From Parasitoids to Gall Inducers and Inquilines

Morphological Evolution in Cynipoid Wasps

BY

HEGE VÅRDAL
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Abstract

One of the large lineages of parasitic wasps, the Cynipoidea, exhibits three distinctly different life modes. Slightly more than half of the about 3000 species are parasitoids in insect larvae, whereas the remaining species are associated with plants, either as gall inducers or as inquilines (guests feeding on plant tissue in galls). The main focus of this thesis has been to identify morphological changes associated with the shifts between life modes. Particular attention was paid to structures believed to be important in gall initiation. Comparative anatomical studies of the egg, larva and venom apparatus were performed, including representatives of parasitoids, gall inducers and inquilines. Examination of gross morphology and ultrastructure revealed that the eggs of the gall inducers are larger and surrounded by a thicker shell than the parasitoid eggs. These differences may be related to the fact that the gall inducer egg contains sufficient egg yolk for the embryo during the entire egg period, whereas the parasitoid egg often absorbs nutrients through the eggshell. Furthermore, the gall inducer egg is probably more exposed to desiccation and therefore a thicker and more resistant eggshell is crucial. Comparing the terminal-instar larvae of about 30 species of parasitoids, gall inducers and inquilines, extensive morphological variation was found, particularly in the head and mouthpart features. The variation was summarized in 33 morphological and one life-history character and parsimony analyses were performed. The resulting phylogenetic estimates were largely in accordance with previous analyses of adult morphology and molecular data. The larval data point to a single origin of the inquilines, in agreement with adult morphology but in conflict with molecular data. The venom apparatus was found to be quite uniform in structure among a sample of 25 species of cynipoide species. It consists of a very short venom duct, a reservoir and a single unbranched venom gland. With few exceptions, the venom apparatus is conspicuously larger relative to the female metasoma in the gall inhabiting species than in the parasitoids. We found little evidence of anatomical structures that could facilitate chemical communication between the gall-inducer embryo and the surrounding plant tissue through the thick eggshell. On the other hand, the enormous venom glands and reservoirs, which are apparently not used for defence, suggest that the adult female plays a significant role in gall induction by injecting secretions into the host plant when laying eggs.

Keywords: gall inducer, parasitoid, inquiline, gall induction, morphology

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List of Papers

This thesis is based on the following papers, which will be referred to by their Roman numerals:


IV  Vårdal, H. Venom gland and reservoir morphology in cynipoid wasps. *Manuscript*.

Reprint of paper I was made with the kind permission of the publisher.

All four papers were written primarily by the author of this thesis, with assistance from the co-authors in paper I-III. The work leading to paper I-III was planned together with co-authors. In paper I, HV completed all the morphological studies. In paper II, JLNA did the majority of the specimen collecting and about 50% of the SEM imaging, whereas the remaining SEM examinations were done together with HV, who also did the phylogenetic analyses and wrote the first draft of the manuscript.
Contents

INTRODUCTION ..................................................................................................................3
BIOLOGY OF THE CYNIPOID WASPS .............................................................................5
DISTRIBUTION, CLASSIFICATION AND PHYLOGENY ......................................................7
IMMATURE STAGES .......................................................................................................12
GLANDS ASSOCIATED WITH THE FEMALE REPRODUCTIVE ORGANS .....................14

CHOICE OF TECHNIQUES AND TAXA .......................................................................16
TECHNIQUES FOR STUDYING MORPHOLOGY ...............................................................16
ESTIMATES OF PHYLOGENETIC RELATIONSHIPS .........................................................17

FINDINGS AND IMPLICATIONS ....................................................................................18
EVOLUTION AND MORPHOLOGY OF THE CYNIPOID EGG .......................................18
COMPARATIVE ANATOMY OF THE TERMINAL-INSTAR LARVA ..................................21
THE PRE-PUPAL AND PUPAL PHASES .........................................................................23
THE VENOM APPARATUS .............................................................................................24
PHYLOGENETIC ANALYSES ........................................................................................25

NORSK SAMMENDRAG ...............................................................................................31

ACKNOWLEDGEMENTS ...............................................................................................35

REFERENCES ..............................................................................................................38
Introduction

Did you ever encounter structures resembling pieces of cotton between the leaves of an oak, or bright red moss-like structures on wild rose? Then you know what a gall might look like. A gall is often defined as an abnormal growth in plant tissue caused by a foreign organism. In these two cases they are produced by the oak gall wasp Andricus quercusramuli (Linnaeus) and the rose gall wasp Diplolepis rosae (Linnaeus) respectively. The shape of the gall is species-specific and may vary immensely even between closely related species.

Figure 1 Galls and gall wasps on rose (A-C) and oak (D-G). A- rose gall of (B) Diplolepis rosae, C- rose gall inquiline Periclistus brandtii (Ratzeburg), D- gall of parthenogentic generation of Andricus foecundatrix (Hartig), E- gall and adult gall wasp of parthenogentic generation of Cynips quercusfolii Linnaeus, F- galls of parthenogentic generation of Neuroterus quercusbaccarum (Linnaeus), G- galls of parthenogentic generation of Cynips longiventris Hartig. Drawings from Caspari and Grossman (Kronen Verlag).

Several groups of insects, as well as mites, nematodes, fungi and bacteria are able to induce galls (Schick and Dahlsten, 2003). This thesis is based on work on one of the families of gall-inducing insects, the gallwasps (Cynipidae) and their insect-parasitic relatives in the superfamily Cynipoidea.
A gall is formed around the developing larva some time after the adult female has laid the egg on a carefully selected part of a particular plant and development to an adult insect is completed within the gall.

In the Middle Ages the formerly mentioned gall of the rose gall wasp, *Diplolepis rosae*, also called the bedeguar gall, was held in high repute in medicine for its astringency, the ability to contract soft body tissues to control blood flow or fluid secretion and its supposed power of inducing sleep if placed under the pillow at night. Derivates of other cynipid galls were used for ink production (Kinsey, 1935) and as photographic developers (Schick and Dahlsten, 2003). Today the gall wasps are mostly seen as pests, although there are very few species that seriously damage economically important plants (Kinsey, 1935). One of them is the chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu, which induces galls on young shoots of chestnuts (*Castanea* spp.) in Japan and North America (Stone *et al.*, 2002) and the European oak gall wasp *Plagiotrochus amenti* Kieffer, which was introduced into North America with the cork oak, *Quercus suber* (Kinsey, 1935; known by him under the name *P. suberi*, a junior synonym of *P. amenti*). Among the parasitoid species closely related to the gall wasps, there are some economically important groups that attack and eventually kill insect pests of crops. Examples include *Trybliographa rapae* (Westwood) (Eucoilinae, Figitidae), a parasitoid on larvae of anthomyiid flies of the genus *Hylemya* (Diptera) attacking cruciferous crops (Wishart and Monteith, 1954) and *Ibalia* spp. (Ibaliidae), parasitoids on woodboring horntail larvae (Hymenoptera: Siricidae) that make extensive damage on pine, *Pinus* spp. *Ibalia* has been used successfully in management programmes of *Sirex* in Australia and New Zealand (see Liu and Nordlander, 1994, for a review). Thus, potentially important biological control agents can be found among the parasitic taxa belonging to the cynipoid wasps.

Comparative morphological studies often form the basis for systematic, taxonomic and phylogenetic work. In this thesis, I have compared character systems like the egg and eggshell, larval mandibles, larval head sclerites and the venom apparatus of the adult female over a number of cynipoid taxa representing the major evolutionary lineages were used both to test existing estimates of phylogenetic relationships and to reconstruct evolutionary changes associated with the shifts between parasitoids, gall inducers, and inquilines. The choice of taxa for the labour-intensive studies of these character systems was guided by previous phylogenetic studies of cynipoid wasps. The guiding principles were to select sets of species that maximized the chances of finding support or conflict with existing estimates of phylogenetic relationships, and were likely to allow accurate reconstruction of the morphological changes associated with the shifts in life mode.

Molecular techniques have become increasingly popular in systematic studies, and recently also in phylogenetic analyses of cynipoid wasps. This does not mean that morphological studies are becoming obsolete. On the
contrary, molecular results help increase the precision with which ancestral states and past character change in morphology can be reconstructed. And morphological changes are still uniquely informative about past evolutionary events and how they affected the design of organisms. Thus, maintaining and developing techniques to gain information about an organism’s morphology is likely to become a critical asset in the near future, as large numbers of biologists are now turning their focus to molecular and genetic problems.

The work included in this thesis was aimed at studying the evolution of cynipoid anatomy, particularly the changes associated with major shifts in life history. The presence of three distinctly different life modes in the superfamily Cynipoidea makes them particularly well suited for this type of study. The ancestral life mode is insect-parasitic: gall inducers probably evolved from parasitoids developing on hosts inside plants, and the gall inducers later gave rise to the inquilines (Ronquist, 1994, 1995a, 1999). The external morphology of cynipoid adults is relatively well-studied (Ronquist and Nordlander, 1989; Ronquist, 1994, 1995a, 1999; Liljeblad and Ronquist, 1998), making it possible to identify many of the changes in these character systems associated with the origin of the gall inducers and the inquilines. However, many of the critical morphological changes are likely to have occurred in the immature stages and in the internal reproductive organs of the adult females. The morphology and ultrastructure of immature stages, as well as internal anatomy, are virtually unexplored sources of data in the Cynipoidea. Thus, comparative examinations of eggs and larvae representing most of the major lineages of Cynipoidea were performed, as well as examinations of the anatomy of the venom apparatus of the adult female. As the mechanism of gall-induction in the phytophagous true gall wasps is still an unsolved enigma, particular attention was paid to systems and structures potentially involved in this process.

Biology of the cynipoid wasps

Females of the parasitoid members of the Cynipoidea lay their eggs in early-instar larvae of the insect orders Hymenoptera, Coleoptera, Neuroptera and Lepidoptera. As far as is known, all cynipoid parasitoids are koinobionts, meaning that the development of the host continues at least for a while after it has been attacked (Askew and Shaw, 1986; Quicke, 1997), in some cases after an initial short phase of paralysis following oviposition (James, 1928). The parasitoid larva feeds on its host larva and there is normally only one parasitoid larva present within the same host specimen, although several may occur. Normally the parasitoid is in one of its late larval instars when it exits its host and eats the remains of the latter from the outside. It does not form a cocoon before pupating (Chrystal, 1930; Haviland, 1921; Huzimatu, 1940; Jenni, 1951; Wishart and Monteith, 1954).
The gall inducing species spend most of their life cycle inside the gall. The adult female selects a particular part of the host plant, and lays one or more eggs on or in the tissue. Galls have been found in roots, stems leaves and fruits of plants, but a particular gall wasp species normally utilizes only one part of the plant. Some gall wasps, notably the species associated with oaks, have alternating sexual and parthenogenetic generations that may develop on different plant parts. For instance, the parthenogenetic generation of *Biorhiza pallida* (Olivier) induces galls on roots of oaks, whereas the sexual generation makes large potato-like galls among the leaves on young shoots of oaks. A larva ecloses from the egg, and a gall develops around the larva, which will then feed on the nutritious plant cells lining the interior of the gall chamber. The gall wasp stays in the gall during its larval and pupal period, and a peculiar feature is that the larva only defecates once since its midgut and hindgut are disconnected during most of the larval development, and are joined only just prior to pupation (Roth, 1949). The gall wasp emerges from the pupa as an adult, and escapes the gall through a perfectly circular exit hole made by its strong mandibles.

The mechanisms of gall induction and development are still unidentified despite large efforts from researchers from the end of the 19th century and onwards. Neither is it fully understood which gall wasp developmental stages are responsible for the process, although the general view is that the larva plays a major role. Modifications in the host plant tissue around the egg have, however, been observed (Beyerinck, 1883; Magnus, 1914, personal unpublished observation). A few large comparative studies of cynipid larvae have investigated how the larva is involved in the gall induction (Rössig, 1904; Roth, 1949). Both arrived at the conclusions that gall induction probably arises as a response to chemical cues transferred to the host plant via larval secretions. The potential origin of the larval secretions was explored by examining and comparing internal organs and glands. The malpighian tubules, which normally function as excretion organs in insects, were found to be particularly large in gall wasps and appear to be at the peak of their production during the time of gall development. Therefore, they were hypothesized to be involved in gall induction.

Another hypothesis suggests that symbiotic viruses injected by the ovipositing female could facilitate gall induction (Cornell, 1983). Virus-like particles suppressing the cellular immunity of the host larva have been reported in the parasitoid cynipoid *Leptopilina heterotoma* (Thomson) (Figitidae) (Rizki and Rizki, 1990). So far, similar virus-like particles have not been reported from gall wasps. However, Ronquist and Liljeblad (2001) reported remarkable patterns of parallel independent radiation onto the same set of unrelated host plants in unrelated lineages of gall-inducing cynipids. These patterns could possibly be explained by horizontal transfer of gall-inducing symbionts but other explanations are also possible.
More recently, researchers have identified proteins in the nutritive tissues of the gall chamber. At least one of the proteins detected is otherwise only involved in seed or pollen development, suggesting that the basis for gall induction in cynipids may be the ability to switch on part of a “seed-development-pathway” (Schönrogge et al, 2000, Harper et al, in press). Establishing suitable bioassays for plant tissue responses in galling systems, distinguishing the galller’s signal molecules from similar molecules present in the plant naturally and separating the cascade of plant responses to a galling organism from the normal developmental pathways in the plant are some of the major challenges in identifying the signal molecules involved in the gall induction process (Stone and Schönrogge, 2003).

A special biological feature can be found among the gall inducing cynipoids. The oak and maple gall wasps (Cynipini and Pediaspidini) show alteration between sexual and parthenogenetic generations (heterogy), and there is normally one parthenogenetic and one sexual generation per year. The galls of the two generations may be dissimilar in appearance and be situated on different parts of the host plant or more rarely on two different species of host plants (heteroecy). Other groups, like some rose gallers in the genus *Diplolepis*, have a mainly parthenogenetic life cycle in which unfertilized eggs give rise to all-female broods (thelytoky) (Quicke, 1997). Nevertheless, males are occasionally encountered in these species. Hymenopteran wasps otherwise typically display arrhenotoky, meaning that unfertilized eggs give rise to haploid (with one set of chromosomes) males and fertilized eggs develop into diploid (with two sets of chromosomes) females. Most of the herb gallers (Aylacini) have a life cycle with one yearly arrhenotokous sexual generation only.

The inquilines represent another type of life mode among the cynipoid wasps. They are phytophagous gall guests, meaning that the adult female oviposit in already existing galls, made primarily by cynipid gall wasps. Although phytophagous, they often kill the larva of the gall inducer during oviposition (Shorthouse, 1980). Several inquiline species retain the ability to modify the galls to suit their own needs (Shorthouse, 1980). It was, for instance, demonstrated that the inquiline *Periclistus pirata* (Osten Sacken) formed several smaller chambers within rose galls induced by *Diplolepis polita* (Ashmead) (Shorthouse, 1980). Except for their inability to initiate galls, their development does not seem to differ much from that of the true gall inducers.

Distribution, classification and phylogeny

The subject of the investigations reported in this thesis is the superfamily Cynipoidea, one of the major lineages of parasitic Hymenoptera with about 3,000 described species distributed worldwide. One reason that makes it
difficult to estimate the species number is the large areas of high potential species richness that remain largely unexplored. Also, for many species of the oak gallers (Cynipini) with alternating generations, the sexual and parthenogenetic generations have yet to be linked, meaning that the current species number is too high (Stone et al., 2002). The Cynipoidea have been shown to form a monophyletic group based on morphological evidence, the most apparent being the laterally compressed metasoma and the displacement of the Media wing vein anteriorly as well as the absence of the antennal radicle (Ronquist, 1995a). Table 1 shows the current classification and biology of the cynipoid groups.

Table 1: A list of current extant families of Cynipoidea indicating the number of genera, species, biology and host relationships. Table based largely on Ronquist (1999) and Liljeblad, (2002) with additions from Diakontshuk (2001), Ronquist and Nieves-Aldrey (2001), Liu (2002), Pujade-Villar et al. (2002), Allemand et al. (2002); Nieves-Aldrey and Parra (2003) and Ros-Farré et al., 2003.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Genera</th>
<th>Species</th>
<th>Biology</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austrocynipidae</td>
<td>1</td>
<td>1</td>
<td>Parasitoids</td>
<td>Lepidoptera: Oecophoridae</td>
</tr>
<tr>
<td>Ibaliiidae</td>
<td>3</td>
<td>20</td>
<td>Parasitoids</td>
<td>Hymenoptera: Siricidae</td>
</tr>
<tr>
<td>Liopteridae</td>
<td>10</td>
<td>175</td>
<td>Parasitoids</td>
<td>Coleoptera: Cerambycidae, Buprestidae</td>
</tr>
<tr>
<td>Cynipidae:</td>
<td>76</td>
<td>1335</td>
<td>Gall inducers and inquilines</td>
<td>Oak (Quercus spp.), rose (Rosa spp.), other herb families (table 3)</td>
</tr>
<tr>
<td>Figitidae:</td>
<td>132</td>
<td>1427</td>
<td>Parasitoids</td>
<td>Diptera, Neuroptera, Hymenoptera</td>
</tr>
<tr>
<td>Total</td>
<td>222</td>
<td>2958</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The cynipoid wasps can be divided into two groups, the macrocynipoids, consisting of generally larger insects belonging to the families Austrocynipidae, Ibaliiidae and Liopteridae, and microcynipoids, constituting the two remaining families Cynipidae and Figitidae, which typically are smaller wasps (Ronquist, 1995a). Figure 2 shows a recent phylogenetic estimate of the relationships between the families. The macrocynipoids are all parasitoids of wood- or cone-boring insect larvae. The Austrocynipidae, consisting of a single species, Austrocynips mirabilis Riek, are only known from very few specimens collected in Australia (Riek, 1971). Specimens were reared from cone-boring Lepidoptera larvae of the family Oecophoridae.

The Ibaliiidae include three genera of which at least one (Ibalia) comprises parasitoids of siricid larvae (Hymenoptera) boring in hardwoods or conifers (Liu and Nordlander, 1994). Ibalia and Heteribalia have a mainly Holarctic distribution, whereas the monotypic genus Eileenella is known from New Guinea (Ronquist and Nordlander, 1989; Ronquist, 1995a,b).

The third macrocynipoid family, the Liopteridae, occur in most geographical regions (Ronquist 1995b). Their biology is largely unknown, but some species belonging to the subfamily Mayrellinae are apparently parasitoids of beetles (Buprestidae, Cerambycidae, Curculionidae) boring in
twigs and stems of deciduous trees and bushes (Ronquist, 1995b). In phylogenetic estimates (Ronquist, 1995a) the macrocynipoid families fall into three lineages forming a paraphyletic grade basal to the monophyletic microcynipoids, the Cynipidae and Figitidae (Fig. 2).

Figure 2 Phylogenetic relationships between the cynipoid families (based on Ronquist, 1999). The shadings of the branches indicate life mode, whereas the size of the triangles represent the species diversity of the families.

The microcynipoids consist of parasitoids and gall inhabitants and the large majority of the cynipoid species belong here. At least two major transitions have occurred within this group, firstly from a parasitoid to a gall inducing life mode, and secondarily from gall inducing to inquilinism (Fig. 2; Ronquist, 1994, 1995a, 1999). The Figitidae are as far as we know parasitoids in larvae of insects belonging to the orders Hymenoptera, Diptera and Neuroptera. Distinct synapomorphic morphological features, such as the Rs + M vein of the wing issuing from the posterior end of the basal vein and the distinct point of weakness on the ninth tergum of the female, point to the monophyly of the Figitidae (Ronquist, 1995a). Table 2 gives an overview of figitid classification, distribution, adult synapomorphies and host relationships.

A cladistic analysis of higher-level figitid relationships was presented by Ronquist (1999) indicating a close relationship between the “core figitids” consisting of Emargininae, Aspicerinae, Eucoilinae, Figitinae and Pycnostigminae, which are all most likely parasitoids of Diptera. Lower-level cladistic analyses have been performed for Anacharitinae and
Aspicerinae (Ros-Farré et al., 2000) and Eucoilinae (Fontal-Cazalla et al., 2002).

Table 2 A list of current subfamilies of Figitidae (Ronquist, 1999) indicating the approximate number of genera and species, distribution, some diagnostic features and hosts. Taxa within quotation marks are most probably not monophyletic (Ronquist, 1994, 1995a, 1999). Table based largely on Ronquist (1999) with additions from Liu (2002), Pujade-Villar et al. (2002), Allemand et al. (2002), Ros-Farré et al. (2000), Ronquist and Nieves-Aldrey (2001) and Ros-Farré et al. (2003).

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Genera</th>
<th>Species</th>
<th>Distribution</th>
<th>Diagnostic features</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parnipinae</td>
<td>1</td>
<td>1</td>
<td>Mediterranean</td>
<td>Dull mesoscutum, lack of horizontal mesopleural furrow</td>
<td>Hymenoptera: Cynipidae</td>
</tr>
<tr>
<td>Thrasorinae</td>
<td>5</td>
<td>11</td>
<td>Australia, N. and S. America</td>
<td>Swollen metacoxa</td>
<td>Reared from cynipoid and chalcidoid galls</td>
</tr>
<tr>
<td>Charipinae</td>
<td>7</td>
<td>139</td>
<td>Worldwide</td>
<td>Scutellum without sculpture</td>
<td>Hymenoptera: Braconidae, Chalcidoidea</td>
</tr>
<tr>
<td>Anacharitinae</td>
<td>8</td>
<td>74</td>
<td>Worldwide</td>
<td>Anterior pronotal plate, elongate petiole derived from pet. neck</td>
<td>Neuroptera: Chrysopidae</td>
</tr>
<tr>
<td>‘Figitinae’</td>
<td>13</td>
<td>138</td>
<td>Worldwide</td>
<td>Lack characteristics of the other subfamilies</td>
<td>Diptera: Cyclorrhapha</td>
</tr>
<tr>
<td>Aspicerinae</td>
<td>8</td>
<td>99</td>
<td>Worldwide</td>
<td>Facial depression, unique pronotal plate</td>
<td>Diptera: Syrphidae, Chamaemyiidae</td>
</tr>
<tr>
<td>Emargininae</td>
<td>5</td>
<td>15</td>
<td>Australia, Japan, SE Asia, Africa, S. America</td>
<td>Strongly laterally compressed metasoma, bilobed fore wing</td>
<td>Possibly Diptera larvae in ant nests</td>
</tr>
<tr>
<td>Pycnostigma</td>
<td>3</td>
<td>3</td>
<td>Africa, Caucasus</td>
<td>Pseudoperostigma, fused abd. terga 3-5</td>
<td>Unknown, but presumably Diptera</td>
</tr>
<tr>
<td>Eucoilinae</td>
<td>82</td>
<td>947</td>
<td>Worldwide</td>
<td>Elevated scutellar plate</td>
<td>Diptera: Cyclorrhapha</td>
</tr>
</tbody>
</table>

The second group of microcynipoids is the gall wasps (Cynipidae). Their unique phytophagous habit and a number of synapomorphic characters of the adult skeletal morphology strongly suggest that the Cynipidae are a natural group (Liljeblad and Ronquist, 1998; Ronquist, 1999). The Cynipidae currently are classified into six tribes (Nieves-Aldrey, 1994; Ronquist, 1994, 1995a) (Table 3), based on host choice and phylogenetic analyses based on adult morphology (Liljeblad and Ronquist, 1998; Ronquist, 1999). Several studies (Ronquist, 1994; Liljeblad and Ronquist, 1998; Ronquist, 1999) show that the mainly herb-galling Aylacini are not monophyletic. The phylogenetic estimates split the tribe into two or three lineages, of which one probably includes the group that subsequently lost the ability to induce galls, the inquilines (Synergini). The larvae of the species belonging to Synergini or inquilines develop inside galls induced by woody-rosid gallers (attacking woody members of the rosid clade of eudicot angiosperms) of the tribes Diplolepidini and Cynipini, but also in galls of Aylacini gall inducers of the...
genus *Diastrophus*. Their monophyly is well supported in recent phylogenetic estimates based on morphological data (Ronquist, 1994; Liljeblad and Ronquist, 1998; Ronquist, 1999; Paper III), but molecular analyses point to multiple origins of the inquilinous life mode (Nylander *et al,* 2004) in accordance with an older hypothesis that cynipid inquilines form a polyphyletic group (Askew, 1984), even though the molecular data do not support a close relationship between each inquiline clade and its hosts as hypothesized by some champions of the polyphyletic origin hypothesis (Gauld and Bolton, 1988).

Table 3 A list of current tribes of Cynipidae with the approximate number of taxa, distribution, some diagnostic features and hosts. The taxa in quotation marks are probably not monophyletic (Ronquist, 1999). Table based largely on Ronquist (1999) and Liljeblad (2002) with additions from Diakontshuk (2001) and Nieves-Aldrey and Parra (2003).

<table>
<thead>
<tr>
<th>Tribes</th>
<th>Genera</th>
<th>Species</th>
<th>Distribution</th>
<th>Diagnostic feature</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Aylacini' (herb gallers)</td>
<td>22</td>
<td>159</td>
<td>Primarily known from temperate regions of the Northern Hemisphere</td>
<td>Fusion of terga not common, lack of facial sculpture</td>
<td>Asteraceae, Rosaceae, Lamiaeae, Papaveraceae, Apiaceae, Valerianaceae, Smilaceae, Brassicaceae</td>
</tr>
<tr>
<td>Synergini (inquilines)</td>
<td>7</td>
<td>176</td>
<td>Mainly Holartic</td>
<td>Terga 3 and 4 often fused, facial sculpture</td>
<td>Cynipidae/Aylacini, Diplolepidini, Cynipini</td>
</tr>
<tr>
<td>Diplolepidini (rose gallers)</td>
<td>2</td>
<td>58</td>
<td>Holartic</td>
<td>Ploughshare-shaped hypopygium, longitudinal depression on mesopleuron</td>
<td><em>Rosa</em> (Rosaceae)</td>
</tr>
<tr>
<td>Eschatocerini</td>
<td>1</td>
<td>3</td>
<td>South America</td>
<td>Antennal insertion high on face, reduced wing venation</td>
<td><em>Acacia, Prosopis</em> (Fabaceae)</td>
</tr>
<tr>
<td>Pediaspidini</td>
<td>3</td>
<td>3</td>
<td>Nepal, Europe, South America</td>
<td>Dorsal impression of scutellum</td>
<td><em>Acer</em> (Sapindaceae), <em>Nothofagus</em> (Nothofagaceae)</td>
</tr>
<tr>
<td>Cynipini</td>
<td>41</td>
<td>936</td>
<td>Mainly temperate regions of the northern Hemisphere</td>
<td>Petiolar hump</td>
<td>Fagaceae (mostly <em>Quercus</em>)</td>
</tr>
</tbody>
</table>

The remaining cynipid tribes, the Diplolepidini, Eschatocerini, Pediaspidini and Cynipini, constitute the woody-rosid gallers, which presumably is a monophyletic group inducing galls on woody representatives of the eudicot subclass Rosidae (Liljeblad and Ronquist, 1998).

Recent phylogenetic studies based on numerous morphological characters (Liljeblad and Ronquist 1998; Liljeblad, 2002) suggest that either Diplolepidini or Pediaspidini is the sister group of Cynipini and that at least two large lineages (the *Neuroterus* group and the *Cynips* group) within the Cynipini are monophyletic. On the other hand, there is no convincing support for the monophyly of the genus *Andricus* (Liljeblad, 2002), currently comprising a large proportion of the Cynipini species.
Immature stages

The cynipoid egg, larva and pupa have received little attention in the past, except for a few comparative studies of the internal structures of mature larvae of gall inducing species (Rössig, 1904; Roth, 1949), aimed at evaluating the possible origin of gall inducing secretions. Descriptions of the development of a few economically important parasitoids have also been published.

The external egg morphology has previously been studied for about 20 cynipoid species including representatives of the parasitoid families Ibaliidae (Chrystal, 1930; Spradbery, 1970) and Figitidae (Haviland, 1921; James, 1928; Wishart and Monteith, 1954) as well as of the gall inducers and inquilines in the Cynipidae (Frühauf, 1924; Evans, 1965; Diaz, 1972; Bronner, 1985).

The cynipoid egg is typically oval and elongate and carries a peduncle at its anterior end. The peduncle forms a thin stalk that may be several times longer than the egg body, with a terminal expansion at the distal end. The non-pedunculate posterior pole enters the female’s ovipositor first at oviposition (Beyerinck, 1883; Frühauf, 1924; Bronner, 1985) and since the diameter of the ovipositor is much smaller than that of the egg, the contents of the egg body is pushed into the peduncle, thus allowing the egg body to pass through the ovipositor. When the egg body appears from the distal end of the ovipositor, the refilling of the egg contents into the egg body starts and finally the peduncle arrives and the oviposition is completed (Frühauf, 1924; Bronner, 1985).

The surface of the egg is smooth and apparently devoid of specialized regions resembling aeropyles and respiratory appendages like those seen in eggs of Diptera (Margaritis, 1985; Kambysellis, 1993), Ephemeroptera (Gaino and Mazzini, 1988) and Hemiptera (Hinton, 1961). The micropyle or sperm entrance does not form an easily detectable structure like in eggs of some insect orders (Margaritis, 1985; Sahlén, 1994). The number of eggs in each adult female is high, at least in the woody-rosid gallers (Cameron, 1889; Frühauf, 1924; Schröder, 1967; Paper I), and one female often carries more than 1000 eggs. Some insects are pro-ovigenic, meaning that the female emerges with all eggs mature, as opposed to synovigenic insects, which continue to mature eggs after emergence (Quicke, 1997). The large number of eggs in many gall wasps indicates that they are pro-ovigenic, like most koinobiont parasitoids (Quicke, 1997).

The cynipoid eggshell ultrastructure has not been studied prior to the present investigation (Paper I), but eggs of several other hymenopteran taxa have been examined using scanning and transmission electron microscopes. The insect eggshell typically has two main layers, the inner vitelline envelope, situated adjacent to the oocyte (the eggcell), and the outer chorion, which is further divided into an endochorion and an exochorion (Margaritis,
1985). The chorion is typically anhydropic, meaning that the egg contains a large enough egg yolk to complete the embryonic development. But especially in endoparasitic taxa the chorion is often hydropic, implying that it is able to absorb nourishment directly from its host fluids as the egg contains insufficient yolk to feed the embryo throughout the entire egg stage (Flanders, 1942; Quicke, 1997). Both types of chorions appear to be present in Cynipoidea (Paper I, II), the anhydropic in the phytophagous taxa and the hydropic in at least *Ibalia leucospoides* (Hochenwarth) (Ibaliidae) (Chrystal, 1930) and *Leptopilina boulardi* Barbotin, Carton and Kelner-Pillault (Eucoilinae) (LeRalec, 1991 referred to by Quicke, 1997) but probably throughout the cynipoid parasitoids.

Major differences have been noted in the larval development between the parasitic and the gall inhabiting taxa of Cynipoidea. The parasitoid larva experiences more or less dramatic changes in morphology between the first instars, a feature termed hypermetamorphosis, whereas the gall inhabiting larva essentially keeps the same shape during its entire larval development. In the known ibaliid larvae, the primary instar is characterized by a short cauda and small, paired appendages on body segments 1-12 (Chrystal, 1930; Spradbery, 1970), a larval type called polypodeiform (James, 1928). In the second to fourth larval instars all appendages are lost, except the short cauda, which does, however, gradually decrease in size towards the terminal instar. In most figitid larvae examined to date, the first (Eucoilinae, Figitinae) or second (Charipininae) instar has three pairs of thoracal processes as well as a long caudal appendage (Keilin and Pluvinel, 1913; Haviland, 1921; James, 1928; Jenni, 1951; Huzimatu, 1940; Wishart and Monteith, 1954). This type of larva is called eucoiliform because it was first observed in the eucoline *Trybliographa keilini* (Kieffer) (as *Eucoila*, Keilin and Pluvinel, 1913). The eucoiliform larva is not reported in *Anacharis* sp. (Anacharitinae). Only a larva with dorsal paired, short appendages on most body segments, as well as a short cauda has been illustrated for this subfamily (Miller and Lambdin, 1985; Fergusson, 1986). A similar polypodeiform larva (but with ventrolateral appendages) has been found to occur in the second instar of *Kleidotoma* (Eucoilinae) and *Figites* (Figitinae) (James, 1928). All appendages disappear gradually during the subsequent larval stages and the terminal instar for all known cynipoids is the ventrally curved and apodous hymenopteriform larva, so called because it is the larva commonly found in Hymenoptera (Clausen, 1940). The cuticle is white, smooth and devoid of setae except for a few scattered ones on the anterior face of the head. The number of instars is reported to be four in Ibaliidae (Chrystal, 1930; Spradbery, 1970) and five in some figitids and cynipids (Huzimatu, 1940; Evans, 1965).

Two distinct prepupal phases were described for the ichneumonid *Exenterus abruptorius* Thunberg, namely the eonymphal and the pronymphal stages (Morris, 1937). The eonymphal stage does not differ
much from the terminal instar larva, except that some lobes and swellings disappear and the activity is lost. In the pronymphal stage, on the other hand, the imaginal eyes are becoming visible as well as a distinct constriction in the thoracic region. The prepupal phases are reported for a number of cynipoid species belonging to Ibalidae (Chrystal, 1930), Figitidae (Simmonds, 1952; Ovruski, 1994) and Cynipidae (Shorthouse and Leggo, 2002; Diaz, 1972).

The pupa is exarate, meaning that the appendages are free from the body and not protected inside the pupa, although a thin membrane may envelope them (Beyerinck, 1883; Cameron, 1889; Riedel, 1910). During its development the colour changes gradually from white to dark brown or black, starting with the eyes and the anterior part of the body (Diaz, 1972).

Glands associated with the female reproductive structures

Morphological differences in the reproductive structures of parasitoids and gall inhabitants could be explained as adaptations to completely different types of host organism. The reproductive structures in Cynipoidea typically include the ovipositor apparatus, a pair of ovaries containing the eggs, two oviducts merging into a common oviduct, a spermatheca, possibly with a little gland associated to it, a venom apparatus and one or two pairs of accessory glands issuing from the common oviduct (Frühauf, 1924).

The venom apparatus consists of a short venom duct, which opens to the ovipositor, a reservoir and a single venom gland. Frühauf (1924) noted that while the reservoir and venom gland take up most of the space inside the female metasoma of the parthenogenetic generation of the oak galller Biorhiza pallida, the venom apparatus is almost totally reduced in the rose galller Diplolepis rosae. In addition to these two cynipids, the venom apparatus is described in part or in full for the ibaliid Ibalia leucospoides (Chrystal, 1930) and the figitids Leptopilina heterotoma (Eucoilinae) (Rizki and Rizki, 1984) and Figites anthomyiarum Bouché (Figitinae) (James, 1928).

The venom secretion occurs in the venom gland and in certain groups also in parts of the reservoir, which is mainly, however, a storage container for the venom. Lubrication or coating of the egg to facilitate its passage through the egg canal during oviposition has been suggested as a function of the venom apparatus (Frühauf, 1924; Robertson, 1968).

In the parasitoid species, some interesting functions have evolved for the venom in different taxa, the most apparent being to paralyze the host for a shorter or longer time. This paralyzing function has been described in the figitid Figites anthomyiarum (Figitinae) (James, 1928) among others. The
venom of the cynipoid *Leptopilina heterotoma* (Eucoilinae) was found to contain virus-like particles, which were shown to suppress the cellular immune response of its drosophilid host (Rizki and Rizki, 1984, 1990). In other Hymenoptera groups, like wasps, bees, and ants, the venom is used for defence and it may also be used to kill prey fed to the brood, for instance in the paper wasps (*Polistes* sp.) (Bernard, 1951).
Techniques and taxa

Taxa were selected to be informative about structural changes associated with the transition from parasitoid to gall inducers, and from gall inducers to inquilines (Paper I, IV). Because of the species-richness in Cynipoidea and the relative time consuming dissections (Paper III, IV), I have concentrated on examining relatively few species. The choices were based on results of recent phylogenetic estimates (Ronquist, 1995a, 1999; Liljeblad and Ronquist, 1998; Nieves-Aldrey, 2001), showing among other things that the ancestral gall inducer probably induced galls on Papaveraceae or possibly Lamiaceae (Ronquist and Liljeblad, 2001). Thus, both extant gall inducers on Papaveraceae (*Barbotinia*) and parasitoids on gall inducers on Papaveraceae (*Parnips*) apparently retaining many morphological features of the original gall inducers and their parasitic ancestors were included (Ronquist and Nieves-Aldrey, 2001). Differences between these species were expected to be particularly indicative of changes associated with the origin of gall inducers from parasitoid ancestors.

Representatives from one of the three macrocynipoid families (Ibaliidae), six of the nine figitid subfamilies (Parnipinae, Charipinae, Anacharitinae, Figitinae, Aspicerinae and Eucoilinae) and five of the six cynipid tribes (Aylacini, Synergini, Diplolepidini, Pediaspidini and Cynipini) were examined.

In Paper III, the terminal instar larva was examined, both because of availability and because the comparison between parasitoid taxa and gall inhabiting taxa is preferrably done for this instar, since hypermetamorphosis occurs in the early instars of the parasitoid taxa.

Techniques for studying morphology

All specimens (Paper I, III, IV) were examined in the stereo microscope, under which eggs (Paper I), larval mandibles (Paper III) and venom apparatuses (Paper IV) were dissected.

Slide preparations were made of eggs and venom apparatuses by mounting specimens in Euparal, and compound microscopes were used for examination. Drawings of the eggs (Fig. 3, Paper I) were made with the aid of a drawing tube fitted to a compound microscope. Digital images were
taken during dissections and of the microscope slides of the venom apparatuses as aids for identifying differing characters among the examined taxa and as guides for correct identification of orientation of the gland system inside the metasoma. The drawings of the venom apparatuses (Fig. 1-3) were prepared from microscope slides using a graphic tablet and digital images imported into Photoshop 7.0.

Material used in paper I and IV (Cynipoid collection, EBC, Uppsala University) was preserved in 70 % ethanol, which is suitable for studying external morphology, but tends to dry out softer tissue. One way to avoid dehydration of the tissues is to fix the material in Karnovsky’s solution, which is a mixture of glutaraldehyde and paraformaldehyde. Karnovsky’s solution binds to proteins and other organic molecules and stabilizes cell structures. This solution is buffered and osmotically balanced to minimize shrinkage, swelling, and other damage. Ideally, fresh material should be used for dissections of the internal soft tissue, but only a small part of the dissections done here were performed on fresh material. Post-fixation with osmium tetroxide was done on all electron microscope preparations, except for those in paper III. The SEM preparations were coated with gold and the TEM specimens were embedded in epoxy resin before sectioning.

In paper III, the larvae were examined at low vacuum in the scanning electron microscope, with no prior preparation other than preservation in ethanol (preferably as close to 100% as possible). This technique was superior to the traditional preparations, and gave more detailed images of the larvae and mouthparts.

Estimates of phylogenetic relationships

Morphological characters of the eggs (Paper I) and venom apparatus (Paper IV) were mapped onto recent phylogenetic estimates of Cynipoidea (Ronquist, 1999; Liljeblad, 2002) in order to see how well the evolution of these characters fit the evolution of other known morphological characters of Cynipoidea, and to allow accurate reconstruction of ancestral states. In paper III, Paup* 4.0b (Swofford, 1998) was used for un-weighted standard parsimony and implied weight parsimony analyses of larval morphological data. It has long been argued that differential weighting of characters is justified in phylogenetic analyses (e.g. Farris, 1983) on grounds that all characters are not equally informative about phylogeny. Implied weighting has been put forward as a possible way of implementing differential weights (e.g. Goloboff, 1993) and this technique is used here. MacClade 4.02 (Maddison and Maddison, 2001) was used for tracing ancestral character states and character changes.
Findings and implications

Evolution and morphology of the cynipoid egg

The external morphology of eggs of twenty-one cynipoids including representatives of all three life modes was described (Paper I). The egg body is mostly oval to elongate and most often a thin peduncle is present at the anterior pole of the egg. The peduncle varies in length and was found to be reduced or absent in some of the figitid species (Fig. 3, character 1:1). The eggs of the parasitoid taxa tend to be relatively more slender, narrower and more gradually tapering towards the peduncle (Fig. 3, character 2:0) than the cynipid eggs which are distinctly divided into a stout egg body and a thin peduncle (Fig. 3, character 2:1). The relatively smaller size of the parasitoid egg may be related to at least two factors. Firstly, the slender parasitoid egg may shorten the oviposition time considerably, which is probably important for an insect laying eggs in a host that may otherwise escape. Secondly, the parasitoid egg often does not have to contain much yolk because it is able to absorb nutrients directly from its host during the egg stage. The gall wasp egg, on the other hand must contain sufficient yolk for the entire egg stage.

The surface of the egg body was more or less smooth, with no sculpture or specialized regions. We were not able to detect the entrance of the sperm into the egg, the micropyle, which can be seen at the anterior pole of some Hymenoptera eggs, being exposed as in the chalcidoid Nasonia vitripennis (Walker) (King, 1962) or situated inside a fold of the chorion and visible as a slight bulge on the egg surface like in the ichneumonid Venturia canescens (Gravenhorst) (as Nemeritis, Rotheram, 1972). The micropyle has been reported to be situated on the peduncle in cynipoid eggs of Trybliographa rapae (Eucoilinae), (Wishart and Monteith, 1954), and Cynips (Leuckart, 1855). It is, however, difficult to explain why the sperm entrance would sit at the point of the egg, from which the sperm would have the longest and most narrow path to the oocyte.

The ultrastructure of the eggshell was studied for the first time in cynipoid wasps for a sample of six species, including two parasitoid species representing Ibaliidae and Figitidae, as well as three cynipids, representing one inquiline (Synergini), one herb galling (Aylacini) and one rose galling species (Dipolepidini). The examination of the eggshell showed distinct
structural differences between the parasitoids and the gall inducers/inquiline species, as well as difference in thickness of the eggshell. The exochorion was found to contain crystalline elements, which appeared as electron translucent rods, whereas the endochorion appeared as a thick homogenous layer without the sublayers seen in, for instance, species of Odonata and Diptera (Sahlén, 1994a,b, 1995a,b).

We also compared eggshells of ovarian and post-oviposition eggs in order to determine whether irreversible structural changes happen in the eggshell during oviposition. The only difference detected was the presence of vesicles probably due to stretching effects when the egg passes through the narrow egg channel of the ovipositor.

A striking feature was found in the outer layer of the eggshell of the rose galler Diplolepis. In a narrow region at the non-pedunculate pole of the egg, which is the part of the egg that is embedded in the host plant tissue, the eggshell is less dense and apparently porous, perhaps facilitating chemical communication between the plant tissue and the embryo. In the other examined species, however, we did not find any structures that would allow the embryo inside the egg to play a major role in gall induction mediated by chemical secretions through the egg shell. However, modifications in the plant tissue may occur late in the egg stage of the gall inducer (Beyerinck, 1883; Magnus, 1914; personal unpublished data), at which time changes in eggshell structure might have occurred.
Figure 3 Phylogenetic estimate of Cynipoidea (Ronquist, 1999) with eggs of twenty-one species. Branch colors indicate life mode, and character 1 indicate presence (0) or absence/reduction (1) of peduncle, whereas character 2 indicates egg type (see text).
Comparative anatomy of the terminal instar larva

We compared the terminal instar larva of five parasitoids, twenty-one gall inducers and four inquilines using scanning electron microscopy. Although most larvae are of the ventrally curved hymenopteriform shape (Clausen, 1940) that we expected to find, distinct modifications were detected in some groups.

A group of species of herb gallers belonging to the genera *Phanacis*, *Timaspis* and *Iraella*, inducing galls in stems of their host plants, exhibited larvae that were elongate, straight and almost rectangular in shape (see figure 2E in paper III). Since this type of larva is not shared by all species inducing this particular type of gall, we suggest that the larva type is potentially a synapomorphic trait for this group and not a result of convergent evolution of unrelated taxa as an adaptation to an elongate gall chamber. This provides further support for the results of a phylogenetic estimate based on morphological characters of the adults, in which *Phanacis* and *Timaspis* form a monophyletic group, also sharing affinities with *Iraella* (Liljeblad and Ronquist, 1998).

Furthermore, we found that in the larvae of four of the nine examined species belonging to the oak galler (Cynipini), the three first body segments are enlarged (Fig. 4D). The significance of this feature is not clear, but it was noted that the enlargement became more pronounced prior to the pre-pupal phase and perhaps is a result of the imaginal disc developing into legs and wings.

![Figure 4 SEM micrographs of the larva, anterior view of the head and left mandible of the parasitoid *Leptopilina boulardi* (ABC) the oak galler *Callirhitis glandium* (Giraud) (DEF) and the oak-galler inquiline *Synergus incrassatus* Hartig (GHI). (aa = antennal areas, m = mandible, L = labium, so = salivary orifice) (micrographs by J. L. Nieves-Aldrey and Hege Vårdal).](image)

We found no pronounced differences in larval shape between parasitoid and gall inducing or inquiline taxa. However, among the parasitoid taxa, there
was a tendency for the larvae of the genus *Ibalia*, parasitoids of wood-boring siricids, to be more elongate than the ventrally curved figitid larvae (*Alloxysta, Leptopilina, Parnips*). This is probably due to the different larval habitats, which are cylindrical wood tunnels for the *Ibalia* larvae and more sphaerical structures (galls, aphid mummies, puparia) for the figitid larvae.

**Head and mandibular structure**

The scanning electron microscopy revealed a surprising variation in head features that were not easily visible in the stereo microscope (Fig. 4E). Especially the structures associated with the mouth offered a wide variety of characters potentially informative about phylogenetic relationships. One of the most obvious characters is perhaps the pair of large and conspicuous antennal areas in all oak gallers (Cynipini) (Fig 4E). The antennal areas are smaller and less prominent in most other taxa, perhaps excepting *Leptopilina* (Fig. 4B), and this feature can be used to separate larvae of oak gallers from inquiline and parasitoid larvae found inside a cynipid gall.

The labium showed a tuberculate pattern in a region surrounding the salivary orifice (cf. figs 4B-E in paper III) in a group of herb gallers (Aylacinii: *Neaylax, Lipothences, Aulacidea* and *Isocolus*). Phylogenetic analyses based on adult morphological characters (Liljeblad and Ronquist, 1998) and molecular data (Nylander *et al*, 2004) show some support for a group including these species. The labium character may be the first good morphological synapomorphy indicating that these species are closely related.

It was necessary to dissect the mandible from the mouthparts of the larva to fully appreciate its shape as several of its features are concealed when it is attached to the larval mouth. When not dissected, it appears that the parasitoids have one strong mandibular tooth, whereas the gall inducers have two, and this character has been suggested as a synapomorphy for the gall inducers (Cynipidae) (Ronquist, 1999). The mandibular tooth pattern proved to be more complex and we found the tooth number to vary from one to four even among the gall inhabitants (Cynipidae) (cf. figs. 5F-P and 6 in Paper III). Another major difference was, however, found between the parasitoids and the gall inhabitants. The former have mandibles with one major acutely pointing tooth and either one (or rarely two) smaller secondary teeth (*Ibalia, Parnips*) or a serrated inner margin of the mandible (*Alloxysta, Leptopilina*) (Fig. 4C). The gall inhabitants, on the other hand, normally have mandibles with two or more large and blunt teeth (Fig. 4F, I). It seems likely that the shape of the mandible is largely determined by its usage. The terminal instar parasitoid larva uses its delicate mandibles for cutting an escape hole in the host larva, which is subsequently eaten. The phytophagous gall inhabitants on the other hand feed on plant cells inside the gall. These cells contain cellulose walls and presumably require the larva to have heavy mandibles with blunt teeth to pierce through them.
The inquilines (Synergini) show several unique modifications of the mandibles. The second of the three mandibular teeth is very broad and occasionally even bilobed (Fig. 4I). In addition a pattern of vertical and sometimes horizontal striations can be seen on the base of the mandibles. Two of the four examined inquilines, namely *Synophrus politus* Hartig and *Periclistus brandii*, inquilines in oak galls and rose galls respectively, have strongly asymmetric mandibles, meaning that while the second tooth of the left mandible is similar to the broad second tooth in the other inquilines, the right second tooth is almost totally reduced. Interestingly, the Aylacini species believed to be closely related to the inquilines, *Diastrophus* and *Xestophanes*, galling *Rubus* and *Potentilla* respectively, also have a broad second tooth and asymmetry in the mandibles, although not as strongly as in *Synophrus* and *Periclistus*.

This study revealed more morphological variation than previously known in the larvae, especially in the head features of the terminal instar, potentially giving indications of phylogenetic relationships within the studied groups. Moreover, a number of observed characters will help us identify larvae found inside a cynipid gall more easily. Aided by these characters we may separate not only cynipoid larvae from larvae of gall inhabitants belonging to other insect groups, but also distinguish between different cynipoid groups.

### The pre-pupal and pupal phases

The prepuetal and pupal stages of the cynipoid wasps were not studied in detail in this thesis, but a short overview of what we know of the pre-pupae and pupae (Fig. 5B) of the oak gallers and their inquilines is given in paper II. When dissecting galls for paper III, we found two different pre-pupal phases for the oak-gallers *Neuroterus numismalis* (Geoffroy) and *Plagiotrochus quercusilicis* (Fabricius) fitting well with descriptions of the eonymphal and pronymphal phases in ichneumonids (Morris, 1937). The first phase was almost identical to the larva, but the segmentation was not well defined and the genital development had started at the ventroposterior segments. In the pronymphal stage (Fig. 5A), the imaginal eyes are visible, and the thoracic region is clearly delimited from the abdominal region by a constriction.
The venom apparatus

I dissected between two and seven adult females of twenty-five cynipoid species to look at their venom apparatus, which can be found in association with the well-developed ovipositor apparatus. The function of the cynipoid venom is not clear, although it is used for paralyzing insect hosts by some of the parasitoid species (James, 1928). In another cynipoid parasitoid, *Leptopilina heterotoma* (Eucoilinae), virus-like particles are produced by the venom apparatus and used to suppress the host immune reaction to the parasitoid egg (Rizki and Rizki, 1984, 1990).

I was able to confirm earlier observations that the venom gland is undivided and that the venom reservoir may be conspicuously large in some gall inducing species. The single venom gland is a rare feature among hymenopteran wasps, found occasionally in some groups of parasitic wasps and aculeates. In the phytophagous symphytan wasps, and several parasitic wasps, the venom gland is subdivided into several branches (Robertson, 1968), whereas in other parasitic wasps as well as aculeates (e. g. bees, solitary wasps, ants) it is commonly divided into two main branches. The single venom gland thus appears to be a good synapomorphic trait of the Cynipoidea. Frühauf (1924) did report that the long venom gland of the oak galler *Biorhiza pallida* may be bifurcated occasionally but this is probably best interpreted as an example of atavism. Absence of the Dufour’s gland, which is a single gland normally associated to the reproductive structures in Hymenoptera, is another possible synapomorphy for the cynipoids, although this needs confirmation. There was no apparent difference between the venom apparatuses of the gall inducers and the inquilines in the examined species, but a few more or less consistent venom apparatus features differ
between the eleven parasitoid species and the fourteen gall inhabiting species (Fig. 6).

Figure 6 Morphology of the venom gland and reservoir in A) the poppy galler parasitoid *Parnips nigripes* (Barbotin), B) the oak galler *Cynips quercus* (Geoffroy) and C) the rose galler inquiline *Periclistus brandii*. The drawings are not to scale, but the lines underneath the drawings indicate half the length of the female metasoma of that species. Note the large size of the venom gland and reservoir in the gall inhabitants.

Firstly, the size of the reservoir, which on average measured less than a third of the length of the female metasoma in the parasitoid species (Fig 6A), compared with about half the length in the gall inhabitants (Fig. 6B) (see also table 2, paper IV). The reservoir size is especially large in the oak gellers, whereas the entire venom apparatus is reduced in size in the rose gellers Diplolepidini and the poppy galler *Barbotinia oraniensis* (Barbotin) and, to a lesser extent, in *Aylax papaveris* (Perris) (also a poppy galler). It is tempting to speculate that the size variation is related to host plant defense or sensitivity, although no evidence for this is provided here. The large venom apparatus in gall inhabiting wasps probably indicates production of substances that are used for manipulation of the host plants in different ways. Gall wasps are sluggish, docile animals and there is no indication that they use their venom apparatus for defence.

**Phylogenetic analyses**

In paper III, a set of thirty-three morphological characters was identified and scored based on observations of terminal instar larval specimens of 5 parasitoids, 21 gall inducers and 4 inquilines. One larval life-history character was added to this dataset. Running a parsimony analysis with equal weights yielded a highly unresolved tree, but the search under implied weights gave a fully resolved tree (Fig. 7A), which is largely in agreement with earlier estimates based on adult morphology (Fig. 8A and B) (Liljeblad and Ronquist, 1998; Ronquist, 1999) and molecular data (Nylander et al, 2004).
The rooting of the Cynipidae (gall inducers and inquilines) clade differs between the larval implied-weight tree and the tree based on adult morphology (Liljeblad and Ronquist, 1998, Fig. 8A). In the adult morphology estimate, the insect parasitoid groups (Figitidae and Ibaliidae) attach to the cynipid tree between a group consisting of the inquilines (Synergini) and related herb gallers (Aylacini) and a clade containing the rest of the gall inducers (Aylacini-Diplolepidini-Pediaspidini-Cynipini) (Fig. 8A). In the larval tree, on the other hand, the cynipid tree is rooted (attached to the insect parasitoids groups) between the rose-gallers/herb gallers/inquilines (Diplolepidini, Aylacini, Synergini) and the maple galler/oak galler (Pediaspidini, Cynipini) clade (Fig. 7). Another important conflict between the larval tree and previous estimates is the placement of the poppy gall wasp parasitoid (Parnips, Figitidae), which did not group together with the other figitids (Alloxysta, Leptopilina) in the larval tree as expected.

The difficulty of rooting the cynipid subtree may possibly be caused by the small number of parasitoid taxa included in the analysis, indicating that further studies including more insect parasitoids are probably needed to correctly resolve the basal cynipid relationships.

According to analyses of the larval data the inquilines form a monophyletic group, indicating that the loss of the ability to induce galls occurred once in the cynipids. This is in accordance with previous studies based on adult morphology (Ronquist, 1994; Liljeblad and Ronquist, 1998) but in conflict with phylogenetic analyses based on adult morphological data combined with molecular data (Nylander et al, 2004), in which the inquilines are not monophyletic. Ronquist (1994) evaluated the possibility that the morphological similarities among the inquilines species are due to convergent evolution and concluded that the adult morphology support for inquiline monophyly was not due to convergent similarities in conflict with true relationships. The chance of strong inquiline convergences in larval morphology appears small, considering that the inquiline and the gall inducing larvae essentially live in the same environment. For these reasons, we should not uncritically accept the molecular signal concerning inquiline origins.

In conclusion, the larval data mostly point to the same relationships as previous studies and add support and several synapomorphies to established groups, despite the few conflicts mentioned above.

26
Figure 7: Strict consensus tree resulting from parsimony analysis under implied weights. The analysis is based on larval morphology (Paper III).
Concluding remarks

The work reported in this thesis identified a number of morphological changes associated with major life-history transitions in cynipoid wasps, particularly the shift from parasitoids to gall inducers. That this particular shift stands out is not unexpected, as the life inside an insect larva is probably quite unlike the life in a gall. Modifications in the egg stage correlated with this transition include a change in shape from a slender egg in parasitoids to stout and broad eggs in gall inhabitants, but also a change from a thin plastic eggshell in the former to a rigid thicker eggshell in the latter. As expected, the shape of the terminal instar larva shows no conspicuous differences between the parasitoid and the gall inhabiting taxa. The features of the head and especially the shape of the mandibles, on the other hand, exhibit great differences between the two groups. The larval mandible of the parasitoid taxa is slender and delicate with pointed teeth and sometimes a serrated inner margin, whereas the gall inducers have larger and blunter mandibular teeth of varying number. The gall wasp larva sometimes has patterns of tubercles on the labium (lower lip) and often conspicuous antennal areas and setae, which are most often absent in the parasitoid larva. The venom apparatus, associated with the internal reproductive organs of the adult female, turned out to be relatively uniform in structure within the Cynipoidea. With a few exceptions within the gall inducing taxa (namely the rose gallers (*Diplolepis*) and the poppy gallers (*Barbotinia, Aylax*)), the entire venom apparatus was enlarged in the gall inhabitants compared with the parasitoid taxa. The parasitoid taxa often have a spiral annulation pattern.
on parts of the venom apparatus, which is most often lacking in the gall inducers.

Morphological differences between gall inducers and inquilines were in most cases inconspicuous. The inquiline egg appears to be very similar to the gall inducer egg both in shape and in eggshell ultrastructure, and the same appears to be true for the gross morphology of the venom apparatus. The inquiline larva did, however, show a number of modifications in head features and mandibular structures relative to the gall-inducing larva. This is perhaps unexpected as the larvae of these two groups practically occupy the same environment. Thus, the modifications in the inquiline larva seem more likely to indicate a common origin of the inquilines rather than convergent adaptation to an environment differing from that of the related gall inducers.

Some of the most important morphological changes are discussed above, but how useful are the new characters in phylogenetic inference? The larval data agree with estimates of relationships based on other types of data. Several of the characters identified in the larval study offer additional putative synapomorphies for already established groups. The pattern of tubercles on the larval labium (lower lip) seems to be the first good synapomorphy for a group of herb gallers (*Neaylax-Aulacidea-Isocolus-Liposthenes*), which was suggested to be closely related by molecular data (Nylander et al., 2004) and partly also by morphological data (Liljeblad and Ronquist, 1998). Two blunt mandibular teeth have previously been suggested as a possible synapomorphy for the Cynipidae, as opposed to one in the parasitoid cynipoids (Ronquist, 1999). It is true that the teeth are blunter and larger in the cynipid larvae and delicate and pointed in the parasitoid larvae, but the number of mandibular teeth varies, especially among the gall inhabitants. Nevertheless, there are probably two or three relatively strong teeth in the cynipid ground plan and only one dominant tooth in the cynipoid parasitoids. Thus, this character is largely confirmed as a cynipid synapomorphy.

So what conclusions do the morphological changes point to regarding the mechanisms of gall initiation? The fact that most gall inducers have a larger venom apparatus than the parasitoids suggests that the venom is involved in the gall initiation process, particularly since the venom is apparently not used for defence in cynipoids. If this is true, however, then gall inducers in the genus *Diplolepis* must have evolved a different initiation mechanism since their venom apparatus is strongly reduced. It is possible that some of the other glands associated with the female reproductive system have taken over the role of the secretion of the venom gland in *Diplolepis*; indeed, some of these other glands are considerably larger in *Diplolepis* than in other cynipoids. But why, then, is the venom apparatus equally large in the inquilines and the gall inducers? Intuitively, one would expect the inquilines to have lost the ability to initiate galls and the anatomical adaptations associated with this capacity. Since the inquilines often modify galls,
however, it is perhaps possible that the function of the venom has changed slightly in the inquilines to redirect gall formation instead of initiating a new gall, and that this explains why the apparatus has remained large. In any case, the link between gall induction and large venum apparatuses appears sufficiently strong to motivate detailed studies of the composition of the cynipid venom.

Judging from morphological observations, it seems unlikely that the egg is important in gall initiation because the gall inhabitant eggshell appears to be quite impermeable, making chemical communication between embryo and plant tissue difficult. If, and how, the young cynipid larva uses chemical secretions to manipulate the host plant to form a gall is still an open question. Older studies (Rössig, 1904; Roth, 1949) suggest that the Malphigian tubules might be involved but the cynipoid phylogeny was not known then so it was impossible to accurately reconstruct ancestral morphological changes associated with the origin of the cynipid gall inducers. Young larvae were not examined in this thesis but they are an obvious priority for future studies of comparative morphology and anatomy of gall wasps. The morphological characters of fully-grown cynipoid larvae described in this thesis proved to be a good source of information for phylogenetic reconstruction but they are probably irrelevant with respect to gall induction since the gall is fully formed well before the larva is mature.

De planteparasittiske medlemmene av Cynipoidea kalles galleveps, fordi de fleste av dem har en spesiell evne til å manipulere planten den lever på (vertsplanten) til å danne en struktur som vepsen kan bo og utvikle seg i. Plantestrukturen kalles galler og finnes i uendelige variasjoner i former og størrelser (se figur 1 på side 3). Galler kan se ut som små epler, bomullsdotter eller poteter mellom løvet på eik, eller store røde, hårete baller på nyperoser, men de kan også være skjulte inni stenglene på ulike urter eller under jorden på plantenes retter. De fleste galleveps lever på eik, mens rundt en femtedel av artene lever på rosebusker (Rosa) eller urter som valmue (Papaver) eller krypmure (Potentilla). Vanligvis kan hver av disse vepsartene bare leve på en bestemt vert, slik at en rosegalleveps (Diplolepis) alltid lever på nyperoser og de insekt-parasittiske vespene er like verts-spesifikk.


En liten gruppe av disse galleboende veps, inkvilinene (Synergini), har mistet evnen til å danne galler selv, men legger sine egg i galler dannet av
ekte galleveps. De insekts-parasittiske medlemmene av Cynipoidea, legger sine egg i larver av andre veps (Hymenoptera), tovinger (Diptera), biller (Coleoptera), sommerfugler (Lepidoptera) eller nettvinger (Neuroptera). Parasittlarven spiser opp sin vertsarve innifra, og når næringstilgangen begyner å ta slutt, biter den hull i vertsarvens kroppsvegg, kryper ut og spiser resten før den forpupper seg. Tiden som puppe kan vare et par uker eller over vinteren. Det voksne insektet klekker så og jakten på en partner begynder slik at neste generasjon kan utvikle seg.

Tidligere slektskapsforskning tyder på at stamfaren til Cynipoidea var insekt-parasittisk, og at evnen til å danne galler oppsto senere (Ronquist, 1995a, 1999). Tap av denne evnen ga senere opphav til inkvilingrupper (Ronquist, 1994).

Denne avhandlingen er basert på undersøkelser av ulike stadiar av livet til insekts-parasitter, galleveps og inkviliner i gruppen Cynipoidea. Målet med disse undersøkelsene var å sammenligne morfologiske strukturer og belyse anatomicke endringer asosiiert med skiftet fra et liv som insekt-parasitt til livet i en plantegalle. Siden vi ikke vet noe om årsakene eller mekanismene for galledannelse har jeg forsøkt å se spesielt nøyde på strukturer som kan være inblandet i denne prosessen.

Jeg har fokusert på de tidligere livsstadiene og kjertler som finnes i forbindelse med reproduksjonsprosessen som jeg har disseert ut og undersøkt ved hjelp av lysmikroskop og elektronmikroskop. Noen av strukturerne har jeg snittet opp i tynne skiver og undersøkt av et spesielt gjennomlysende elektronmikroskop. De tidligere stadiene (egg og larver) er en tidligere utviklings kilde til informasjon som kan gi oss indikasjoner om hvordan de ulike artene av veps er i slekt med hverandre. Jeg har gjort sammenlignende undersøkelser av egg og larver av insekts-parasitter, galleveps og inkviliner og i tillegg har jeg sett på den voksne hunnenes giftapparat for de samme gruppene.

Artikkel 1 viste at eggene hos veps i gruppen Cynipoidea oftest består av en oval til avlang eggkropp med en lang eller kort stilk i framenden (se figur 3). Eggene til insekts-parasittene var generelt mindre og smalere enn gallevepseggene, og dette kan kanskje delvis forklares av de ulike strategiene forbundet med deres levesett. Man kan for eksempel anta at det tar kortere tid å for et lite og smalt egg å forflytte gjennom det lange tynne eggleggingsrøret til den voksne hunnen, og eggleggingstiden er antagelig en begrensande faktor for et insekt som skal legge sine egg på et annet insekt som kan forsvinne eller bryte seg løs om det tar for lang tid. Det er altså antagelig en fordel at egget til en insekts-parasitt er smalt. Vi undersøkte også eggskallets struktur i seks av artene, og det viste seg at parasittegget var tynnere enn galleveps- og inkvilinervepskallet og det finnes også store strukturelle forskjeller. Igen kan tykkelsen av skallet avhenge av eggets miljø, slik at insekts-parasittenes eggeskall er tynt fordi egret absorberer flytende næringsstoffer fra sine omgivelser, i motsetning til galleveps/inkvilin-
egget. I tillegg er ofte galleveps’egget mer utsatt for tørke da egget kan legges mellom vevein i en bladnøkkel, mens insekt-parasitten lever i sin verts kroppsvæske.

Artikkel 2 er et bokkapittel som gir en oversikt over de tidlige stadiene (egg, larver og pupper) til galleveps som lever på eik og deres inkviliner. Etter eggstadiet kommer fem larvestadier som ser ganske like ut for eikegalleveps og deres inkviliner. Det siste larvestadiet utvikler seg til det første av to pre-puppe faser som kalles den eonymfale fasen, og er ganske likt larven bortsett fra at genitaliene begynner å bli synlige og at segmenteringen blir mindre tydelig. Det andre pre-puppe stadiet kalles den pronymfale fasen og kan sees på figur 5A. Nå begynner øynene å bli synlige og kroppen er tydelig inndelt i en mellomkropp og en bakkropp.

I artikkel 3 sammenlignet vi siste-stadie larver av 5 inseks-parasitter, 21 galledannere og 4 inkviliner (se figur 4) i scanning elektronmikroskop. Larven har ingen ben eller behåring og er oftest litt buet i formen fordi den lever i et rundt kammer i gallen. Vi fant lite forskjeller i selve kroppformen, men det par arter som lever i smale avlange kryptiske galler inni urtestengler, var avlange og nesten rektangulære i formen. Hos noen eikegalleveps var kroppsegment 1-3, som blir til mellomkroppen i det voksne insektet oppblåste i forhold til dr. Årsakene til dette er uklare, men hos enkelte arter ble dette tydeligere jo nærmere larven kom til pupperstadiet, noe som kan tyde på at områdene (imaginalsikvene) for ben og vinger (som kommer ut fra mellomkroppen først i det voksne insektet) har begynt å vokse.

Den største variasjonen fant vi imidlertid i larvenes hoderegion. Eikegallevepslarvene skillte seg tydelig fra de andre ved at de har et par store hevelser i antenneområdet (se figur 4E (aa)). Vi dissekerte ut deres tyggeverktøy (mandibler) (se figur 4C, F og I) og observerte at insekt-parasitt larvene vanligvis har en skarp, relativt tynn hovedtann og enten 1-2 mindre sekundærtaller eller en sag-tann rad av skarpe små tenner langs hele innsiden av mandibelen (se figur 4C). Gallevepslarvene har 1-4 større og butte tenner på sine mandibler (se figur 4F). Inkvilinenes mandibler skillte seg ganske mye fra gallevepsenes. Hos disse var det andre av de tre mandibletallene den bredeste og på basen av inkvinmandiblene kunne man se et tydelig mønster av riller i overflaten (se figur 4I). Hos noen av inkvinartene var de to mandiblene veldig asymmetriske i forhold til hverandre.

Basert på alle observasjoner vi gjorde hos larvene, utførte vi en slekskapsanalyse ved hjelp av et dataprogram som regner ut hvilke arter som sannsynligvis er nærmest i slekt med hverandre. Det resulterende slektskapstree stemte ganske bra overens med tidligere slektskapshypoteser basert på morfologiske karakterer hos det voksne insektet og molekylære data.

I artikkel 4 sammenlignet jeg giftapparatet til 25 arter av Cynipoidea. Mange har sikkert stiftet bekjentskap med giften til enkelte andre veps eller
maur som tilhører samme insektsgruppe. De fleste av disse giftene inneholder maursyre, som brukes kommersielt blant annet som ensileringsmiddel for å styre gjæringsprosessen i gress som lagres i silo, i avisningssvæske på flyplasser og i miljøvennlig borevæske som brukes ved utvinning av olje og gass. Maur og mange veps bruker selv sin etsende gift til forsvar mot angripere eller til å bedøve eller drepe sin vert eller byttedyr. Man vet imidlertid ikke riktig hva gallevepsene bruker sin “gift” til, eller om den virkelig inneholder giftige komponenter. Vi vet at de sprøyer den inn i sin vert sammen med eggene og derfor er det foreslått at giften manipulerer verten på et eller annet vis. Minst en av de insekts-parasittiske vepsene i Cynipoidea har gift som inneholder virus-lignende partikler som brukes til å undertrykke vertens immunsystem. Jeg har beskrevet den generelle strukturen i cynipoid-giftapparatet som var ganske lik for alle de studerte artene, bortsett fra at hele giftapparatet var mye større i forhold til den voksne hunnens størrelse i nesten alle gallevepsene og spesielt hos eikegallevepsene (se figur 6) enn hos de inseks-parasittiske gruppene. Giftapparatet består av en lang enkel giftkjertel der produksjonen av giften hovedsaklig foregår, og et reservoir der giften lagres.

I det foregående har jeg forsøkt å oppsummere de morfologiske endringer som antagelig har oppstått i evolusjonen av galledannere og inkviliner fra insekts-parasitter innen Cynipoidea. Disse forskjellene er nyttige både når man skal identifisere insektene i egg, larve og puppestadiet og dessuten kan de være informative om slektskap mellom artene i denne gruppen av veps. Når det gjelder galledannelse, virker det lite sannsynlig at embryoet inni det tilsynelatende uugjennomtrengelige eggeskallet. Men man skal ikke se bort fra at giften eller andre emner som overføres med eggene fra gallevepshunnen til vertsplanten er innblandet i initieringen av gallen, spesielt med tanke på at giftapparatet er enormt stort i de fleste galleveps og at giften mest sannsynlig ikke brukes til forsvar.

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37


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