the biology of gall insects and the development of their galls; however, the mechanisms used by insects to gain control of the development of their host plants are still poorly understood. Even more elusive is the means by which multiple, taxonomically related species manage to induce galls, often on the same host plant, that are structurally distinct from one another as was the case with galls of *D. polita* and *D. nebulosa* studied n this thesis. With the availability and widespread use of molecular techniques today, determining the chemical make-up of the stimulus that is passed from insect to plant to initiate species-specific galls should now be possible. However, before biologists can undertake such studies, they must have the ability to identify the inducer, ensure that galls found in the field at various phases of development are induced by the same species, and perhaps learn enough about their life history strategies that galls can be cultured in the laboratory.

Before the stimuli that cynipids use to induce their galls can be identified, it is necessary to choose ideal systems to study. It is also necessary to have a clear understanding of the patterns of gall development from initiation to maturation as well as the types of gall cell

types that differentiate. Two such systems were identified in this thesis and by carefully following the development of the galls induced by the two species of *Diplolepis* using histological techniques, a clearer understanding of the events in gall development has been revealed compared to previous studies in the literature. The approach of comparing the development of the galls of two closely taxonomically related species proved beneficial as important details of gall development and biology were revealed that likely would have been overlooked if only a single species of galler was examined. In addition, the presence of *Periclistus* inquilines within galls of both species provided an opportunity for the first time to compare the effects of cynipid inquiline modification in two gall systems. By carefully monitoring galls at multiple study sites, all stages of inquiline modification of galls of both *Diplolepis polita* and *D. nebulosa* were obtained. As with the galls inhabited by only *Diplolepis*, having the opportunity to compare modification strategies and developmental events associated with inquiline modification by two species of *Periclistus*, revealed more than if only the impact of one species of *Periclistus* was examined.

For galls of both *D. polita* and *D. nebulosa*, and those *of D. polita* inhabited by *Periclistus* 1 and galls of *D. nebulosa* inhabited by *Periclistus* 2, this study has revealed that the developmental events initiated by *Diplolepis* and *Periclistus* are more complex than has previously been reported (Meyer and Maresquelle 1983; Rohfritsch 1992; Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001; Sliva and Shorthouse 2005; Leggo and Shorthouse 2006). The ability of *D. polita* to gain control of immature leaflets within closed buds of *R. acicularis,* and likewise of *D. nebulosa* to gain control of maturing leaves of *R. blanda* and induce the differentiation of plant cells foreign to the

attacked organ is nothing less than spectacular. Furthermore, the ability of *Periclistus* inquilines to essentially 'shut down' the development of their host galls and dramatically redirect the development of tissues induced by another insect is equally spectacular.

Histology has proven to be an effective technique in revealing the extent of the control cynipid wasps have over their host plants and the diversity in patterns of gall development and anatomy. Stone and Schonrögge (2003) suggested that diversification of outer gall tissues (cortex and epidermis) was responsible for the numerous gall structures observed today; however, this thesis showed that there is extensive diversity within galls at the tissue and cellular level. This is particularly true of the nutritive and sclerenchyma cells of inducer-inhabited galls of *D. polita* and *D. nebulosa.* Difference in the thickness of the layer of nutritive cells is also a reflection of the level of control the respective larva has over its host plant.

The proliferation of nutritive cells, as described by Rohfritsch (1992), is a result of stimuli from the inducer larva and gall induction does not occur when only eggs are present (Roth 1949). Thus, live and actively feeding larvae are necessary for gall development. The ability of *D. nebulosa* to induce a thick layer of nutritive cells that circumscribes the larval chamber soon after hatching suggests that this stimulus is present in the early larval instars. In contrast, *D. polita* induces a thin layer of patchy nutritive cells that does not increase in density or thickness until gall maturation, suggesting that the stimulus may be weak or absent early in gall development and increases once larvae are protected by the hard sclerenchyma layer in the maturation phase. Interestingly, *Periclistus* 1 and 2 induce nutritive cells that follow a similar developmental pattern as galls of *D. polita*, where nutritive cells are small and sparse in galls occupied by

immature *Periclistus* larvae and increase in size and density once larvae are enclosed within sclerified larval chambers.

The diversity of sclerenchyma cells induced by cynipids was also highlighted for the first time in this thesis. Previous studies have not considered differences in size, shape, and arrangement of sclerenchyma cells within galls induced or modified by cynipids. Here, the importance of examining galls from a histological perspective as a variety of species-specific characters within galls was demonstrated. The appearance of sclerenchyma can only be illustrated by using this classic histological technique. Sclerenchyma plays an important role in gall biology and its role as a protector of cynipid larvae from their parasitoids enemies has been suggested (Stone and Schonrögge 2003; Meyer and Maresquelle 1983). It also increases the structural integrity of the gall (Stone and Schonrögge 2003; Csóka *et al.* 2005), and may enhance the overwintering ability of inhabitants by preventing ice formation within the chambers (Williams *et al.* 2003). Given the variety of possible functions that sclerenchyma tissues serve within cynipid galls, examining this tissue type further could provide new clues as to its function. It is likely that sclerenchyma plays several roles within cynipid galls; however, it is also likely that different selective pressures over evolutionary time have caused diversification at the cellular level to increase the effectiveness of one or more of the suggested functions in galls induced by different species. For example, galls of *D. polita* are heavily attacked by inquilines and parasitoids and have a survival rate of less than five percent (Shorthouse 1973; Shorthouse 2010). The sclerification pattern within cells induced by *D. polita* was found to be unique among all other galls of *Diplolepis* previously examined (Brooks and Shorthouse 1998; LeBlanc and Lacroix 2001; Sliva and Shorthouse 2005; Leggo and

Shorthouse 2006). Even with a thick layer of unique sclerenchyma, and survival of less than five percent, the species continues to sustain populations from year to year. It is possible that the characteristics of this sclerenchyma layer represents a 'ghost of competition past' and once prevented parasitoids from penetrating with their ovipositors but species of parasitoids have since developed longer and stronger ovipositors.

When discussing the extent of host plant modification by gallers and inquilines, it is also important to consider the vascular tissues that deliver nutrients from the rest of the plant to each developing larva. Cynipid galls have been shown to act as physiological sinks (Bagatto and Shorthouse 1994; St. John and Shorthouse 2000). Even more remarkable is that the galls examined in this thesis are induced on leaves which are sources for assimilates soon after they expand to their full size. However, when galls are present, they are converted to physiological sinks, each supported by a network of vascular tissues that connect to the host plant. Interestingly, the degree of vascularisation within galls of *D. polita* and *D nebulosa* differs significantly. Galls of *D. polita* have few vascular bundles and those present are small with poorly defined xylem and phloem. In contrast, the vascular bundles in galls of *D. nebulosa* are much more abundant and each has well defined xylem and phloem. Thus, despite both species inducing single-chambered, similarly sized galls on leaves of roses, they differ substantially in the extent and anatomy of their vasculature. Even more intriguing, is that the vascularisation of *Periclistus*-modified galls is more extensive than that in tissues of inducer-inhabited galls. That is, there are more vascular bundles in tissues of galls inhabited by Periclistus 1 and 2 than there is in galls inhabited by *D. polita* or *D. nebulosa*. This was also shown by Brooks and Shorthouse (1998) and LeBlanc and Lacroix (2001). It is suspected that an increase

in the number of cynipid larvae per gall increases the demand for nutrients, they are stronger physiological sinks, and as a result, additional vascularisation is needed. Although it is hypothesized that *Periclistus* and other inquilines lost their ability to induce galls of their own (Ronquist 1994), their remarkable ability to induce extensive vasculature with well defined xylem and phloem demonstrates that they have maintained strong relationships with the tissues of their host plants.

This thesis also provided detailed phenological data of the *Diplolepis*, *Periclistus*, and their host plants. These data will not only be important for future studies of galls of *Diplolepis* and those modified by *Periclistus* as the ability to locate galls at the appropriate phase of development in the field is essential, but have also provided clues as to broader biological patterns and ecological interactions among *Diplolepis* gallers, *Periclistus* inquilines, and their host roses.

Diplolepis wasps exit their host galls throughout the spring depending on the species, and typically only live for a few days (Shorthouse 2010). During this period, locate their host plants and developing leaf buds that are suitable for oviposition and the initiation of their galls. It is suspected that the availability of suitable oviposition sites fluctuates from year to year as slight differences in weather patterns would alter the synchrony of the phenology of the *Diplolepis* and their host plants. For example, adults exiting galls when air temperatures are above or below normal would likely result in host plant tissues that are too mature for gall induction, or the presence of undeveloped leaf buds that had not reached a suitable stage for gall initiation. In addition, rain or strong winds at the time of emergence would interfere with oviposition success.

Despite being faced with a variety of hurdles at the time of oviposition, populations of *Diplolepis* managed to persist from year to year at the study sites which likely is attributed to a variety of traits of both the wasps and their host roses. *Diplolepis* are weak flyers; however, there is evidence that they disperse great distances, likely carried by the wind (Shorthouse 2010). It is common to find small isolated patches of *R. acicularis* in central Ontario with a small number of galls. More remarkable is that it is common to find such galls occupied by *Periclistus* and a couple species of parasitoids. It is likely that *Diplolepis* and *Periclistus* are blown away from their host plants by the wind and by chance must come down on their host plants. It was noticed that patches of roses have leaf buds present in varying numbers from year to year and their period of development varies slightly, thus extending the period of time when suitable oviposition sites are available. This is particularly important for *D. polita* which oviposit into closed leaf buds of within a narrow developmental window in contrast to *D. nebulosa* that is less restricted and finds leaf tissues suitable for oviposition for periods of about six weeks. As a result, populations of immature galls of *D. polita* appear within a one week period whereas; immature galls of *D. nebulosa* appear over a period of six to seven weeks. In addition, *Periclistus* 1 associated with galls of *D. polita* have a narrow oviposition window of one week compared to *Periclistus* 2 associated with galls of *D. nebulosa* which have a much longer oviposition period of five to six weeks.

This thesis also demonstrated that subtle differences in oviposition sites among gallers results in major difference in the location and orientation of galls. Galls of *D. polita* are initiated on the palisade mesophyll cells of leaves of *R. acicularis* whereas, galls of *D. nebulosa* re initiated on the spongy mesophyll of leaves of *R. blanda.* This was also

hypothesized by Shorthouse *et al.* (2005) who showed the partitioning of oviposition sites of five species of *Diplolepis,* and likely the entire genus, has occurred and likely played a role in radiation of rose gallers.

Gall wasps may be the most conservative phytophagous insects with their host plant use, making approximately one host shift per hundred speciation events (Ronquist and Liljeblad 2001). Despite few shifts over evolutionary time, each shift is thought to have been major (shifting to remarkably distantly related hosts) (Ronquist and Liljeblad 2001). Thus, it is likely that many taxonomically unrelated plant families are susceptible to gall initiation by cynipids, yet few plant families are used by the majority of species. The largest radiations of gall wasps occurred on oaks (*Quercus*) and the second largest on wild roses (Csóka *et al.* 2005). It is interesting to consider what characteristics of host plants are responsible for cynipid diversity, in particular, the characteristics of roses that have lead to the radiation of the genus *Diplolepis*. Somehow, the growth form and life-history strategies of roses have made them an ideal host.

Diversification of *Diplolepis* has undoubtedly been influenced by characteristics of their host plant. The genus *Rosa* is composed of about 20 species in North America (Wissemann 2003), many of which have overlapping ranges and habitats, suggesting that sympatric speciation occurred within the genus *Diplolepis* in the past. In addition, species diversity of galling insects is typically higher in temperate than tropical regions, which has been attributed to gall inducers requiring meristematic tissue to initiate their galls (Espirito-Santo and Fernandes 2007). In temperate regions, there is an abundance of reliable meristematic tissues available for gall inducers each spring, as is the case with all species of roses. Roses are hardy plants that are resistant to damage such as fire and the

consumption of their upper branches. To compensate, roses continuously develop new meristematic tissues throughout the growing season and increase the amount of branching, both of which proves beneficial to *Diplolepis.* Roses are poorly defended against insects by secondary compounds, yet they are rarely defoliated. This could also be attributed to their vigour, and rather than investing in the production of costly defence compounds, they allocate their resources to repair and growth. It is possible that plant hardiness is a key characteristic selected for by cynipid gall inducers. This quality could be especially advantageous for *Diplolepis* as their wounding the plant would only stimulate more vigorous growth. For example, stem galls of *D. triforma* induced the previous year will develop several leaf buds in the spring (Leggo and Shorthouse 2006). Thus, plants attacked by *D. triforma* typically have increased branching which is beneficial for the plant as there is an increased biomass. *Diplolepis* would also benefit as bushier plants mean more oviposition sites for future generations. Plant architecture also affects the diversity of phytophagous insects feeding on them (Lawton 1983). For example, the bushy *R. woodsii* in the grasslands of Canada is attacked by five *Diplolepis* leaf gallers, three stem gallers, and one root galler (Shorthouse 2010), each of which are phenologically distinct and oviposit onto slightly different cells within the plant (Shorthouse *et al.* 2005). Thus, rose plant architecture could have provided *Diplolepis* with an ideal platform of low inter-specific competition for oviposition sites, and could help to explain the large radiation of cynipid gallers on roses compared to other plants used by the family.

Based on the general trends in the evolution of cynipids (references within Csóka *et al.* 2005) and the results of this thesis, it is hypothesized that the first *Diplolepis* galler was

capable of initiating galls on many or all species of roses and oviposited into a variety of rose organs. Güçlü, *et al.* (2008) noted that galls induced by *D. fructuum* (Rübsaamen) were capable of inducing galls on a variety of organs of *R. canina* L. Over evolutionary time, it is possible that environmental factors or niche partitioning could have influenced the phenology of rose gall wasps, and thus certain populations or species became restricted to oviposting into plant tissues available at different times of the season. For example, *D. fusiformaans* oviposits into elongated shoots late in the spring well after leaves have developed, whereas *D. spinosa* oviposits below the meristematic tissue within closed leaf buds early in the spring (Shorthouse 2010). It is likely that becoming associated with different host organs brings about physiological and physical constraints. For example stem galls of *D. spinosa* are large, heavy, and inhabited by many inducer larvae and galls such as this would be difficult to sustain on leaves or petals. Thus, it is speculated that *Diplolepis* gallers evolved strategies to best exploit their host organs and to avoid enemy attack, which contributed to the diversification in gall structure.

There is still much to learn regarding the means by which cynipid gall inducers take control of their host plants and stimulate the appearance of developmentally and morphologically complex, species-specific structures. This is the first study to directly compare the galling strategies and development of galls induced by two closely taxonomically related species of cynipids as well as the first to compare the modification strategies and developmental events associated with modification by inquilines. The findings from this thesis were derived from a combination of phenological data collected in the field as well as detailed histological data collected from sectioning thousands of inducer-inhabited and inquiline-modified galls from initiation to maturation. This

combined approach revealed further complexities and diversity of the developmental events of cynipid galls and inquiline modification that have been previously overlooked and has contributed to the understanding of gall biology as a whole. It is hoped that the results of this thesis will encourage future researchers to use *Diplolepis* galls as model systems to study the molecular mechanisms by which cynipid gallers and their inquiline relatives manipulate plant tissues.

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