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Research

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Morphology and Ontology

Comparative anatomy of venom glands suggests a role of maternal secretions in gall induction by cynipid wasps (Hymenoptera: Cynipidae)

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Many herbivorous insect species are capable of hijacking plant development to induce novel plant organs called galls. In most groups of galling insects, the insect organs and molecular signals involved in gall induction are poorly understood. We focused on gall wasps (Hymenoptera:Cynipidae), the second largest clade of gall inducers (~1,400 spp.), for which the developmental stages and organs responsible for gall development are unclear. We investigated the female metasomal anatomy of 69 gall-inducing and 29 non-gall-inducing species across each of the major lineages of Cynipoidea, to test relationships between this lifestyle and the relative size of secretory organs. We confirmed that the venom apparatus in gall-inducing species is greatly expanded, although gall-inducing lineages vary in the relative size of these glands. Among these gallers, we measured the largest venom gland apparatus relative to body size ever recorded in insects. Non-galling inquiline species are accompanied by a reduction of this apparatus. Comparative microscopic analysis of venom glands suggests varying venom gland content across the lineages. Some oak gallers also had enlarged accessory glands, a lipid-rich organ whose function remains unclear, and which has not been previously studied in relation to gall formation. Together, the massive expansion of secretory organs specifically in gall-inducing species suggests a role of these secretions in the process of gall formation, and the variance in size of venom glands, accessory glands, and the contents of these glands among gallers, suggests that gall formation across this clade is likely to employ a diversity of molecular strategies.

Key words: morphology, gall, venom gland, accessory glands, ovary

Introduction

Herbivory is the dominant feeding style in insects, and its evolutionary success is partly due to the evolved ability of herbivorous insects to interact with and manipulate plant development and defense (Zhu-Salzman et al. 2005). Plant galls—atypical growths in plants induced by a parasite—represent one of the most spectacular cases of such manipulation. Several different lineages of insects, including species of moths, aphids, midges, and wasps, have evolved strategies to induce these growths. Each galling species produces galls on specific tissues of specific host plant species and these galls tend to have species-specific morphologies and chemistry that house, feed, and protect the insects from predators and parasitoids. For instance, gall wasp (Hymenoptera: Cynipidae) *Disholcaspis quercusglobulus* (Fitch, 1859) induces round galls on stems of white oaks, whereas the gall wasp *Acraspis erinacei* (Beutenmüller, 1909) induces spiny, round "hedgehog" galls on white oak leaves (Fig. 1). Building such predictable structures, which match no other structures in the plant, requires specific alteration by the insect of plant developmental and growth pathways.

Many herbivorous insect species produce and secrete substances in their salivary glands that alter plant physiology and modulate the immune reaction of the host plant to the benefit of the insect

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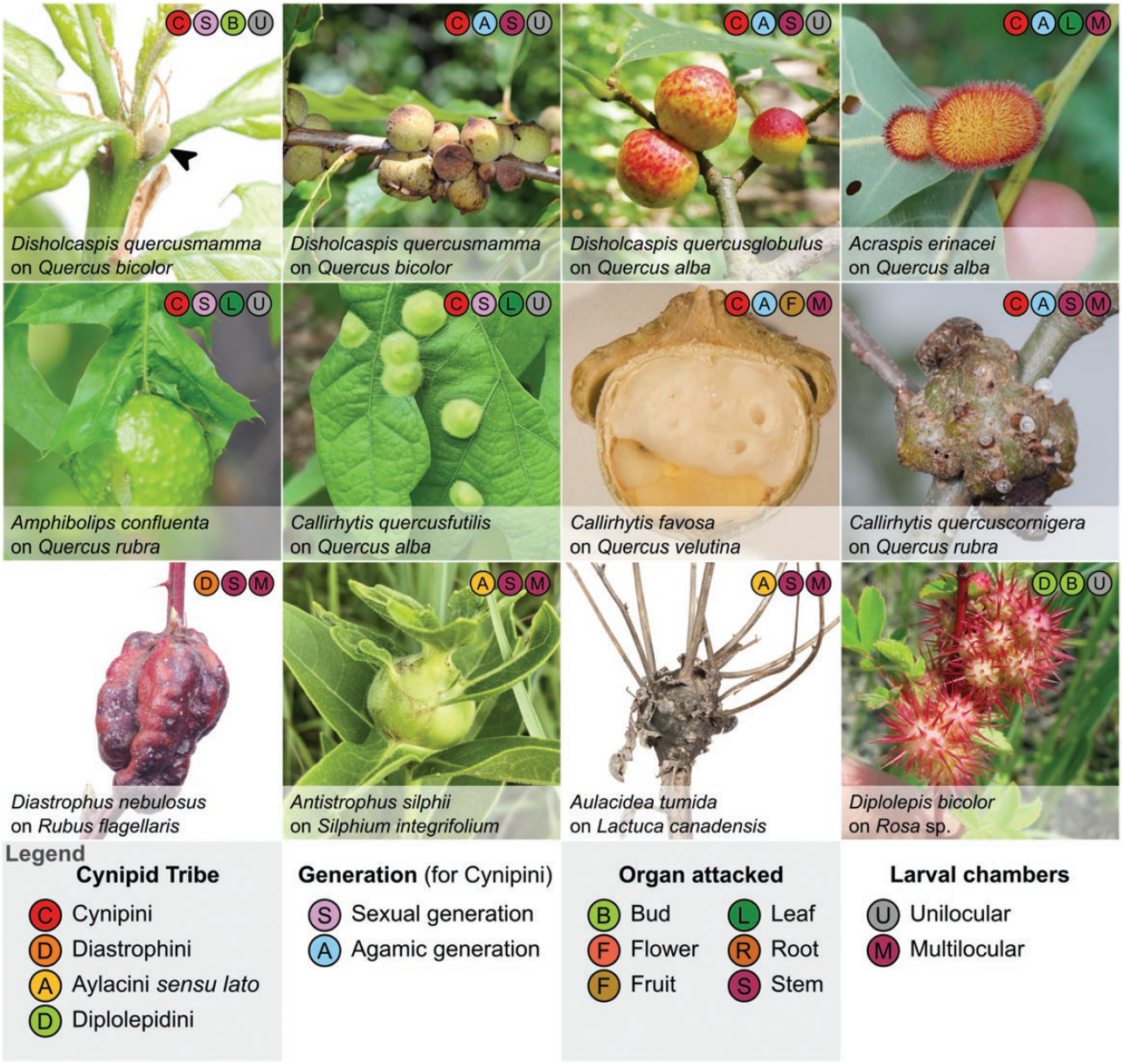

Fig. 1. Diversity of galls induced by Cynipid wasps. Four clades of Cynipidae contain gall-inducing species: the rose-gallers (Diplolepidini), the herb gallers (Aylacini *sensu lato*), the non-rose Rosaceae gallers (Diastrophini), and the oak gallers (Cynipidini). Cynipidini alternate two generations, sexual and agamic, that differ in their phenotype. Cynipid galls can be induced in various organs (bud, flower, fruit, leaf, root, or stem), and contain one larval chamber (unilocular gall) or several (multilocular gall). Photo credits: Tom Murray (*Disholcaspis quercusmamma*), Matthew Wills (*Acraspis erinacei*), Jeremy Collison (*Amphibolips confluenta*), Bill MacIndewar (*Callirhytis quercusfutilis*), Jeff Skrentny (*Antistrophis silphii*), and Erin Faulkner (*Diplolepis bicolor*).

(Felton et al. 2014). Insect oral secretions have been suspected to be involved in gall initiation and induction as well (Hori 1992). Studies have identified phytohormones involved in plant growth and development (e.g., auxins or cytokinins) in oral secretions and bodies of galling insect species (Mapes and Davies 2001, Tooker and Helms 2014). Secreted proteins are also suspected to play a role in gall induction. Several such effector genes have been implicated in gall-inducing insect species (Zhao et al. 2019), and it has been suggested that massive gene families may have evolved for this purpose (Zhao et al. 2015, Korgaonkar et al. 2021).

After gall midges (Diptera: Cecidomyidae), the ~1,400 species of gall wasps (Hymenoptera: Cynipidae) represent the most diverse and specialized lineages of gallers (Ronquist et al. 2015) (Fig. 1). These wasps evolved herbivorous galling from an ancestral parasitic wasp condition (Ronquist et al. 2015, Blaimer et al. 2020), whereby wasps oviposit into the tissue of other insects and utilize substances in their venom to regulate host defense. Gall wasps instead oviposit into specific tissues of the plant—usually undifferentiated meristem tissue—which triggers subsequent developmental alteration and growth of galls (Fig. 2). Cynipidae exhibit a large diversity of gall structures, with each species generating galls of specific morphologies in one or a few host plant species and in specific tissues within a plant. Cynipid galls can affect all plant organs, be solitary or clustered, range from cryptic to the size of an apple, and range from a simple tissue swelling to complex internal structures comprising air chambers or ant-attracting tissues (Stone and Schönrogge 2003, Warren et al. 2022). Cynipidae comprises an early diverging lineage that galls roses (Rosaceae: *Rosa* spp.) (Diplolepidini, 60 spp), a subsequent

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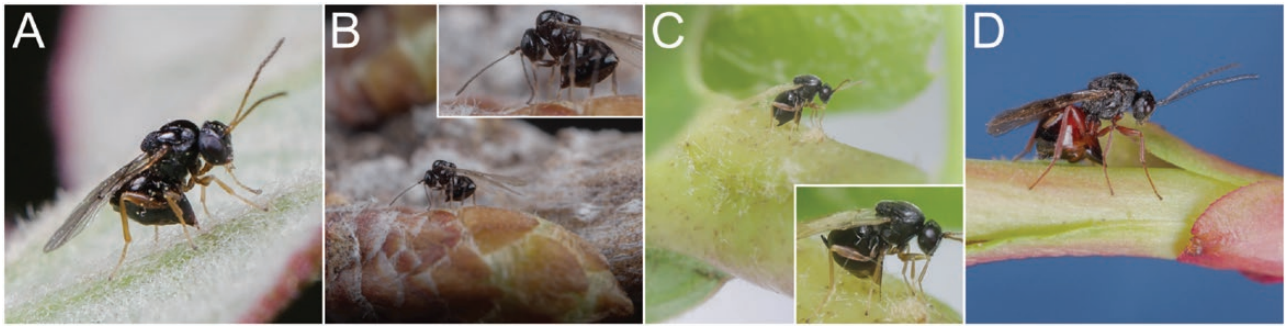

Fig. 2. Examples of oviposition in Cynipidae. Unidentified Cynipini wasps laying eggs (A) in the secondary vein of a red oak (*Quercus rubra*) and (B) in the bud of a white oak (*Quercus alba*). (C) Inquiline cynipid wasp (*Ceroptres* sp.) laying eggs into the petiole gall of the sexual generation of *Melikaiella tumifica* on *Q. rubra*. (D) *Diplolepis nodulosa* (Diplolepidini) ovipositing in a rose bud.

radiation that galls primarily herbaceous Asteraceae, Lamiaceae, and Papaveraceae (170 spp., Aylacini sensu lato), a lineage that galls primarily *Rubus (Rosaceae)* bramble berries (Diastrophini, 20 galling spp.), and a radiation of oak (Fagaceae) gallers (Cynipini, 1,000 spp.) that harbors most of the group's diversity. The oak gallers have further evolved sexual and agamic generations, each of which generates different gall types on different tissues of the host plant (Ronquist et al. 2015). Within Cynipidae three inquilinous lineages have evolved (Ceroptresini, 21 spp.; Diastrophini, 24 inquiline spp.; and Synergini, 180 spp.) that do not induce their own galls but instead are oviposited into and feed upon the developing galls of other gall wasp species. Intralineage comparisons of galling and non-galling species in this clade are highly valuable for discerning the mechanisms responsible for galling.

In gall-inducing cynipids, it remains unclear whether substances during oviposition, or produced by the embryo and developing larvae, are involved in gall induction. While substances involved in oviposition were first suspected to contain gall-inducing factors (Malpighi 1687), the majority of research has concentrated on the gall-inducing role of larval secretions. For example, for *Liposthenes glechomae* (Linnaeus, 1758) (Aulacideini), a herb galler that attacks a species of mint (Lamiaceae), larval ablation arrested further gall development, suggesting larvae were the source of an effector of gall growth (Rohfritsch 1971). Auxin and cytokinins were detected in larvae of 2 oak gallers (*Cynips quercusfolii* Linnaeus, 1758 and *Dryocosmus kuriphilus* Yasumatsu, 1951), suggesting that larvae may use these plant hormones to affect gall formation (Kaldewey 1965, Wood and Payne 1988). Triggerson (1914) found that *A. erinacei* larvae secreted an anal substance likely from the precursors of Malpighian tubules (or iliac glands) only during the stages of larval development when galls grow, which was corroborated by other observations (Rössig 1904, Roth 1949). In contrast, some observations suggest that maternal secretions or the egg itself are involved in initiation. For example, gall formation by the herb galler *Phanacis hypochoeridis* (Kieffer 1887) (Phanacidini) starts before egg hatching, supporting a role for oviposition substances (Lintott 1975). Furthermore, oviposition by *Diplolepis* species is quickly followed by cell hypertrophy and lysis (Leggo and Shorthouse 2006, Sliva and Shorthouse 2006) that cannot be explained only by mechanical wounding (Magnus 1914), and may result from substances deposited during oviposition (Bronner 1985, LeBlanc and Lacroix 2001). Moreover, secretions accompanying oviposition by the oak galler *Biorhiza pallida* (Olivier 1791) have proteolytic, cellulolytic, and pectinolytic activities, suggesting that they could play a role in gall induction and/or formation (Bronner 1973).

The venom gland is considered the most likely candidate for oviposition secretions. In Tenthredinidae, an early diverging Hymenoptera family, glands homologous to venom glands were shown to contain high concentrations of phytohormones and to contribute to the gall induction in galling lineages (McCalla et al. 1962, Yamaguchi et al. 2012). Parasitoid Hymenoptera lineages, which include cynipid ancestors, secrete venom during oviposition that paralyzes hosts and suppresses their immunity. As such the venom gland actively produces these substances that are stored in the venom reservoir prior to secretion. Despite the loss of parasitoidism and gain of herbivory, previous research has shown that gall wasps have not only retained these glands but they are especially large (Vårdal 2006). Comparison of venom-gland morphology from 25 cynipoid species, including 12 gallers, 11 parasitoids, and 2 inquilines revealed that, relative to non-gall-inducing parasitoids and inquilines, the oak gall wasps (Cynipini) have exceptionally large venom glands that occupy much of their metasoma (Vårdal 2006). Producing a large gland has metabolic costs and when features become non-functional they tend to become vestiges (smaller). Enlargement of these glands in gall wasp species suggests they may have been co-opted for facilitating interactions with plants during galling.

In this study, we improve understanding of the role of venom glands in gall induction and/or formation by expanding on this previous work. We compare the anatomy of venom and reproductive apparati across a greater diversity of lineages, including 69 galler species belonging to 4 independent lineages, 18 non-galler species from all known inquiline lineages, and 11 outgroup lineages, and we test how venom gland and reservoir size relates to galling strategy and gall type. Larger size in this case, especially when it occupies a substantial portion of the metasoma, would implicate venom in having an important role, and relating this size to galling life history strategy thus reveals the importance of venom to galling. Furthermore, our examination of the internal metasomal anatomy of these wasps revealed in several taxa relatively large accessory glands, a structure that has received little attention in gall wasps (Frühauf 1924). Accessory glands, also named oviductal or colleterial glands, are lipid-rich organs that, among other functions, can secrete lubricant to facilitate oocytes transport through the ovipositor (Sturm 2012). To assess the possible role of these glands in galling, relative to their life history traits, we compared the size and morphology of this structure across 42 species. We also examined the correlation of these traits with each other, and with body size and egg load to test the alternate hypothesis that wasp secretions play a role in egg laying. Finally, to assess the contents of these glands, we utilize high-resolution imaging techniques to reveal differences in microscopic secretory strategies across cynipid species.

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Table 1. List of taxa examined or for which data was obtained from previous work, with their classification and lifestyle. Tissue: plant tissue targeted by the wasp female to lay eggs in; Chamber: quantity of larval chamber in the gall induced by the wasp female, ranging for one chamber or unilocular gall (Uni) to several chambers or Multilocular (Multi); Generation: generation of the wasp female dissected, sexual or agamic; N: number of individuals dissected; P: species included in Blaimer et al. 2020 and/or in Ward et al. 2022 phylogenies. A hyphen designates a category not relevant for the species and question marks indicate missing information. The host species of inquilines is indicated in brackets.

Family	Tribe	Species	Lifestyle	Tissue	Chamber	Generation	*N*	*P*	
Cynipidae	Aylacini s.l.	*Antistrophus jeanae*	Galler	Stem	Uni	-	2		*This study*
Cynipidae	Aylacini s.l.	*Antistrophus laciniatus*	Galler	Flower	Multi	-	3	*P*	*This study*
Cynipidae	Aylacini s.l.	*Antistrophus lygodesmiaepisum*	Galler	Stem	Uni	-	2		*This study*
Cynipidae	Aylacini s.l.	*Antistrophus minor*	Galler	Stem	Uni	-	1	*P*	*This study*
Cynipidae	Aylacini s.l.	*Antistrophus rufus*	Galler	Stem	Uni	-	2	*P*	*This study*
Cynipidae	Aylacini s.l.	*Antistrophus silphii*	Galler	Flower	Multi	-	2	*P*	*This study*
Cynipidae	Aylacini s.l.	*Antistrophus* sp.	Galler	Stem	Uni	-	1		*This study*
Cynipidae	Aylacini s.l.	*Aulacidea* sp.	Galler	Stem	Multi	-	2	*P*	*This study*
Cynipidae	Aylacini s.l.	*Aulacidea tragopogonis*	Galler	Root	Uni	-		*P*	Vårdal 2006, Vårdal et al. 2003
Cynipidae	Aylacini s.l.	*Aulacidea tumida*	Galler	Stem	Multi	-	2	*P*	*This study*
Cynipidae	Aylacini s.l.	*Aylax hypecoi*	Galler	Fruit	Uni	-			Stojanova & Draganov 2008
Cynipidae	Aylacini s.l.	*Aylax papaveris*	Galler	Fruit	Multi	-			Vårdal 2006, Vårdal et al. 2003
Cynipidae	Aylacini s.l.	*Barbotinia oraniensis*	Galler	Fruit	Multi	-			Vårdal 2006, Vårdal et al. 2003
Cynipidae	Aylacini s.l.	*Isocolus lichtensteini*	Galler	Stem	Multi	-		*P*	Vårdal 2006, Vårdal et al. 2003
Cynipidae	Ceroptresini	*Ceroptres* sp. 1 *(Zapatella quercusphellos)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Ceroptresini	*Ceroptres* sp. 2 *(Cecidomyiidae)*	Inquiline	-	-	-	4	*P*	*This study*
Cynipidae	Ceroptresini	*Ceroptres* sp. 3 *(Andricus quercuspetiolicola)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Ceroptresini	*Ceroptres* sp. 4 *(Philonix fulvicollis)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Ceroptresini	*Ceroptres* sp. 5 *(Neuroterus rileyi)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Ceroptresini	*Ceroptres* sp. 6 *(Kokkocynips rileyi)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Cynipini	*Acraspis erinacei*	Galler	Bud	Multi	Agamic	5	*P*	*This study*
Cynipidae	Cynipini	*Acraspis pezomachoides*	Galler	Bud	Multi	Agamic	3	*P*	*This study*
Cynipidae	Cynipini	*Amphibolips acuminata*	Galler	?	?	Sexual	1		*This study*
Cynipidae	Cynipini	*Amphibolips confluenta*	Galler	?	?	Sexual	2	*P*	*This study*
Cynipidae	Cynipini	*Amphibolips nubilipennis*	Galler	Fruit	Uni	Sexual	1	*P*	*This study*
Cynipidae	Cynipini	*Amphibolips quercuscoelebs*	Galler	?	?	Sexual	2		*This study*
Cynipidae	Cynipini	*Amphibolips quercusinanis*	Galler	Bud	Uni	Sexual	3	*P*	*This study*
Cynipidae	Cynipini	*Amphibolips quercusostensackenii*	Galler	?	?	Sexual	1	*P*	*This study*
Cynipidae	Cynipini	*Amphibolips quercusrugosa*	Galler	?	?	Sexual	2		*This study*
Cynipidae	Cynipini	*Andricus curvator*	Galler	Leaf	Uni	Sexual			Sanderson 1988
Cynipidae	Cynipini	*Andricus kollari*	Galler	Bud	Uni	Agamic	3		*This study*, Sanderson 1988
Cynipidae	Cynipini	*Andricus quercuscalicis*	Galler	Flower	Uni	Agamic			Sanderson 1988
Cynipidae	Cynipini	*Andricus quercuspetiolicola*	Galler	?	?	Agamic	4	*P*	*This study*
Cynipidae	Cynipini	*Andricus quercusradicis*	Galler	Stem	Multi	Sexual			Vårdal 2006, Vårdal et al. 2003
Cynipidae	Cynipini	*Andricus quercusstrobilanus*	Galler	?	?	Agamic	3	*P*	*This study*
Cynipidae	Cynipini	*Andricus robustus*	Galler	?	?	Agamic	1		*This study*
Cynipidae	Cynipini	*Andricus texanus*	Galler	?	?	Agamic	4		*This study*
Cynipidae	Cynipini	*Atrusca unica*	Galler	?	?	Agamic	2	*P*	*This study*

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Table 1. Continued

Family	Tribe	Species	Lifestyle	Tissue	Chamber	Generation	*N*	*P*	
Cynipidae	Cynipini	*Biorhiza pallida*	Galler	Bud	Multi	Agamic			Cambier et al. 2019, Vårdal 2006, Vårdal et al. 2003, Sanderson 1988
Cynipidae	Cynipini	*Biorhiza pallida*	Galler	Root	Multi	Sexual			Sanderson 1988
Cynipidae	Cynipini	*Callirhytis clavula*	Galler	?	?	Agamic	3		*This study*
Cynipidae	Cynipini	*Callirhytis favosa*	Galler	Fruit	Multi	Sexual	2	*P*	*This study*
Cynipidae	Cynipini	*Callirhytis flavipes*	Galler	?	?	Sexual	1	*P*	*This study*
Cynipidae	Cynipini	*Callirhytis quercuscornigera*	Galler	Leaf	Uni	Agamic	1	*P*	*This study*
Cynipidae	Cynipini	*Callirhytis quercusfutilis*	Galler	Leaf	Uni	Agamic	3	*P*	*This study*
Cynipidae	Cynipini	*Callirhytis quercusfutilis*	Galler	Root	Multi	Sexual	3	*P*	*This study*
Cynipidae	Cynipini	*Callirhytis quercusoperator*	Galler	Fruit	Uni	Sexual	1	*P*	*This study*
Cynipidae	Cynipini	*Callirhytis quercuspunctata*	Galler	Leaf	Uni	Agamic	1	*P*	*This study*
Cynipidae	Cynipini	*Callirhytis seminator*	Galler	?	?	Agamic	9	*P*	*This study*
Cynipidae	Cynipini	*Callirhytis seminator*	Galler	Stem	Multi	Sexual	2	*P*	*This study*
Cynipidae	Cynipini	*Callirhytis* sp. 1	Galler	?	?	Sexual	1		*This study*
Cynipidae	Cynipini	*Callirhytis* sp. 2	Galler	Bud	?	?	1		*This study*
Cynipidae	Cynipini	*Cynips divisa*	Galler	Leaf	Uni	Agamic			Sanderson 1988
Cynipidae	Cynipini	*Cynips quercus*	Galler	Bud	Uni	Agamic			Vårdal 2006, Vårdal et al. 2003
Cynipidae	Cynipini	*Disholcaspis cinerosa*	Galler	Bud	Uni	Agamic	2	*P*	*This study*
Cynipidae	Cynipini	*Disholcaspis quercusglobulus*	Galler	Bud	Uni	Agamic	2	*P*	*This study*
Cynipidae	Cynipini	*Disholcaspis quercusmamma*	Galler	Bud	Uni	Agamic	10	*P*	*This study*
Cynipidae	Cynipini	*Disholcaspis quercusmamma*	Galler	Stem	Uni	Sexual	8	*P*	*This study*
Cynipidae	Cynipini	*Druon quercusflocci*	Galler	Bud	Uni	Agamic	2	*P*	*This study*
Cynipidae	Cynipini	*Holocynips maxima*	Galler	Bud	?	Agamic	1	*P*	*This study*
Cynipidae	Cynipini	*Kokkocynips imbricaria*	Galler	?	?	Agamic	1	*P*	*This study*
Cynipidae	Cynipini	*Loxaulus quercusmammula*	Galler	?	?	Sexual	1	*P*	*This study*
Cynipidae	Cynipini	*Melikaiella tumifica*	Galler	Fruit	Multi	Sexual	1	*P*	*This study*
Cynipidae	Cynipini	*Neuroterus numismalis*	Galler	Leaf	Uni	Agamic			Vårdal 2006, Vårdal et al. 2003, Sanderson 1988
Cynipidae	Cynipini	*Neuroterus numismalis*	Galler	Leaf	Uni	Sexual			Sanderson 1988
Cynipidae	Cynipini	*Neuroterus quercusbaccarum*	Galler	Flower	Uni	Agamic			Sanderson 1988
Cynipidae	Cynipini	*Neuroterus quercusbaccarum*	Galler	Leaf	Uni	Sexual			Sanderson 1988
Cynipidae	Cynipini	*Neuroterus quercusbatatus*	Galler	Stem	Multi	Sexual	1	*P*	*This study*
Cynipidae	Cynipini	*Neuroterus quercusverrucarum*	Galler	Leaf	Uni	Agamic	2		*This study*
Cynipidae	Cynipini	*Neuroterus vesicula*	Galler	?	?	Sexual	1	*P*	*This study*
Cynipidae	Cynipini	*Neuroterus washingtonensis*	Galler	Stem	Uni	Sexual	3	*P*	*This study*
Cynipidae	Cynipini	*Philonix fulvicollis*	Galler	Bud	Uni	Agamic	5	*P*	*This study*
Cynipidae	Cynipini	*Zapatella quercusphellos*	Galler	?	?	Sexual	1	*P*	*This study*
Cynipidae	Diastrophini	*Diastrophus cuscutaeformis*	Galler	Stem	Uni	-	5	*P*	*This study*
Cynipidae	Diastrophini	*Diastrophus kincaidii*	Galler	Stem	Multi	-	4	*P*	*This study*
Cynipidae	Diastrophini	*Diastrophus nebulosus*	Galler	Stem	Multi	-	4	*P*	*This study*
Cynipidae	Diastrophini	*Xestophanes potentillae*	Galler	Stem	Multi	-			Vårdal 2006, Vårdal et al.2003
Cynipidae	Diastrophini	*Periclistus brandtii*	Inquiline	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Cynipidae	Diastrophini	*Periclistus* sp.	Inquiline	-	-	-	2	*P*	*This study*

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Table 1. Continued

Family	Tribe	Species	Lifestyle	Tissue	Chamber	Generation	*N*	*P*	
Cynipidae	Diastrophini	*Synophromorpha sylvestris*	Inquiline	-	-	-	3	*P*	*This study*
Cynipidae	Diplolepidini	*Diplolepis bicolor*	Galler	Bud	Uni	-	2	*P*	*This study*
Cynipidae	Diplolepidini	*Diplolepis eglanteriae*	Galler	Leaf	Uni	-			Sanderson 1988
Cynipidae	Diplolepidini	*Diplolepis mayri*	Galler	Bud	Multi	-		*P*	Vårdal 2006, Vårdal et al. 2003
Cynipidae	Diplolepidini	*Diplolepis rosae*	Galler	Bud	Multi	-		*P*	Cambier et al. 2019, Sanderson 1988, Vårdal 2006, Vårdal et al. 2003
Cynipidae	Synergini	*Synergus albipes*	Inquiline	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Cynipidae	Synergini	*Synergus* sp. 1 *(Philonix fulvicollis)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Synergini	*Synergus* sp. 2 *(Acraspis erinacei)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Synergini	*Synergus* sp. 3 *(Neuroterus sp.)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Synergini	*Synergus* sp. 4 *(Callirhytis favosa)*	Inquiline	-	-	-	3	*P*	*This study*
Cynipidae	Synergini	*Synergus* sp. 5 *(Disholcaspis quercusglobulosus)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Synergini	*Synergus* sp. 6 *(Andricus quercusstrobilanus)*	Inquiline	-	-	-	1	*P*	*This study*
Cynipidae	Synergini	*Synergus* sp. 7 *(Philonix fulvicollis)*	Inquiline	-	-	-	3	*P*	*This study*
Cynipidae	Synergini	*Synergus* sp. 8 *(Amphibolips quercusjuglans)*	Inquiline	-	-	-	1	*P*	*This study*
Figitidae	Anacharitinae	*Anacharis immunis*	Parasitoid	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Figitidae	Anacharitinae	*Xyalaspis* sp.	Parasitoid	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Figitidae	Aspicerinae	*Prosaspicera* sp.	Parasitoid	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Figitidae	Charipinae	*Alloxysta victrix*	Parasitoid	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Figitidae	Charipinae	*Phaenoglyphis villosa*	Parasitoid	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Figitidae	Eucoilinae	*Kleidotoma dolichocera*	Parasitoid	-	-	-			Vårdal 2006, Vårdal et al. 2003
Figitidae	Eucoilinae	*Leptopilina boulardi*	Parasitoid	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Figitidae	Figitinae	*Figites* sp.	Parasitoid	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Figitidae	Figitinae	*Xyalophora* sp.	Parasitoid	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Ibaliidae	Ibaliinae	*Ibalia rufipes*	Parasitoid	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003
Figitidae	Parnipinae	*Parnips nigripes*	Inquiline	-	-	-		*P*	Vårdal 2006, Vårdal et al. 2003

Material and Methods

Selection of Taxa

We examined gland morphology in 166 specimens belonging to 69 species of Cynipoidea (Hymenoptera) (Table 1 and Supplementary Table S1). We dissected 1–10 specimens per species, with 2.3 dissections on average. We also included data available in the literature from venom glands of 26 species and egg loads of 33 species (Sanderson 1988, Vårdal et al. 2003, Vårdal 2006, Stojanova and Draganov 2008, Cambier et al. 2019). We note that after examination, the glands of *Diplolepis rosae* that are described as "venom gland" in Cambier et al. (2019) actually present all the morphological characteristics of accessory glands and therefore are treated as such in our study. Together, our data include 98 species that belong to 6 well-supported clades of Cynipoidea (Supplementary Table S1; Blaimer et al., 2020): 47 species of Cynipini (oak gallers), 6 Ceroptresini (inquilines), 7 Diastrophini (non-rose Rosaceae gallers and inquilines), 4 Diplolepidini (rose gallers), 9 Synergini (inquilines), and 14 herb gallers from Aulacideini, Aylacini, and Phanacidini that we grouped as "Aylacini s.l." (Ronquist et al. 2015), and 11 parasitoid cynipoids from Ibaliidae and Figitidae.

Terminology

For the purposes of this work, we distinguish between gall induction and gall formation based on the roles played by insects and plants. We define "induction" broadly as actions taken by insects to initiate galls and cause them to grow. In contrast, we define "formation" as the process of gall growth accomplished via the machinery of the plant, even though it occurs at the direction of the insect. We acknowledge there is some uncertainty in where induction stops and formation begins, but for our purposes these definitions allow us to attribute the work of the insects to induction. The terminology of the female venom apparatus follows Vårdal (2006): we use the term *venom gland* for the filiform organ that secretes the venom and the term *venom reservoir* for the sac that stores the venom. We use the term

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accessory glands for the pair of long tubular glands connected to the oviduct, also called colleterial glands (Copland and King 1971). We designate as *accessory sacs* the pair of round glandular structures at the base of the ovary and the accessory gland, described by Frühauf (1924) as "the paired, bean-shaped oviduct glands or synonym uterine glands, lubricating glands" ("die paarigen, bohnenförmigen Eileiterdrüsen oder synonym Uterusdrüsen, Schmierdrüsen"). Our terminology for organ anatomy is compatible with the Hymenoptera Anatomy Ontology (Yoder et al. 2010). For gland histology, we use the terminology of Noirot and Quennedey (1974). We designate the gland cells as *secretory cells*, and the secretion organelle that these cells contain, sometimes called vesicular organelle or rough canal, as *end apparatus* (Dierckx 1899, Ratcliffe and King 1969, van Marle and Piek 1986, Ferrarese et al. 2009). The secretory cells are connected to the central lumen of the gland by ducts, or smooth canals (Noirot and Quennedey 1974, Ferrarese et al. 2009). These ducts are surrounded by ductule cells or intimal cells (Noirot and Quennedey 1974, Ferrarese et al. 2009).

Dissections and Measurements

We collected galls in the United States and France between March 2020 and October 2021 and reared them in the laboratory until adult emergence. One or 2 days after emergence, we dissected each female metasoma in phosphate-buffered saline (1 × PBS) under a stereomicroscope, isolating the venom gland and its associated reservoir, the accessory glands and sacs, and ovaries. Each organ was spread and photographed separately using a camera attached to the microscope (CellSens Entry 1.18, Olympus). From these images, we measured the isolated organs as well as the length of the metasoma and the ovipositor. The length of the accessory gland was measured only when visible beyond the length of the sac. As in previous studies on venom apparatus morphology (Martinson et al. 2014), areas of the venom reservoirs were determined from images, whereas areas of metasomas and accessory sacs were approximated as circles with diameters from their measured length. The length of venom glands and areas of venom reservoirs from the literature were measured from figures (Vårdal 2006). All length and area measurements as well as egg counts were performed in ImageJ (Schneider et al. 2012). When glands were broken during dissection, their lengths were measured segment by segment. When reservoirs were accidentally pierced, reservoir length was estimated but area was not calculated. We counted the eggs of 1 ovary. As a surrogate for body size, we preferred metasoma length over forewing length, as it was available for the entire dataset, which included several brachypterous or apterous species.

Statistical Analyses

We examined morphological data in the context of phylogeny and life history traits, including sexual/agamic generation, location of gall, lifestyle (inquiline/galler/parasitoid), whether a gall is uni- or multilocular (chambered), and host plant species. For gall assignments to generation, we are recognizing which galls are being induced, rather than the gall from which the individual emerged. We extracted the tribal phylogeny from Blaimer et al. (2020) and life-history traits (i.e., plant tissue attacked, and generation for Cynipini) from the literature and a website on gall-inducing insects (Gallformers 2022). We performed statistical analyses using the software program *R* (R Core Team 2020). Body part dimensions and ratios were analyzed using one-way ANOVA, after confirming the data met necessary assumptions. Categories with less than 3 data points were excluded from the comparisons. The comparisons were performed with Tukey's Honest Significant Differences Test for post hoc multiple comparisons. Correlation was tested with the Pearson correlation coefficient that was calculated in *R* with the cor() function and *P* values were calculated with the cor.mtest() function.

Using a phylogenetically corrected generalized least squares regression (PGLS) in the nlme R package, we investigated whether the absolute and relative size of the venom apparatus, the metasoma size (length and area), and the egg load in cynipoids are significantly related to galling strategy after correcting for phylogeny, whether the venom apparatus size is correlated to the egg load or the metasoma size, and whether the venom gland length and venom reservoir area are correlated (Pinheiro et al. 2023). For this analysis, we obtained a time-calibrated phylogeny by merging recently published Cynipoid and Cynipini phylogenies (Blaimer et al. 2020, Ward et al. 2022). Species absent from the dataset were pruned from both trees. The nodes between *D. kuriphilus* and *Disholcaspis quercusmamma* (Walsh and Riley, 1869) and between *Callirhytis quercusoperator* (Osten Sacken, 1862) and *D. quercusmamma* of Blaimer et al 2020 were used to time calibrate the Cynipini phylogeny obtained from Ward et al. 2022 by Maximum Likelihood using the "chronos" function of the R package ape (Paradis and Schliep 2019). Once time-calibrated, the Cynipini tree from Ward et al. (2022) was grafted at the root of Cynipini in the Blaimer et al. (2020) time-calibrated phylogeny. The obtained phylogeny was fully dichotomous, except for *Ceroptres*, *Synergus*, and *Aulacidea*, in which each species was attached to the same node. The PGLS regressions were performed using the "gls" function in the R package nlme (Pinheiro et al. 2023). Only the taxa represented in these published phylogenies were able to be analyzed (71 of 98 were included). To further assess this trait in an evolutionary context, we mapped the evolution of relative size of the venom gland apparatus on the branches of the phylogeny using the "phenogram" function in phytools (Revell 2012). The phenogram generated with this method projects the phylogeny related to a phenotype trait, in our case the relative venom gland length and the relative venom reservoir area.

Microtomography

We used microtomography to visualize and image the whole interior morphology of a representative cynipid metasoma with the goal of understanding the respective sizes and position of each major organ in situ. For this, we scanned the metasoma of a female of the agamic generation of *D. quercusmamma* using a Zeiss Xradia 620 Versa micro-CT scanner, using 80 kV, a power of 10 W, an exposure time of 0.65 s and a voxel size of 1.70 µm. Tomographic slices were converted to 8 bit. In the software Avizo (Avizo Light 9.0, FEI), venom gland, accessory gland, gut, and ovaries were segmented manually.

Confocal Microscopy

We utilized confocal laser scanning microscopy (CLSM) to examine the anatomy of the venom gland and reservoir, accessory gland and sac, and ovaries, with the aim of understanding the structure of the secretory apparatus and the involvement of protein and lipids in the respective structures. For this, we dissected in 1 × PBS the reproductive system of female *D. quercusmamma* and stained it with various compounds. To view actin filaments and intracellular lipid droplets, we stained them with Alexa Fluor 488 Phalloidin or Nile Red, respectively. Dissections of venom apparatuses of *D. quercusmamma* were also stained with Alexa Fluor 488 Phalloidin. All dissections were simultaneously stained with Hoescht. Dissected organs were placed in a 1 × PBS droplet in a concave slide under a cover glass and imaged with a Leica SP8 DIVE confocal/multiphoton

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microscope system (Leica, Germany) using 405- and 488-nm lasers at the Microscopy and Cytometry Facility at The Pennsylvania State University. Fluorescence was collected using three channels assigned contrasting pseudocolors (420–520 nm, blue; 490–520 nm, yellow; and 570–670 nm, red). Volume-rendered images and media files were generated using FIJI (Schindelin et al. 2012). The three-dimensional reconstruction for the *D. quercusmamma* venom gland was obtained with 3D Slicer 4 (Fedorov et al. 2012).

Transmission Electron Microscopy

To assess the internal contents and microstructures of glands across lineages as they relate to secretion, we performed transmission electron microscopy on representatives of 3 galler lineages and 1 inquiline lineage. Previously fixed venom glands of *A. erinacei*, *Amphibolips confluenta*, *Antistrophus laciniatus*, *Callirhytis seminator*, *Diastrophus nebulosus*, *Dischocaspis quercusmamma*, and *Synergus* sp. were stained with osmium tetroxide for 2 h and uranyl acetate for 12 h, dehydrated through an ethanol series, and embedded in eponate (protocol available at https://doi.org/10.6084/m9.figshare.4993793). Blocks were trimmed and sectioned using a Leica UCT ultramicrotome. Sections were collected on slot grids and then double-stained with lead citrate and uranyl acetate. Sections were imaged with a JEOL 1200 transmission electron microscopy (TEM) at the Microscopy and Cytometry Facility at The Pennsylvania State University. Semithin sections were collected on VWR VistaVision HistoBond adhesive slides and stained with toluidine blue (0.1% toluidine blue and 0.1% sodium tetraborate) for 25 s and safranin (0.1%) for 45 s. Sections were imaged using an Olympus CX41 microscope equipped with a Canon EOS 70D camera.

Serial Block Face Scanning Electron Microscope

To better understand the spatial relationships between subcellular elements and identify the delivery strategies of the gland extract and gland type, we performed Serial Block Face SEM (SBFSEM). This method allows the generation of serial sections with high Z resolution (70 nm thick section width). Specimens stained for SBFSEM can also be used for TEM without performing grid staining making the EM-based examinations of subcellular structures easier to perform. For SBFSEM, we collected live specimens of *A. erinacei* and *Acraspis quercushirta* Bassett, 1864 in University Park, PA, USA, and dissected them in 0.1M cacodylate buffer. The venom gland, reservoir and accessory glands were fixed with 2.5% glutaraldehyde and 6% sucrose in 0.1M cacodylate buffer for 1 h at room temperature and 24 h at 4°C. Following fixation, specimens were stained with osmium tetroxide, potassium ferrocyanide, thiocarbohydrazide, uranyl acetate, and lead aspartate. Specimens were then dehydrated through an ethanol series and embedded in eponate (Protocol available at: https://doi.org/10.6084/m9.figshare.4993796.v1). Resin blocks were trimmed and sectioned using a Leica UCT ultramicrotome, then mounted into a Zeiss SIGMA VP-FESEM with a Gatan 3View2 accessory for sectioning and imaging. Data were processed in Avizo (version 9.1.1). The images were aligned and cropped in ImageJ (Version 2.0.0) and then imported into Avizo (version 9.1.1). Images were stacked and volume-rendered, and then each unique morphological component was marked as an individual label field and modeled through manual outlining and interpolation. The generated surface model was smoothed, and the polygon points were simplified to make the file more manageable. The images were then converted into a Graphics Interchange Format file (.gif) and respective jpeg images that allowed cross-sectional viewing of the models. For TEM, resin blocks containing specimens used for SBFSEM were trimmed and sectioned using a Leica UCT ultramicrotome. Sections were collected on slot grids and imaged with a JEOL 1200 TEM at the Microscopy and Cytometry Facility at The Pennsylvania State University.

Results

General Anatomy of Metasomal Organs

Dissections and micro-tomography of agamic females of *D. quercusmamma* revealed 2 apparati connected to the ovipositor: the venom apparatus and the reproductive apparatus (Fig. 3, Supplementary Movie S1). The venom apparatus consisted of the venom gland, which was thin and linear, with a duct exiting into a venom reservoir, itself connected to the ovipositor by a small duct (Fig. 1). The reproductive apparatus comprised 2 symmetric organ complexes each made up of an ovary, a thick linear accessory gland, and a discoidal organ, the accessory sac, each connected to the oviduct (Fig. 3). Both oviducts merged close to the intersection point of the accessory sacs and form the common oviduct. We found this anatomy in all cynipoids, with some organs being absent or reduced in some groups. In the microtomography reconstruction of the *D. quercusmamma* metasoma, the venom apparatus represented 29% of the metasoma volume, the accessory apparatus 11%, and the ovaries 6%.

Comparative Size and Anatomy of the Venom Apparatus

Dissection of the venom apparatus of 99 Cynipoid species revealed strong interspecies variation in organ size (Fig. 4). In the oak gallers (Cynipini), the length of the venom gland exceeded metasoma length in all species except *Neuroterus numismalis* Geoffroy in Fourcroy, 1785 (agamic generation), with an average venom gland length/metasoma length (VG/M) ratio of 3.49 ± 1.71. *Disholcaspis cinerosa* (Bassett, 1881) (agamic generation) had the highest VG/M ratio with 10.38. The reservoir area exceeded 5% of the metasoma area (VR/Ma ratio) in all but 4 species. The average VR/Ma in Cynipini was 14.8 ± 8.8%. The highest VR/Ma ratio was found for an unidentified *Callirhytis* sp. and for *Atrusca unica* (Weld, 1926) (agamic generation) for which we measured a VR/Ma ratio close to 40%.

By contrast, the inquiline tribe Ceroptresini had much-reduced venom apparati, with venom gland lengths that were about half of metasoma length, and reservoir areas that were consistently under 5% of metasoma area (Fig. 4). Similarly, in the inquiline tribe Synergini, all venom glands were shorter than the metasoma and all reservoirs were smaller than 5% of the metasoma area. The contrast in size between inquilines and gallers was especially marked in Diastrophini, in which the 3 gall-inducing species had venom glands that were longer than their body, with a maximum VG/M ratio of 8.11 for *Diastrophus kincaidii*, Gillette, 1893, whereas the three inquilines had VG/M ratio close to or less than 1 [e.g., 1.32 for *Periclistus brandtii* (Ratzeburg, 1831)]. Species of the genus *Diastrophus* (gallers) had the largest reservoirs of the tribe, with an area close to 15% of their metasoma area whereas inquilines had smaller reservoirs (<5% of the metasoma for *Periclistus* sp.).

The herb gallers, which possibly represent a more ancestral galling condition, had more variable venom-gland size relative to metasomal size (Fig. 4). In the herb gallers in Aylacini s.l., venom gland length exceeded metasoma length in all *Antistrophus* and in 2 *Aulacidea*, with a maximum ratio of 8.91 for *Antistrophus silphii* (Gillette, 1891). However, gland length was shorter than the

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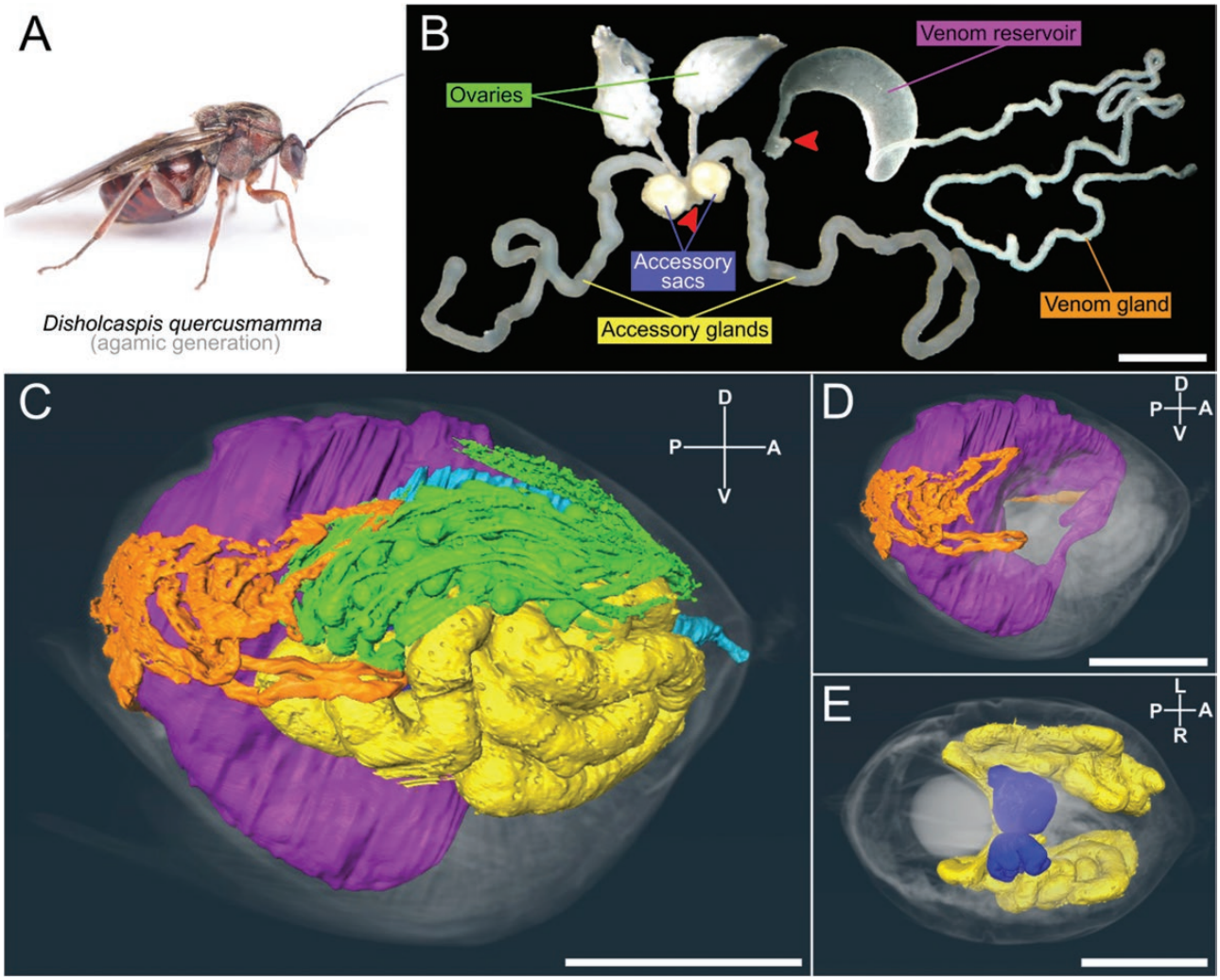

Fig. 3. Anatomy of the metasoma of the agamic female of *Disholcaspis quercusmamma* (Cynipini). (A) Photograph of the adult of the agamic generation. (B) Exocrine and reproductive organs of the metasoma studied in this article. (C–E) Reconstructed internal structures of the metasoma using micro-tomography. (C) Right-lateral view. (D) Detail of the venom apparatus in lateral view. e) Detail of the accessory gland complex in dorsal view. Legend: red arrow: insertion point of the gland into the reservoir; orange, venom gland; purple, venom reservoir; yellow, accessory gland; dark blue, accessory sac; green, ovaries; and light blue, gut. Axis: D, dorsal; V, ventral; A, anterior; P, posterior; L, left; R, right. Scale bar: 1 mm.

metasoma in 4 species, suggesting considerable variance in the size of the venom gland in this lineage. The venom glands of the rose gallers in Diplolepidini could not be measured due to their extremely reduced size which did not allow them to be distinguished from the atrophied reservoir. Similar to inquilines, the reservoir area of the 2 measured species represented less than 5% of the metasoma area. Finally, in parasitoid cynipoids, venom glands of all species were shorter than the metasoma, and only 3 species out of the 11 had a venom reservoir representing more than 5% of the metasoma area.

Reservoir morphology also varied among species (Fig. 5). Cynipini appeared to have the richest diversity of shapes. In the genera *Disholcaspis*, *Acraspis*, *Atrusca*, and *Philonix*, and in species *Andricus robustus* (Weld, 1926) and *Amphibolips quercusrugosa* (Ashmead, 1881), reservoirs were among the largest we measured (Supplementary Table S1) and were crescent shaped. The reservoirs of *Andricus quercuspetiolicola* (Bassett, 1863) and *A. kollari* (Hartig, 1843) were spherical. The reservoirs of the remaining Cynipini species were ovular, with insertion points of the ovipositor duct and the gland duct at the opposite extremity. In Diastrophini gallers, the reservoir of both *Diastrophus* species was bean-shaped, and the exit duct connected at 1 extremity, whereas the gland was inserted in the shape concavity as has been noted for *Xestophanes potentillae* (Retzius, 1773) (Vårdal 2006). Among Aylacini s.l., most species had oval reservoirs as found in previous studies (Vårdal 2006), but *A. laciniatus* (Gillette, 1891) and *A. silphii* had reservoirs with 2 regions: a narrow and curved portion connected to the gland that expanded into an enlarged portion joined with the ovipositor (Fig. 5). Finally, the reservoirs of the 3 groups of inquilines shared the same oval elongated shape (Fig. 5).

While the venom contained in the reservoirs was generally translucent, its appearance differed in several species (Fig. 6), suggesting that the molecules secreted into the reservoirs varied in composition across species. The venom color of the sexual generation of the *Amphibolips* genus ranged from pale yellow to dark brown for *Amphibolips acuminata* (Ashmead, 1896), including orange for *A. confluenta* (Harris, 1841) and *A. quercusinnanis* (Osten Sacken, 1861). Both generations of *C. seminator* (Harris, 1841) appeared to produce red venom. The venom of *A. unica*, *Philonix fulivicollis* (Fitch, 1859), and *Kokkocynips imbricaria* (Ashmead, 1896) were yellowish. The venom in *A. laciniatus* and *A. silphii* reservoirs was

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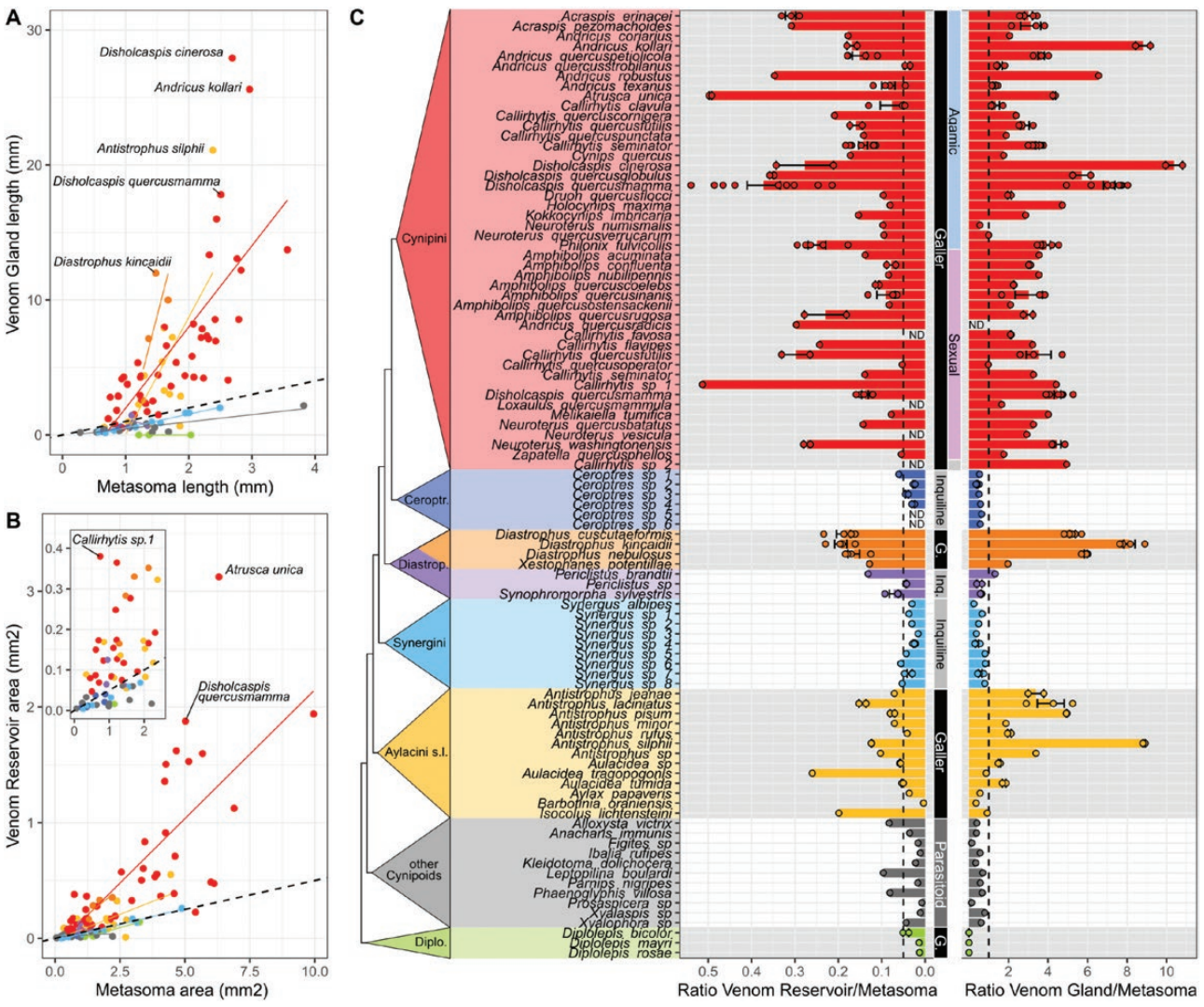

Fig. 4. Relative size of the venom apparatus in Cynipoids. (A) Venom gland length relative to metasoma length, with the names of the species having the largest venom gland over metasoma ratio indicated. (B) Venom reservoir area relative to metasoma area, with the names of the species having the largest venom reservoir over metasoma ratio indicated. (C) The ratios of venom reservoir area over metasoma area, and venom gland length over metasoma length for each species dissected. The dendrogram is adapted from Blaimer et al. (2020). For venom gland graphs, the dashed lines correspond to the venom gland length equaling the metasoma size, and to the reservoir area equaling 5% of the metasoma area for venom reservoir graphs. The central band indicates the species lifestyle (galler, inquiline, or parasitoid) and the generation for Cynipini (sexual, agamic, or unknown). The colors carry through from (A)–(C) and indicate the cynipoid tribes outlined in (C). Ceropt. = Ceroptrini, Diastrop. = Diastrophini, Diplo. = Diplolepidini, G. = Galler, Inq. = Inquiline, ND = Not dissected.

yellowish to reddish and slightly opaque. Finally, the reservoir *A. robustus* contained needle-like crystallizations similar to protein crystals.

Relationships between Venom Gland Size and Other Morphological Traits

Using ANOVA, we found that mean metasoma lengths of 6 of the tribes were not significantly different ($F_{7,88}$ = 2.009, P = 0.063, Supplementary Fig. S1) and our phylogenetic correction tests did not find a significant correlation between metasoma length or area and galling lifestyle (respectively, P = 0.32 and P = 0.55, Table 2), thus results on venom gland size are not attributable to clade-specific differences in body size. For all species combined, venom gland length correlated positively with metasoma length (n = 95, r = 0.62, P < 0.001 uncorrected; n = 75, P < 0.0001 when correcting for phylogeny) as well as the venom reservoir area and the metasoma area (n = 90, r = 0.62, P < 0.0001 uncorrected; n = 70, P < 0.0001 when correcting for phylogeny). The strongest correlation coefficients between metasoma length and venom gland length were detected for inquiline-containing lineages Ceroptresini (n = 6, r = 0.93) and Synergini (n = 10, r = 0.90), followed by Diastrophini (n = 6, r = 0.84) and cynipoid parasitoids (n = 11, r = 0.86). Cynipini (n = 39, r = 0.78) and Aylacini s.l. (n = 14, r = 0.68) (the main galling lineages) had the weakest positive correlation, suggesting a decoupling of these gland sizes from typical isometric growth in gallers. These correlations, however, point to the need for venom gland/reservoir size assessments to be corrected for body size, as was done in our comparisons here (e.g., venom gland length to metasomal length; VG/M).

For all species combined, we found no correlation between egg load and venom reservoir area (n = 67, r = 0.21, P = 0.028 uncorrected; n = 51, P = 0.99 when correcting for phylogeny). The correlation between egg load and venom gland length was significant without correction for phylogeny (n = 71, r = 0.31, P = 0.007) but

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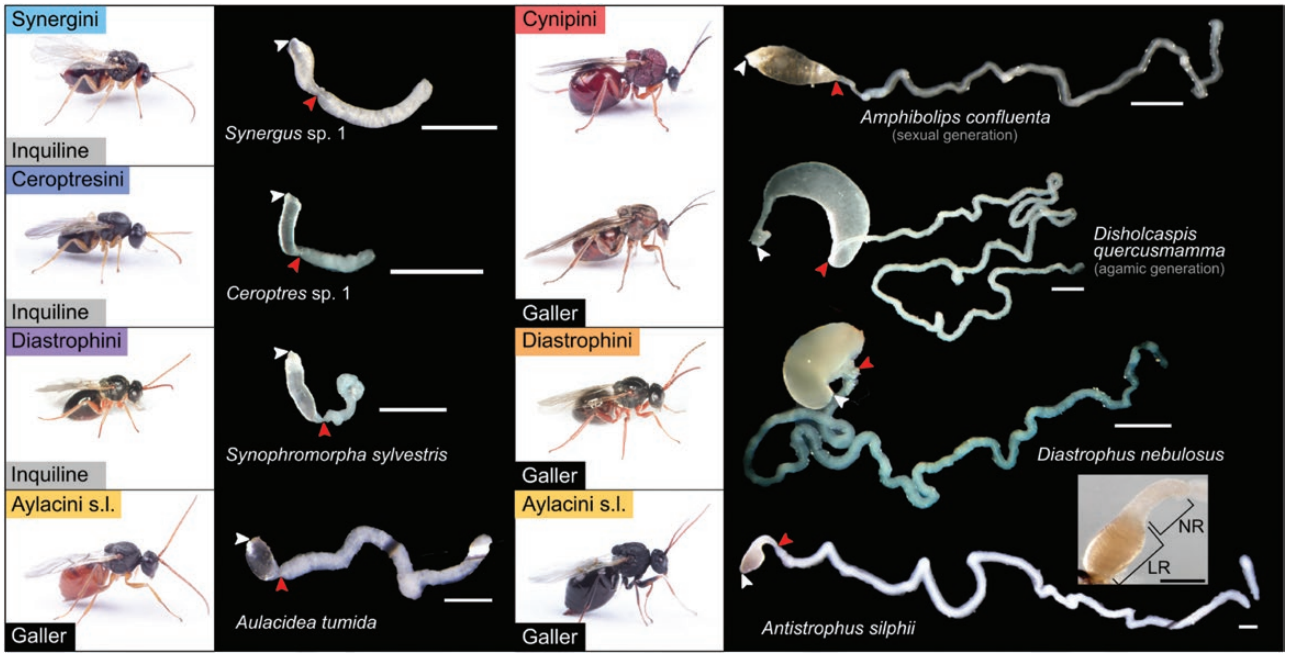

Fig. 5. Venom apparatus anatomy representative of different Cynipid tribes. Scale bar = 0.5 mm. White arrow: insertion point of the reservoir into the ovipositor; red arrow: insertion point of the gland into the reservoir. The insert for *Antistrophus silphii* shows the detail of the reservoir morphology, with division between the narrow region (NR) and the large region (LR).

not when correcting for phylogeny ($n = 55$, $P = 0.6$). The strength of correlation varied by lineage, with correlation coefficients very low for Cynipini, Ceroptresini, and Synergini ($r = 0.04$; $r = 0.10$; $r = -0.28$), higher for Aylacini s.l. and cynipoid parasitoids ($r = 0.52$, $r = 0.63$), and very high for Diastrophini ($r = 0.995$). When considered across all species, we found a positive correlation between the length of venom glands and the area of their reservoirs ($n = 89$, $r = 0.72$, $P < 0.0001$ uncorrected; $n = 70$, $P < 0.0001$ when correcting for phylogeny), thus supporting that longer venom glands promote more venom production.

Relationships between Venom Gland Size and Life History Traits

We tested the relationship between venom gland lengths and several nonmorphological or taxonomic traits (Fig. 7). Most notably, ANOVA supported the VG/M and VR/Ma ratios of gallers as significantly larger than that of inquilines and parasitoids ($F_{2,92} = 20.92$, $P < 0.001$, $F_{2,87} = 13.92$, $P < 0.001$, Fig. 7A). This strong relationship remained when correcting for phylogeny (respectively, for VG/M and VR/Ma, $P = 0.0001$, $P = 0.01$, Table 2). The VG/M ratio of Cynipini and Diastrophini gallers is higher than the inquiline lineages Ceroptresini and Synergini, and the parasitoid cynipoids ($F_{7,87} = 9.95$, $P < 0.001$), and the VR/Ma ratio of Cynipini is higher than other tribes with the exception of Diastrophini ($F_{7,82} = 7.234$, $P < 0.001$, Fig. 7B). The time-calibrated phenogram (Fig. 8) showed that expansion of the VG/M and VR/Ma ratio occurred in most gall-inducing clades but not Diplolepidini. Expansion of the venom gland apparatus appears to have occurred at the root of each gall-inducing clade, and extreme relative lengths have evolved repeatedly.

Venom gland and venom reservoirs ratios did not differ significantly by plant tissue (e.g., bud, stem, and leaf) in which the gall is induced (respectively, $F_{3,38} = 1.833$, $P = 0.158$, $F_{3,37} = 2.587$, $P = 0.0676$, Fig. 7C), nor according to the number of larval chambers in a given gall (respectively, $F_{1,42} = 0.338$, $p = 0.564$, $F_{1,42} = 0.186$, $p = 0.668$, Fig. 7D). Furthermore, among Cynipini, there were no significant differences in both VG/M and VR/Ma ratios between the sexual and the agamic generations, despite different galls of different size induced in different plant tissues for most of these species ($F_{1,43} = 0.968$, $P = 0.331$, $F_{1,41} = 0.657$, $P = 0.422$, Fig. 7E).

Histology and Ultrastructure of the Venom Apparatus

Based on SBFSEM and CLSM, venom glands of the Cynipini *A. erinacei*, *A. quercusbirta*, and *D. quercusmamma* contained 2 cell types: cells located at the periphery and characterized by large polyploid nuclei and an actin-rich region, and an intimal cell layer surrounding the central lumen comprising cells with small nuclei (Fig. 9A). The same 2 cell types were encountered in the venom glands of parasitoid cynipoids and corresponded, respectively, to secretory cells and intimal layer cell (Ferrarese et al. 2009). Secretions thus derive from secretory cells and travel through channels to a central lumen which carries secretions to the venom reservoir. In secretory cells, the actin-rich region is called the "end apparatus" and corresponds to the actin filaments associated with the microvilli typically encountered in the secretory region of class III gland cells (Noirot and Quennedey 1974, Ferrarese et al. 2009). Contrary to parasitoid cynipoids whose secretory regions are ≤ 10 µm long and straight and perpendicular to the lumen axis (Ferrarese et al. 2009), the end apparatus of the secretory cells of *D. quercusmamma* venom gland was curved and longer than 20 µm (Fig. 9A, B). The venom gland of *A. erinacei* was similar (Fig. 9C).

We describe the end apparati of 6 species using TEM (Fig. 10). The chitin-lined canal of the ductule cell extended inside the secretory cells to form class III gland cells, and the end apparati of these cells were at the level of and surrounding the cell nuclei of the gland cells in all species. In *D. quercusmamma*, the width of the end apparati ranged from 0.5 to 1 µm (Fig. 10A). They were surrounded by secretory vesicles containing 50 nm electron-dense

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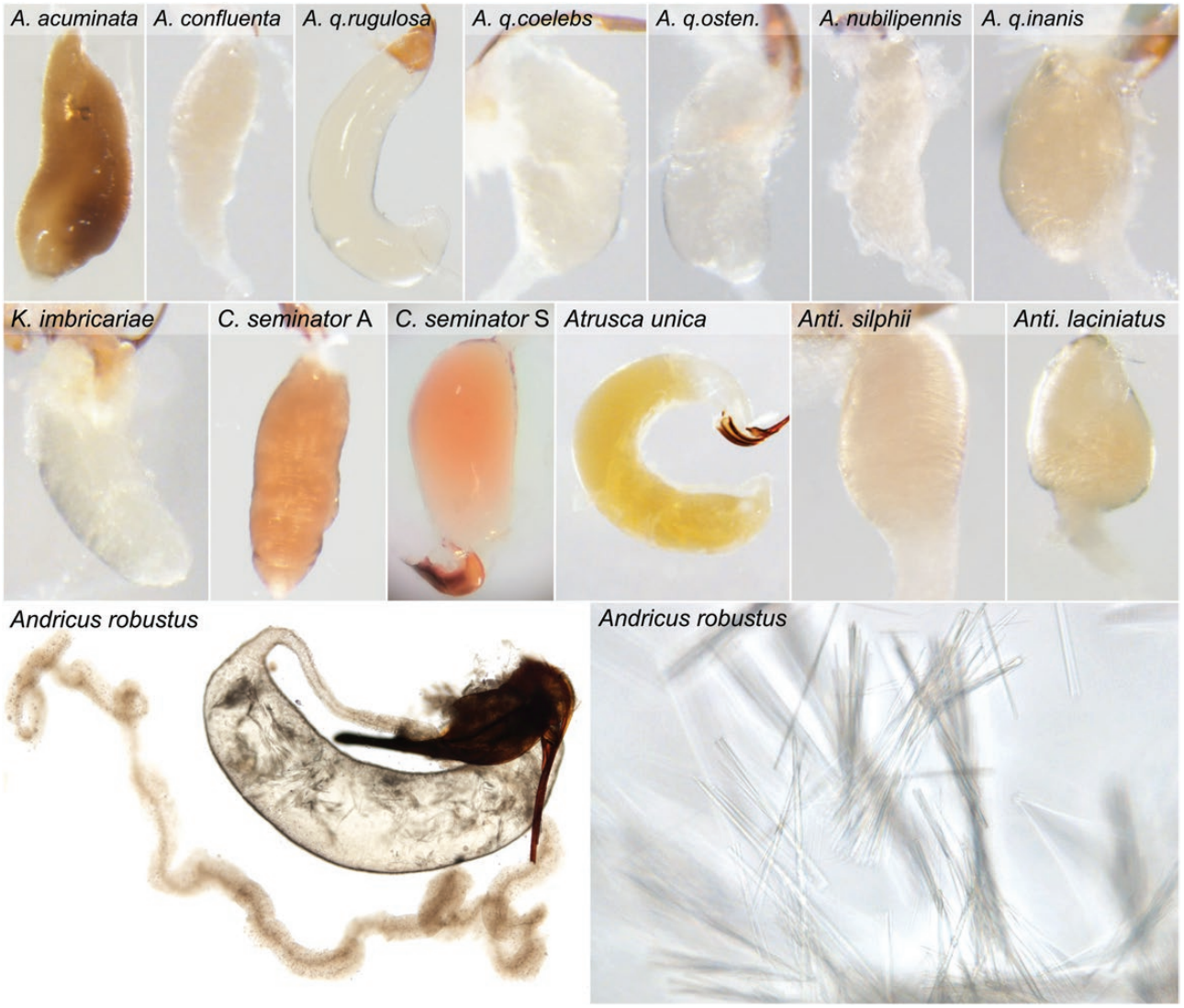

Fig. 6. Divergent appearances of Cynipid wasp venoms. The variable venom coloration in the sexual generation of the genus *Amphibolips*, ranging from light yellow in most species to dark brown in *A. acuminata* (first line). Other venom coloration observed in gall wasps (middle line). The agamic generation was dissected for *Kokkocynips imbricariae* and *Atrusca unica*. For *Callirhytis seminator*, A: agamic generation, S: sexual generation, Anti.: Antistrophus. The needle crystals found in the venom reservoir of *Andricus robustus* (bottom line).

particles (Fig. 10A, A', Supplementary Fig. S3). Particles of similar size and electron density connected to fiber-like elements found in the end apparatus, indicating these particles are secreted. The secretory region of *A. erinacei* was similar to *D. quercusmamma*, with a lower density of secretory vesicles that did not contain visible particles (Fig. 10B, B'). There were electron-dense particles bound to fiber-like structures in the end apparatus, but these were smaller (20–25 nm) than for *D. quercusmamma*. In *A. confluenta*, we found large electron-dense granules ranging from 1 to 2 µm diameter close to secretory regions (Fig. 10C). Smaller-sized nanometric particles accumulated in the end apparatus and migrated to the chitin-lined duct (Fig. 10C'). The secretory region of *D. nebulosus* (Osten Sacken, 1863) was lined by 1-µm-long microvilli and contained large vesicles merging with the extracellular membrane (Fig. 10D). These vesicles carry 2 visible components: 250-nm-diameter particles that were electron dense and lamellar bodies (Fig. 10D', D"). The vesicle composition suggests the 250 nm particles result from smaller nanometric particle agglomeration (Fig. 10D'). The dark particles were also encountered in the extracellular medium (Fig. 10D"). In *A. laciniatus*, we found that secretory vesicles and dense granules accumulated around the end apparatus (Fig. 10E, E'). Finally, the secretory region of the inquiline *Synergus* sp. was the smallest observed, with microvilli shorter than 1 µm and a central canal about 0.5 µm wide (Fig. 10F). The lighter color of the cytosol and vesicles suggests a higher lipid concentration in the secretions and no clear particles were observed. Although exemplary figures are shown (Fig. 10), each of these interpretations is drawn from multiple images, exemplified by the more extensive diagrams presented for *D. quercusmamma* in Supplementary Fig. S3, for *A. confluenta* in Supplementary Fig. S4, for *Synergus* sp. in Supplementary Fig. S5, and for *D. nebulosus* in Supplementary Fig. S6.

Comparative Anatomy of the Reproductive Apparatus

We dissected accessory glands and sacs of 42 species, to which we added data from previous publications for *Diplolepis rosae* (Linnaeus, 1758) (Cambier et al. 2019) (Fig. 11 and Fig. 12, Supplementary Table S1). The sacs were encountered in most tribes

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Table 2. Results of the phylogenetically corrected generalized least squares regression (PGLS).

		Std. error	*t* value	Lambda	*F* value	*P*	
Lifestyle	Metasoma length (M)	0.2914001	−1.00945	0.650	1.018985	0.3161	NS
Lifestyle	Metasoma area (Ma)	0.8951958	−0.60223	0.727	0.362682	0.5489	NS
Lifestyle	Venom gland length (VG)	1.445488	−3.23419	0.249	10.46001	**0.0018**	**
Lifestyle	Venom Reservoir area (VR)	0.1734094	−1.50516	0.373	2.265494	0.1369	NS
Lifestyle	Venom gland relative length (VG/M)	0.6582853	−4.08185	0.476	16.66149	**0.0001**	***
Lifestyle	Venom reservoir relative area (VR/Ma)	0.0335535	−2.64401	0.480	6.990788	**0.0102**	*
Lifestyle	Egg load	26.09203	−8.375	−0.085	70	**<0.0001**	***
VG	Egg load	0.0026541	0.52135	0.380		0.6043	NS
VR	Egg load	0.0002676	0.00878	0.476		0.993	NS
VG	VR	0.9184072	7.40134	0.145		**<0.0001**	***
M	Egg load	0.0002571	2.81895	0.866		**0.0068**	**
M	VG	0.0123048	6.29777	0.803		**<0.0001**	***
Ma	VR	0.3575066	6.2327	0.884		**<0.0001**	***

Significant p-values are in bold and asterisks indicate level of significance (*=<0.05, **=<0.01, and ***=<or =0.0001).

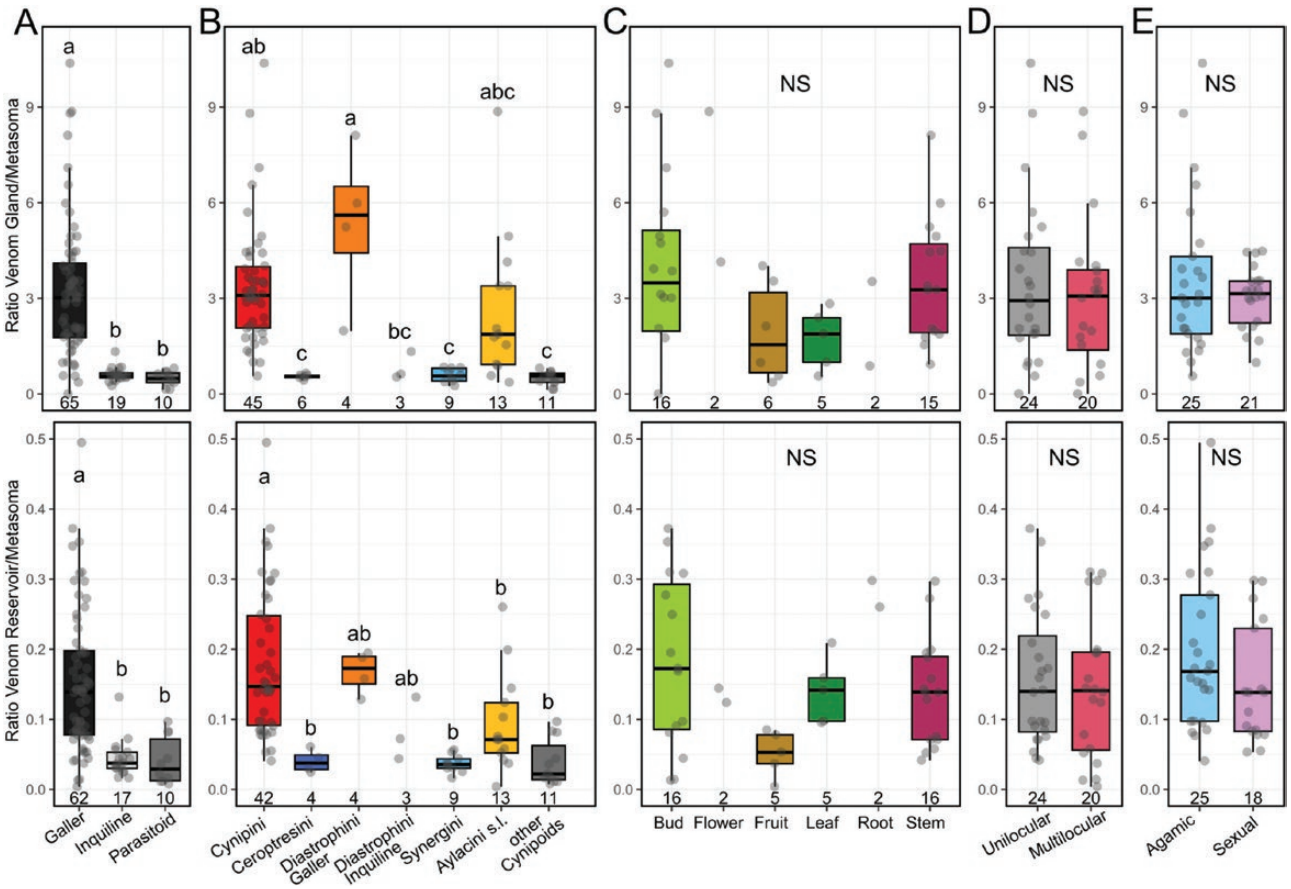

Fig. 7. Boxplots of the relative size of the venom apparatus in Cynipoids in relation to taxonomic group and life-history traits. This includes (A) lifestyle, (B) tribes, (C) plant tissue attacked among gallers, (D) number of larval chambers in the induced galls (uni- or multilocular), and, among Cynipini, (E) generation. Diplolepidini were excluded due to their absence of fully developed venom apparatus. Bars marked with a different letter indicate significant difference, NS indicates a non-significant difference (Tukey post hoc test, $P \leq 0.05$).

dissected but not the 2 earliest diverging lineages: Diplolepidini and parasitoid cynipoids. The diameters of the sacs of non-Cynipini (except *Diastrophus* gallers) did not vary with metasoma size, and were constant around 0.18 mm. On the contrary, the diameters of Cynipini sacs were strongly positively correlated with metasoma length ($n = 32$, $r = 0.64$, $P < 0.0001$). The largest sacs were found in *A. robustus* with a ratio sac length over metasoma length of 0.5. The accessory sacs appeared to contain a white substance, except

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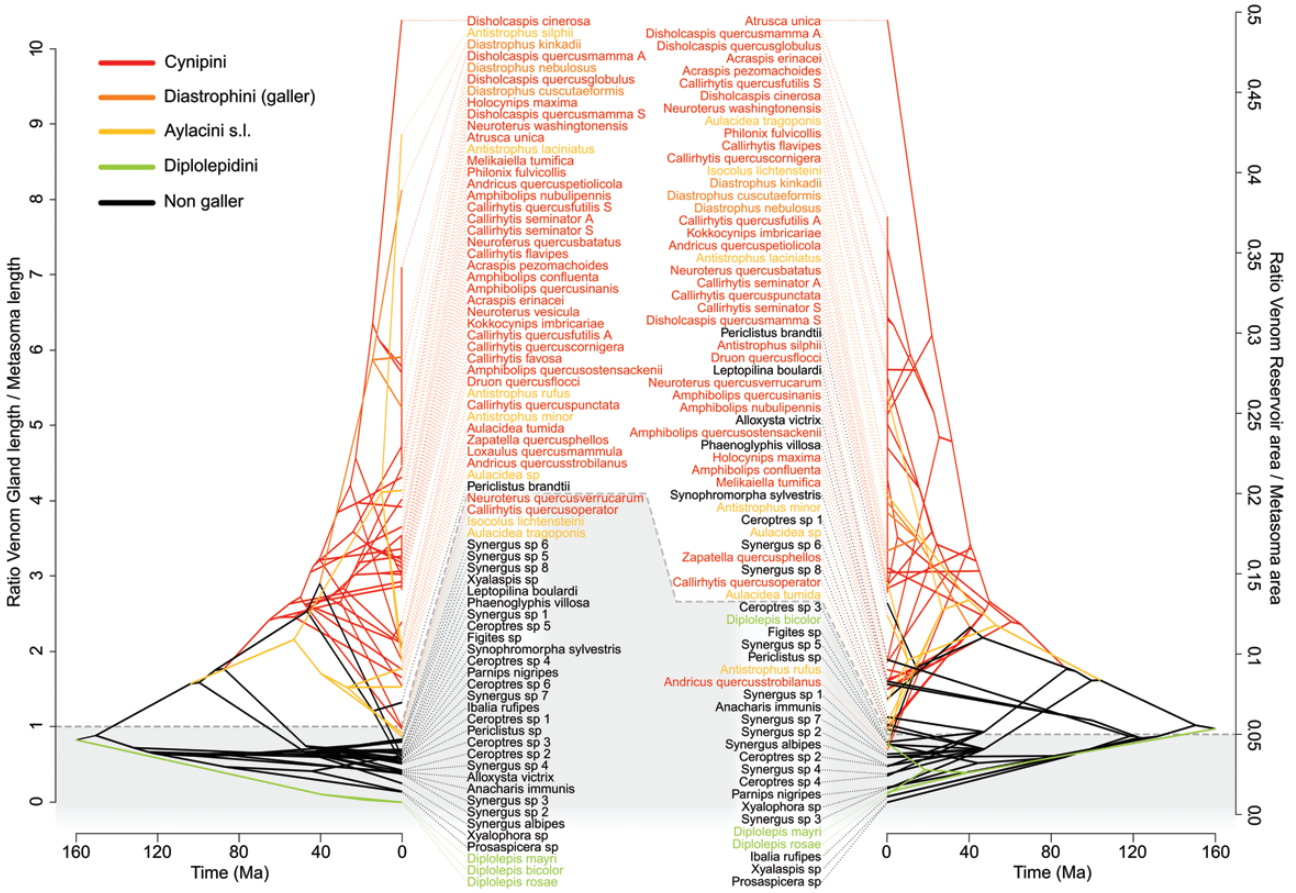

Fig. 8. Evolutionary pattern of the venom apparatus relative size in Cynipoids. Phenograms depicting the relative size of the venom gland on the left (ratio venom gland length over metasoma length) and the relative size of venom reservoir on the right (ratio venom reservoir area over metasoma area), with indication of the four gall-inducing clades. The phenograms are based on a time calibrated phylogeny of Cynipoidea (Blaimer et al. 2020, Ward et al. 2022), and phylogenetic tree tips are plotted according to their phenotypic value. A: agamic generation, S: sexual generation.

in the two inquilines and in *Aulacidea tumida* (Bassett, 1890) (Fig. 12). The accessory glands were visible in only 12 species belonging to the tribes Cynipini, Aylacini s.l., and Diplolepini (Fig. 10B). In *Diplolepis rosae*, while sacs are absent, the glands reached twice the metasoma length. Among Cynipini, the genera *Disholcaspis* (Fig. 3), *Acraspis*, and *Philonix* had the longest glands, with a maximum of 9.6 mm for *D. quercusmamma*, representing about four times the metasoma length. In Aylacini s.l., *A. silphii* had long accessory glands, with 3.4 mm representing 1.3 times the length of the metasoma. Due to an insufficient sample size in different groups, we were not able to statistically compare sac size in the different tribes and life-history groups.

Three-dimensional reconstruction from confocal images of the reproductive apparatus of *D. quercusmamma* (agamic generation) revealed that the accessory gland was covered by a network of actin, the shape of which indicates the presence of smooth muscles on the surface of the gland (Fig. 13A, B). No muscle was found on the surface of the sacs. Accessory gland cells are full of spherical lipid bodies (Fig. 13D, E), whereas the center of the sac contains a homogenous lipid-rich substance (Fig. 13G, H). Sac cells and oviduct cells both contained small lipid vesicles near the nucleus (Fig. 13H, I). TEM of *A. quercushirta* accessory glands revealed an external layer of smooth muscle cells attached to the basal lamina (Fig. 13C) and an internal layer of cells with extensive lamellar bodies (Fig. 13F). We did not find any end apparati in the accessory gland and the accessory sac.

Comparative Anatomy of the Ovaries

Egg load varied considerably among species, with a maximum of 1,800 eggs for *Callirhytis quercuspunctata* (Bassett, 1863) (Supplementary Fig. S2). Egg loads varied significantly with respect to life history ($F_{2,78} = 5.348$, $P < 0.01$ uncorrected; $P < 0.0001$ when corrected for phylogeny) with gallers having significantly more eggs than inquilines (Supplementary Fig. S2D). However, egg load was not significantly different among tribes ($F_{6,72} = 2.228$, $P = 0.0499$, Supplementary Fig. S2E), among plant tissues attacked by gallers ($F_{5,36} = 0.629$, $P = 0.678$, Supplementary Fig. S2F), nor between species inducing unilocular or multilocular galls ($F_{1,42} = 0.098$, $P = 0.755$, Supplementary Fig. S2G) or between Cynipini sexual and agamic generations ($F_{1,29} = 1.742$, $P = 0.197$, Supplementary Fig. S2H). Galler ovarioles contained only mature eggs suggesting adults emerge with mature eggs, with the exception of *A. tumida* and *Antistrophus pisum* (Walsh, 1869), which apparently contain at least some developing eggs (Fig. 14). All inquilines ovarioles contained both mature and developing eggs (Fig. 14).

Discussion

Our results reveal variation in gland size and morphology among cynipid groups that supports the involvement of venom glands and accessory glands in galling. In general, venom glands and accessory glands are larger in galling lineages, supporting their role in this life history trait. Nevertheless, not all galling lineages vary in

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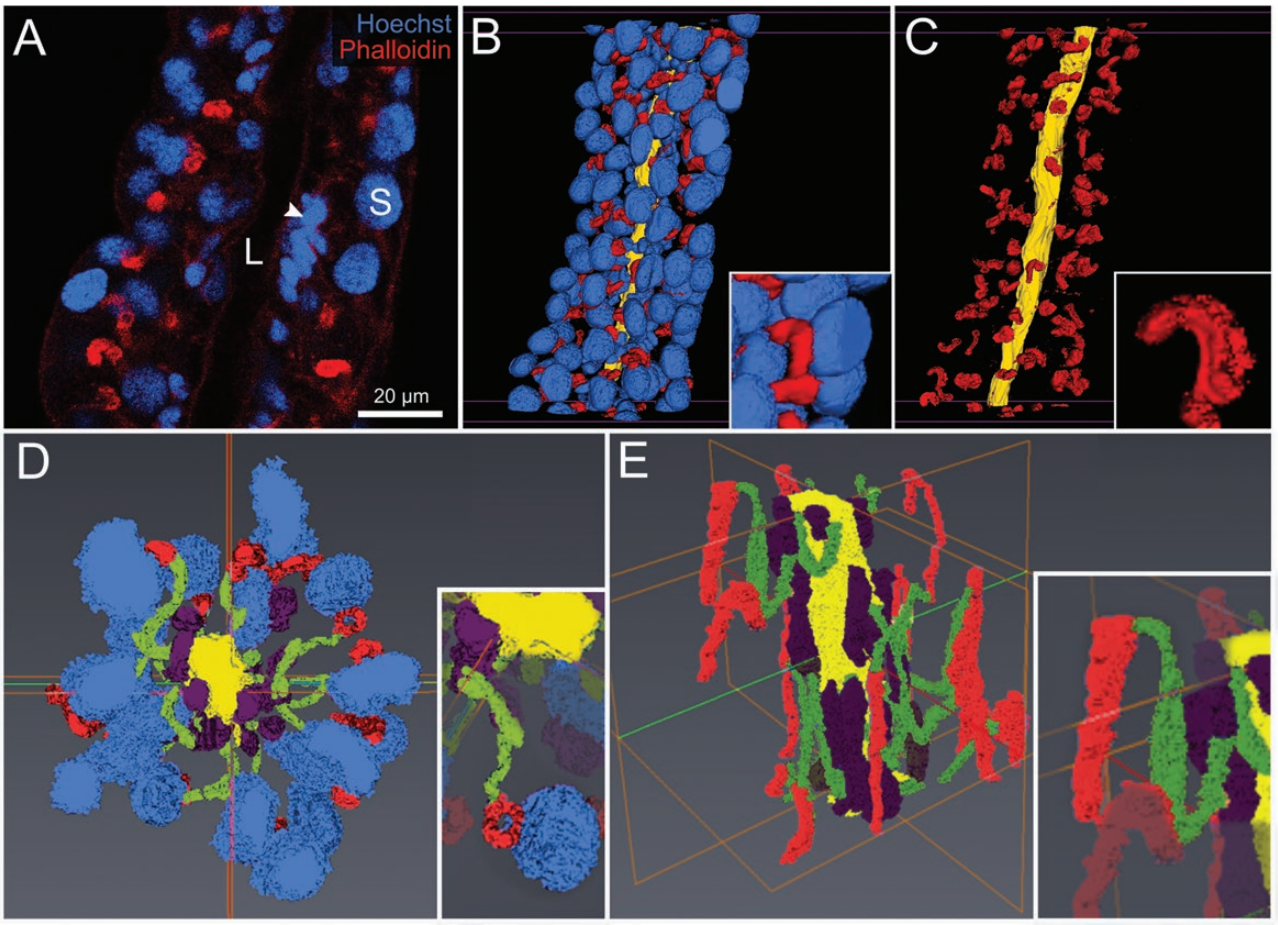

Fig. 9. Ultrastructure of Cynipini venom glands. (A) Transversal section of a venom gland of the agamic generation of *Disholcaspis quercusmamma*, obtained with confocal microscope and stained with Hoechst (DNA) and phalloidin (actin). S: nuclei of secretory cells; L: lumen; white arrow: nuclei of ductule cells. (B, C) Volume rendered micrographs of *D. quercusmamma* agamic generation venom gland. (B) Secretory cell nuclei (blue) and end apparati (represented by red actin canals), with a subfigure showing the proximity between the nuclei and the secretory region. (C) End apparati of secretory cells, with a subfigure showing the curvature of the secretory region. Yellow: lumen. (D, E) Surface rendered micrographs of *A. erinacei* agamic generation venom gland obtained by serial block face scanning electron microscopy (D: top view, E: lateral view). The subfigures show the connections between the subcellular elements. Red: end apparatus; green: duct; yellow: lumen; blue: secretory cell nuclei; and purple: ductule cell nuclei.

these structures in the same way. The diversity of sizes and secretory morphology in these glands across galling lineages of these wasps suggests that they likely employ diverse strategies for gall induction.

Venom Apparatus Enlargement Related to Gall Induction

Specifically for gall-inducing clades, we found enlargement of the venom apparatus beyond expectations given body size. While size does not by necessity suggest function, this result strongly supports the role of venom in gall induction. Consistent with previous work (Vårdal, 2006), we found that Cynipini had the largest apparatus, and we found the largest venom glands occurred in the genus *Disholcaspis*. For *D. quercusmamma*, this gland occupied 29% of their metasoma volume, 5 times larger than the volume of the ovaries and larger by metasoma size than has been recorded in any Hymenoptera; thus, these venoms must play important roles for these wasp species. We also found more pronounced venom-gland sizes in other galler lineages, including Aylacini s.l. and Diastrophini, than has been previously recorded (Vårdal 2006). Most notably, we found that each of the inquiline lineages (Ceroptresini, Diastrophini, and Synergini), as well as cynipoid parasitoids, had significantly smaller venom apparati, with smaller venom glands and reservoirs than, and reduced secretory apparatuses compared to, gall-inducing Cynipidae, consistent with the inability of these groups to induce galls. The only exception was Diplolepidini, a gall-inducing tribe exhibiting a vestigial venom apparatus. A recent phylogeny placed this clade outside of the traditional bounds of Cynipidae (Blaimer et al. 2020), possibly suggesting an independent origin of galling in Diplolepidini. The lack of venom glands in rose gallers suggests they likely do not use venom in the same way as other gallers. *Diplolepis* is the only lineage for which careful observations have been taken of the role of maternal secretions, with data suggesting they may be involved in clearing space through cell degradation for the developing embryo, but unlikely to play the main role in cell proliferation (Brooks and Shorthouse 1998). Given the reduced role of venom in these species, it will be important to compare how venoms may affect plant development in species with more pronounced venom glands.

Although gallers generally have larger venom glands/reservoirs than non-galling Cynipidae, within gall inducers, the weak correlation between venom gland and metasoma lengths suggests that the importance and volume of venom involved in gall induction varies among species. The greatest contrast we encountered was in Aylacini s.l., in which the poppy gallers *Barbotinia* and *Aylax* have greatly

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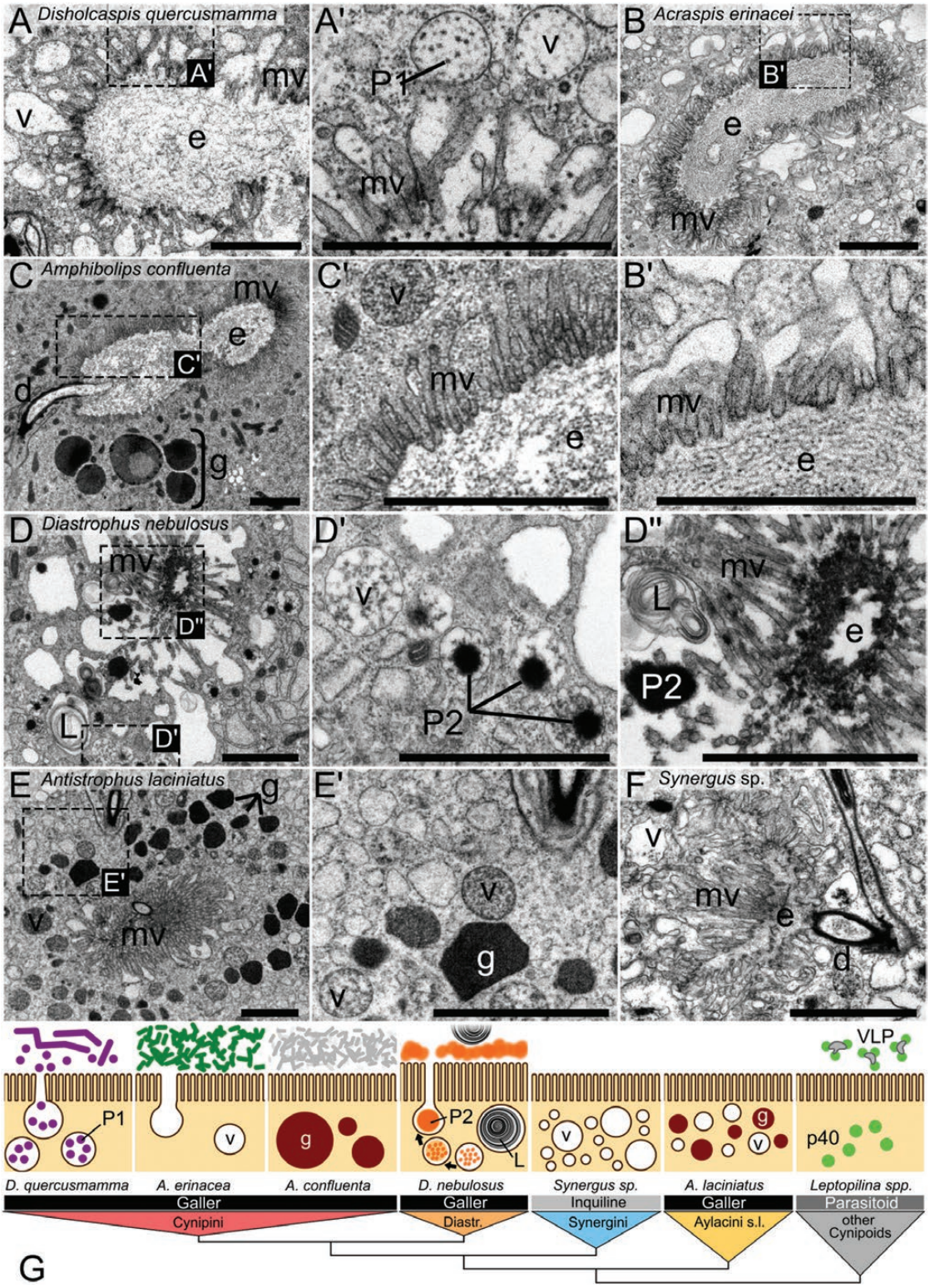

Fig. 10. Transmission electron micrographs of the venom gland secretory region of Cynipoids. (A, A') *Disholcaspis quercusmamma* agamic generation. (B, B') *Acraspis erinacei* agamic generation. (C, C') A*mphibolips confluenta* sexual generation. (D, D', D'') *Diastrophus nebulosus*. (E, E') *Antistrophus laciniatus*. (F) *Synergus* sp. (G) Schematic summarizing the organization of the venom secretory unit inferred from TEM images. The extracellular medium of the 3 tested Cynipini contain high concentrations of nanometric particles. In *D. quercusmamma*, these particles (P1) are also found in secretory vesicles and seem to assemble into fibers once secreted. In *A. erinacei*, some secretory vesicles are observed, without particle content, whereas no vesicle are clearly visible in *A. confluenta*. In this last species, secretory units are surrounded by large granules. In *Diastrophus nebulosus*, nanometric particles seems to assemble into a 250 nm particle (P2) within secretory vesicles. Once secreted these P2 seem to form larger agglomerations. Lamellar bodies are also secreted in this species. The secretory unit is reduced and surrounded by light vesicles that do not contain any particles in the inquiline *Synergus* sp. An accumulation of vesicles and granules is observed around the secretory unit of *A. laciniatus*. In comparison, virus-like particles are secreted in *Leptopilina* sp. (adapted from Ferrarese et al., 2009). Legend: d: duct, e: extracellular medium, v: vesicle, mv: microvilli, g: granules, P1: 50 nm particle, P2: 250 nm particle, L: lamellar body, VLP: viruslike particle, p40: virus-like particle protein p40. Scale bar = 2 μm.

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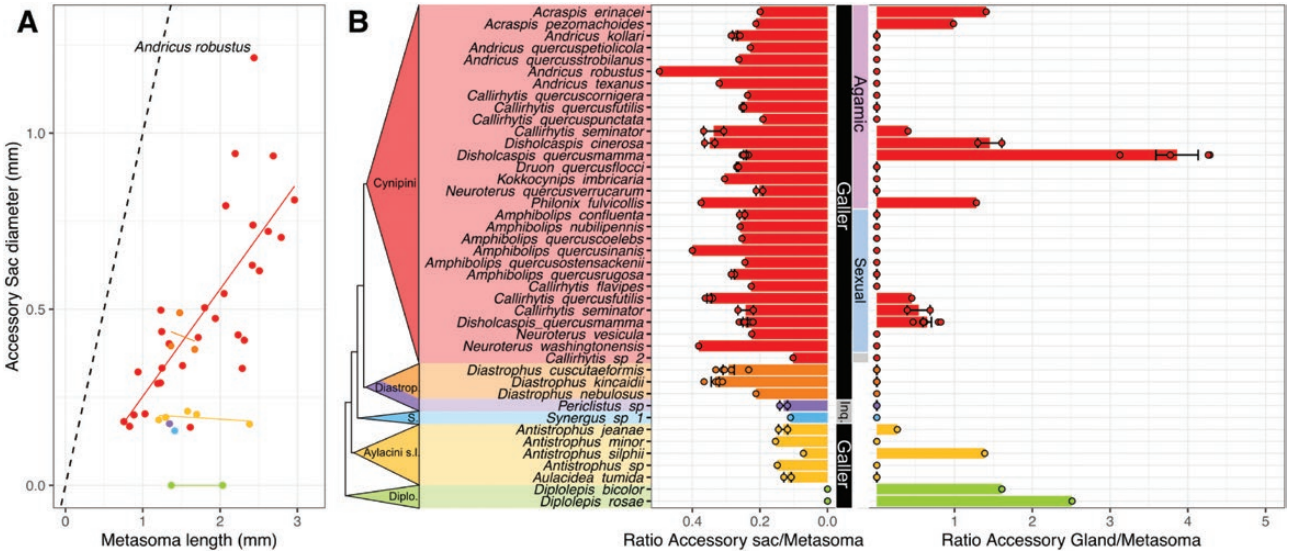

Fig. 11. Relative size of the accessory apparatus in Cynipoids. Accessory sac diameter relative to metasoma length, with the name of the species with the highest ratio. The ratios of (A) accessory sac area over metasoma area and (B) accessory gland length over metasoma length for each species dissected. The dendrogram is adapted from Blaimer et al. (2020). The band indicates the species lifestyle (galler or inquiline) and the generation for Cynipini (sexual, agamic, or unknown). The dot colors in (A) correspond to the cynipoid tribes displayed in the dendrogram in (B).

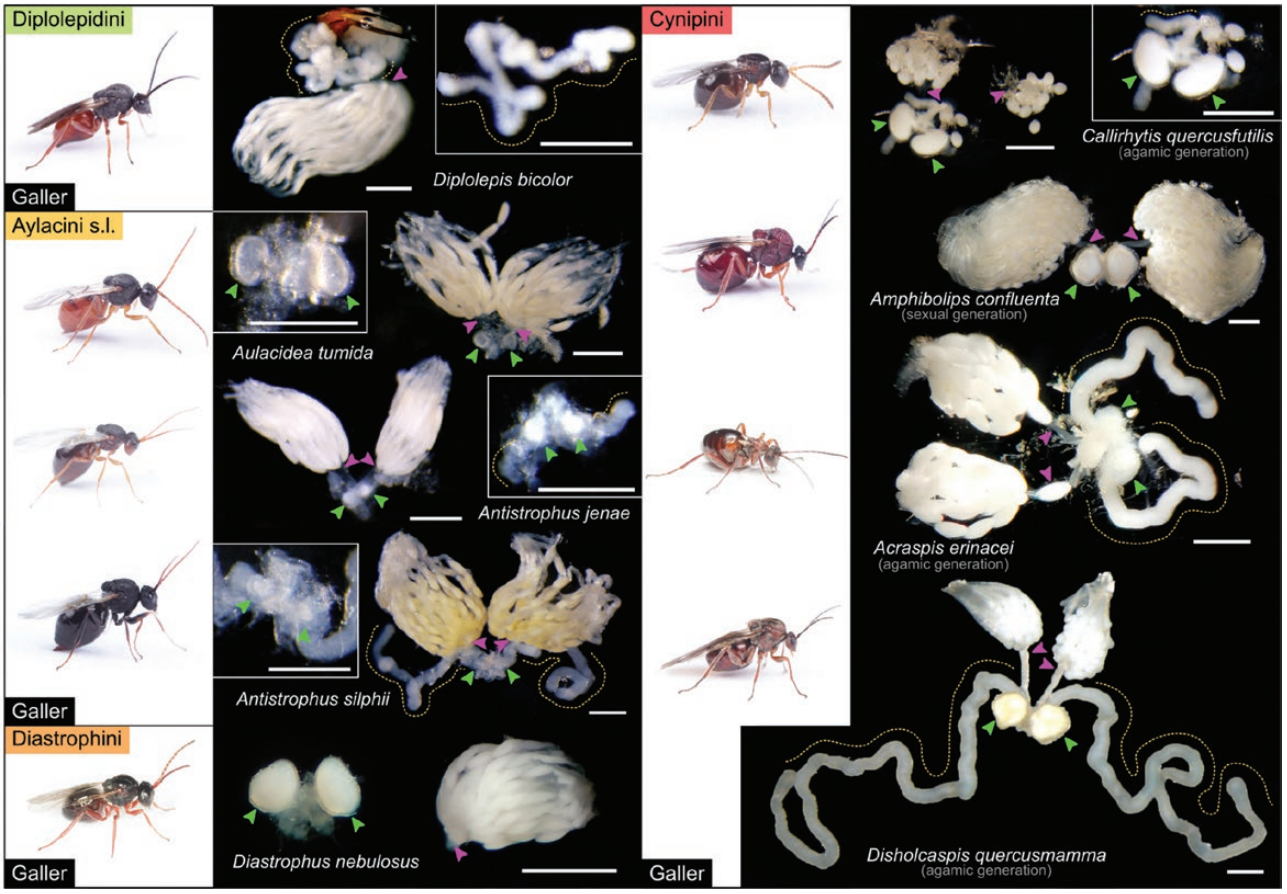

Fig. 12. Reproductive apparatus anatomy of various Cynipids. Scale bar = 0.5 mm. Ovaries and accessory glands of 8 species belonging to three tribes, with insets showing the detail of the accessory sacs when necessary. Green arrow: accessory sacs, pink arrow: ovary extremity, and yellow dashed line: accessory gland.

reduced venom apparatuses, whereas the *Silphium* galler *A. silphii* has one of the longest venom glands we measured. This large difference suggests that even within groups, there is unlikely a single strategy that is employed by all gall inducers and that the mechanism used by each species should be examined separately. The strategy employed may vary based on the targeted plant species, or even the

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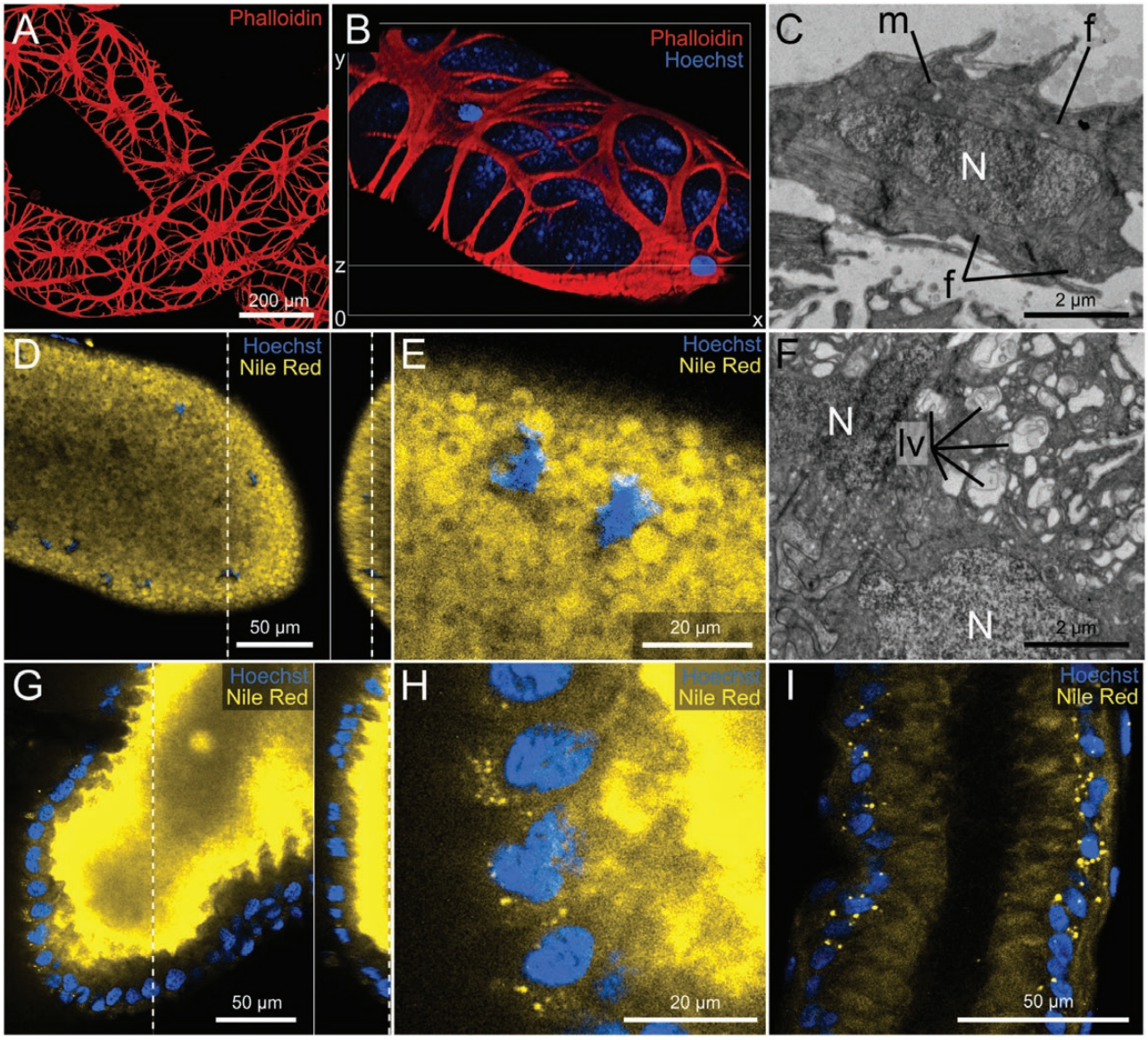

Fig. 13. Ultrastructure of the reproductive apparatus of Cynipini. (A, B) Volume rendered micrographs of accessory gland of the agamic generation of *Disholcaspis quercusmamma* confocal images stained with phalloidin (actin), revealing smooth muscles cells, and Hoescht (blue), revealing nuclei. (C) Transmission electron micrograph of a smooth muscle cell on the accessory gland of the agamic generation of *Acraspis macrocarpae*. (D, E) Confocal images of an accessory gland of *D. quercusmamma* stained with Hoescht (DNA) and Nile Red (lipid). (F) Transmission electron micrograph of the accessory gland of *A. macrocarpae*. (G–I) Confocal images of: (G, H) an accessory sac and (I) an oviduct of *D. quercusmamma* with Hoescht and Nile Red staining. Legend: N: nucleus, f: muscle fiber, m: mitochondria, lv: lipid vesicle.

targeted plant tissue. We did not find significant differences in venom gland and reservoir sizes based on the plant organ targeted by the gallers.

Beyond size, venom apparati also differed in other morphological characteristics that suggest that venom composition varies among species. We found venom to range from translucent to opaque, suggesting different compositions. The most striking difference was in venom color, with *C. seminator* and *Amphibolips acuminatus* producing red and brown pigmented venoms, respectively. We found in 2 species with milder venom pigmentation, *A. laciniatus* and *A. confluenta*, an accumulation of numerous or large granules in secretory cells at the vicinity of the secretory region that may correspond to the pigment storage. Pigments are known to play diverse roles in molecular reactions, such as redox reactions and molecular catalysts, and thus may contain important substances for interacting with plant tissues (Moran and Jarvik 2010). At the microscopic level, we observed the secretion of nanometric particles in Cynipini, whereas *D. nebulosus* nanometric particles seemed to assemble into 250 nm particles that are secreted. Finally, we noticed the secretion of lamellar bodies by the venom gland cells of *D. nebulosus*. Lamellar bodies are large secretory organelles with surfactant properties (Schmitz and Müller 1991). Surfactant properties were found in the oral secretions of caterpillars and help stick the secretion to hydrophobic surfaces (Rostás and Blassmann 2009), which in the case of the venom could help the liquid to stick with the egg surface.

Venoms in aculeate wasps serve as defense against other animals, a function not considered of importance for smaller Parasitica wasps like cynipoids (Poirié et al. 2014). Parasitoid wasps use their venom in part for immunity against host animals, which in this case is not needed. Given what is known about early gall formation,

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Fig. 14. Ovariole anatomy of various Cynipids. Ovarioles of 19 species belonging to five tribes, with insets showing the egg development stage. M: mature egg, D: developing egg, Ga.: galler species, and In.: inquiline species. Square colors correspond to tribe colors introduced in Fig. 2.

conceivable functions of venom for these herbivorous gallers, include cell lysis for initiating galls, defense against plant immunity and oxidative stress, and/or in inducing developmental changes in the plant. A supplementary function of venom that would be unrelated to galling could be as an oviposition lubricant. In Diastrophini, venoms may be involved with both gall induction and lubrication of oviposition because gallers in this taxon had large venom apparati, whereas inquilines did not, and species had strong correlations between venom gland length and egg load. In general, however, egg load did not explain patterns in venom gland size across Cynipidae.

Functions of Accessory Glands and Sacs

Our study provides the first detailed description of the accessory glands of cynipids. We observed a muscular framework covering the accessory glands of *D. quercusmamma* and *Acraspis macrocarpae*. These muscles likely produce gland contraction and release of gland secretions. Similar muscle coverings have been observed for other insect glands, like in salivary glands or Dufour glands (Ratcliffe and King 1969, Abdalla 2006, Guerra et al. 2015, Borella Marfil Anhê et al. 2021). The relative size of accessory glands appears to be quite variable among species and does not correlate with their lifestyle. Three gall-inducing clades, however, had substantially enlarged accessory glands: the clade formed by the genera *Acraspis*, *Disholcaspis*, and *Philonix* in Cynipini, the closely related species *A. laciniatus*/*A. silphii* in Aylacini s.l., and Diplolepidini. Gland function may diverge in these clades. For example, given the lack of venom glands in Diplolepidini, accessory glands could provide an alternative source of maternal secretions that influence gall induction. As previously

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suggested (Adler 1877), our results support that accessory glands are lipid-rich organs. However, contrary to previous suggestions, we did not find longer accessory glands in sexual generations (Adler and Straton 1894), excluding an exclusive role of spermatheca for this organ. In other insect species, accessory gland secretions serve as lubricants that facilitate the transport of oocytes through ovipositors (reviewed in Gillott 2003). The low egg load in the three clades with enlarged accessory gland, and more generally the lack of correlation between accessory gland size and egg load, suggests its function is not limited to facilitating egg transport. *Disholcaspis* have the highest contrast, with the largest accessory gland relative to body size among cynipoids, and possibly among all Hymenoptera, but also with among the lowest egg load of the tribe. In other insect orders, the accessory glands can produce oothecae, structures that enclose eggs deposited by the female and protect them from desiccation, predation, and parasitism (Chapman et al. 2013). The agamic generations of the clade *Acraspis/Disholcaspis/Philonix* have the largest accessory glands and have in common the laying of eggs in oak buds in fall, followed by hatch at the end of winter, a trait which may require desiccation protection. Further investigation should test the presence of such egg secretions in these taxa. Overall, given variation in size of accessory glands, it seems likely that their contents are involved in gall induction and/or formation for some species, but are unlikely to have a generalized gall-induction function.

In this study, we describe the histology of the organ that we named the "accessory sac," previously described as the smear gland (Frühauf 1924). First mentioned in Adler (1877), our results show this organ is a synapomorphy of Cynipidae *sensu* Blaimer et al., (2020). The strong correlation between sac and metasoma sizes in Cynipini suggests a conserved function, whereas the presence of sacs of similar size in inquilines tends to indicate this function may not be related to gall induction. However, gallers (with the exception of *A. tumida*) had sacs that contained a white substance absent from inquilines sacs. According to microscopy, this substance is lipidic and may be produced in the sac itself, given that sac size is independent of the size and presence of the accessory gland.

Egg Load

Our observations of ovariole morphology indicate that inquilines are synovigenic, developing eggs continuously, whereas almost all gallers are proovigenic, emerging with all eggs mature. We confirmed the hypothesis that proovigenic wasps tend to have more eggs than synovigenic (Vårdal et al. 2003). In particular, the absence of developing eggs in Cynipini after emergence is consistent with the lack of feeding by emerged adults and is a common characteristic of proovigenic wasps in general (Quicke 1997). In addition, we noticed a yellow coloration of *A. tumida* eggs that is consistent with the recent discovery of carotenoid biosynthesis and accumulation in *Aulacidea hieracii* (Nikelshparg et al. 2022), suggesting carotenoid production may be a characteristic of the genus *Aulacidea*.

Our overall analyses did not find that egg number correlated significantly with relative venom gland size, but that egg number is lower in inquilines, which also have significantly smaller venom glands. Inquilinism, therefore, is a better explanatory variable of venom gland size than egg number. Nevertheless, the synovigenic nature could be one factor involved in inquilines having smaller venom glands as, if these are involved in oviposition, they could be produced in smaller volumes over time in inquilines.

Possible Role of Symbionts in Gall Induction

Our comparative microscopic study of venom glands revealed secreted particles of various sizes and shapes that should be further characterized. Their characteristics do not match those of virus-like particles produced by cynipoid parasitoids, because they are either too small or too large and do not display any visible repetitive pattern (Ferrarese et al. 2009). We found no bacteria-like structures. This result is consistent with recent genomic investigations that failed to identify any virus-like particle genes in the genome of an oak gall wasp, *B. pallida* (Cambier et al. 2019, Hearn et al. 2019), and failed to find bacteria using microbiota sequencing (Hammer et al. 2021).

Conclusions

Mechanisms by which insect species, like gall wasps, can induce predictable changes in plant development, yielding an extended phenotype, remain an exciting but poorly understood area of research. Our study improves our collective understanding of the morphology and histology of metasomal glands in gall wasps with likely relevance for gall induction. Our comparative morphology data strongly suggests female oviposition secretions play a role in gall induction. Among the three secretory organs possibly involved, the best candidate is the venom apparatus, which shows a massive expansion in galling groups compared to clades with other lifestyles. The accessory sacs, present only in Cynipidae and containing a white substance only in galling species, may also play a role. Reduction of accessory glands in most gallers decreases the likelihood that they have a gall-inducing role in all cynipids; however, the large size of these glands in species with reduced egg loads suggests a function different than facilitating oocyte transport. In particular, the exceptional expansion of the venom gland and accessory glands in *D. quercusmamma*, being among the largest glands of these categories of Hymenoptera, suggests both gland secretions may interact with plant development. Furthermore, our study points to the likelihood that gall induction in gall wasps does not involve just a single universal strategy but the molecular effectors in different lineages likely have shifted over time. The diversity of secretory strategies, from potential roles of pigments and secretory particles of different sizes, and the inferred variable role of venom and accessory glands by clade, suggests that future research on mechanisms of gall formation should expect to find lineage-specific differences.

Supplementary material

Supplementary material is available at *Insect Systematics and Diversity* online.

Specimen Collection Statement

Nagoya Protocol: The authors attest that all legal and regulatory requirements, including export and import collection permits, have been followed for the collection of specimens from source populations at any international, national, regional, or other geographic level for all relevant field specimens collected as part of this study.

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Data availability

Data from this study are available from ScholarSphere (https://doi.org/10.26207/hf9y-py47).

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