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Sweet tetra-trophic interactions: multiple evolution of nectar secretion, a defensive extended phenotype in cynipid gallwasps

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Abstract

Many herbivores employ reward-based mutualisms with ants to gain protection from natural enemies. We examine the evolutionary dynamics of a tetra-trophic interaction in which gallwasp herbivores induce their host oaks to produce nectar-secreting galls, which attract ants that provide protection from parasitoids. We show that, consistent with other gall defensive traits, nectar secretion has evolved repeatedly across the oak gallwasp tribe and also within a single genus (*Disholcaspis*) that includes many nectar-inducing species. Once evolved, nectar secretion is never lost in *Disholcaspis*, consistent with high defensive value of this trait. We also show that evolution of nectar secretion is correlated with a transition from solitary to aggregated oviposition, resulting in clustered nectar-secreting galls, which produce a resource that ants can more easily monopolize. Such clustering is commonly seen in ant guard mutualisms. We suggest that correlated evolution between maternal oviposition and larval nectar-induction traits has enhanced the effectiveness of this gall defense strategy.
Introduction

Many herbivorous insects use sugar-rich secretions to recruit ant guards, which in turn protect the herbivores from their natural enemies. This food reward-based mutualism (trophobiosis) usually involves three trophic levels: the herbivore (trophobiont), one or more guarding ant species, and enemies (predators or parasitoids) (Pierce et al. 2002; Styrsky and Eubanks 2007). In such tri-trophic interactions, honeydew is secreted directly by the herbivore, and derives either from excretion of excess sugars present in a phloem-feeding diet (in homopterans such as aphids, scale insects and treehoppers: Stadler and Dixon 2005; Styrsky and Eubanks 2007) or from the secretions of specialised cuticular glands (in the larvae of some lycaenid butterflies: Pierce et al. 2002). While these interactions have been studied in depth, here we consider patterns in the evolution of a qualitatively distinct and less-studied trophobiosis involving four trophic levels. In this interaction, the herbivores are gall inducing wasps (Hymenoptera: Cynipidae: Cynipini; see Figure 1) that do not produce honeydew-like secretions themselves, but cause nectar to be secreted by their host plant (oaks; Fagaceae: *Quercus* spp.); as a result, they pay none of the direct energetic costs of ant guard recruitment (Washburn 1984; Stadler and Dixon 2005).

Female oak gall wasps lay their eggs into meristematic host tissues (often buds, but also leaves, shoots, fruits or roots), and the eggs and larvae induce the development of characteristic gall structures within which the larvae complete their development. Gall traits are highly specific to the inducing wasp species and can be considered the extended phenotypes of galler genes (Weis and Abrahamson 1986; Stone and Schönrogge 2003). Internal gall tissues provide the developing larva with nutrition, while a range of outer tissues, such as thick woody walls or dense coatings of spines, reduce the mortality imposed by parasitoid enemies (Bailey et al. 2009).
Character state analyses show that while oak gallwasps are conservative in their host plant use (Stone et al. 2009), they easily evolve a diversity of novel gall phenotypes (Stone and Cook 1998; Cook et al. 2002). A small subset of cynipid oak galls produce sweet secretions that are highly attractive to ants (Bequaert 1924; Conway 1980; Washburn 1984; Abe 1988; Abe 1992; Seibert 1993; Fernandes et al. 1999; Inouye and Agrawal 2004: see also Online Supplementary Table S1). Oaks and related Fagaceae have not naturally produced either floral or extrafloral nectar for tens of millions of years (Manos and Stanford 2001), so nectar secretion by gall tissues is evolutionarily novel. As only a small minority of cynipid galls secrete nectar, this trait is also not a necessary consequence of gall induction. Gall nectar secretion can thus be viewed as another extended phenotype of the gall inducer, the adaptive significance of which should be considered in terms of impacts on gallwasp fitness.

Gallwasps commonly experience high rates of attack by natural enemies, particularly chalcid parasitoid wasps (Bailey et al. 2009), and manipulation experiments have shown that ant guards significantly reduce the mortality these parasitoids impose (Washburn 1984; Abe 1992; Seibert 1993; Fernandes et al. 1999; Inouye and Agrawal 2004).

In all ant guard trophobioses, the protective efficacy of ant guards depends on the relationships between rewards offered, guarding effort, and herbivore survival (Cushman and Whitham 1991; Breton and Addicott 1992; Morales 2000; Blüthgen and Fiedler 2002). Both free-feeding herbivores and galls that recruit ants are commonly found in groups (see Figure 1), and while the relationship between herbivore group size and the per capita benefit to herbivores of ant guards varies among systems (e.g. McEvoiy 1979; Cushman and Whitham 1989; Cushman and Whitham 1991; Breton and Addicott 1992; Morales 2000; Blüthgen and Fiedler 2002;
Guerra et al. 2011), aggregated nectar-secreting galls commonly recruit more ants (Washburn 1984; Abe 1992). From the gall inducer’s perspective, aggregated galls may provide a competitive advantage over other nectar secretors when effective ant guards are a limiting resource (Cushman and Whitham 1989; Cushman and Whitham 1991). From the ant’s perspective, aggregated secretors are probably attractive because they provide a temporally predictable resource that can be monopolized (Washburn 1984; Abe 1992; Delabie 2001; Styrsky and Eubanks 2007). These patterns suggest that clustered gall induction (a maternally-expressed trait resulting from aggregated oviposition, with multiple eggs laid in a single oviposition event leading to mature galls touching each other) and nectar secretion (under the control of her individual offspring) may have been selected together in cynipid gallwasps, as is the case with some other gall traits with maternal and larval components (Atkinson et al. 2002; Bailey et al. 2009).

Here we examine patterns in the evolution of nectar secretion in oak gallwasps, across the Cynipini worldwide, and within one North American genus, Disholcaspis, which contains multiple species inducing nectar-secreting galls. We use new multi-locus molecular phylogenies at both taxonomic levels in addition to recently developed Bayesian trait reconstruction methods to address two general questions:

1. What are the evolutionary dynamics of nectar secretion in oak gallwasps? If, as experiments suggest, nectar secretion is an effective defensive phenotype, we predict two patterns. Firstly, we expect that nectar secretion will have evolved repeatedly, as is apparent with other defensive traits in cynipid galls (Stone and Schönrogge 2003; Bailey et al. 2009). This prediction is supported by the recurrent evolution of extrafloral nectaries in many plant taxa and habitats (Marazzi et al. 2013; Weber and Keeler 2013). Secondly, we expect that nectar secretion, once evolved,
will be retained within lineages and hence show a signature of phylogenetic
clustering, as seen in other defensive gall traits (Stone and Schönrogge 2003; Bailey
et al. 2009). This question is addressed in both the Cynipini and within Disholcaspis.

2. Has the evolution of nectar secretion been associated with the evolution of
aggregated oviposition? If aggregation of galls significantly enhances the efficacy of
ant recruitment, we expect evolutionary transitions in these traits to co-vary across the
phylogeny. This prediction is tested using the genus Disholcaspis (Dalla Torre &
Kieffer), a genus of approximately 30 species galling white oaks (Quercus section
Quercus spp.) in North America.

Methods

Sampling and data generation:

Galls were collected for 21 of the 30 species of nectar-secreting gallwasps
(including 10 in Disholcaspis) from across the known Holarctic geographic
distribution of cynipid gallwasps (Online Supplementary Tables S1 and S2, and see
the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.bj82r (Nicholls et al.
2016)). Species were identified using their distinctive gall morphologies (e.g. Figure
1, Stone and Schönrogge 2003) and adult wasp morphology with reference to type
specimens. Fifteen nectar-secreting species, including a subsample of four species
from Disholcaspis, were placed in a Cynipini-wide phylogeny which incorporated a
further 54 species that span the current understanding of Cynipini diversity. The non-
oak gallwasps Pediaspis and Diplolepis were used as outgroups (see Online
Supplementary Table S2).

Taxon sampling for the Disholcaspis study excluded four species currently
classified within the genus Disholcaspis (D. chrysolepidis, D. corallina, D. plumbella
and *D. sulcata*), as both the Cynipini-wide molecular analysis herein (see Results) and morphology (Melika and Abrahamson 2002) indicated these species have been erroneously placed within *Discolcaspis* and in fact represent multiple divergent and unrelated lineages. The final taxon sampling for *Discolcaspis* involved only species from the monophyletic and well-supported lineage (posterior probability of 1.00 in the Cynipini analysis) corresponding to typical *Discolcaspis*, and incorporated 10 nectar-secreting species and 13 non-secreting species from a total of circa 30 known species (following Melika and Abrahamson 2002: Online Supplementary Table S2).

For the Cynipini-wide analysis, sequence data were generated for one individual per species for fragments of three genes: cytochrome *b* (*cytb*), long-wavelength opsin (*opsin*) and the D2 loop of the 28S rRNA locus (D2), following conditions in Stone et al. (2009). For the *Discolcaspis* data set, four gene fragments were sequenced for 1-12 individuals per species: the three fragments above and the second internal transcribed spacer of the ribosomal gene complex (ITS2) using conditions from Campbell et al. (1993). PCR amplicons were cleaned using a standard shrimp alkaline phosphatase protocol and sequenced in both directions using ABI’s BigDye v3.1 terminator chemistry run on an ABI3730 capillary sequencer. Base calling was checked in Sequencher v4.10.1 (Gene Codes Corporation 1995), and alignments for each locus constructed using Muscle v3.8.31 (Edgar 2004). GenBank accession numbers for all sequences are given in Online Supplementary Table S2, and alignments are in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.bj82r (Nicholls et al. 2016).

*Phylogeny reconstruction*
A three-gene species tree analysis for the Cynipini was conducted under a multi-species coalescent model as implemented by BEST v2.3.1 (Liu 2008). Substitution models for each gene were selected using MrModeltest v2.2 (Nylander 2004), with final models for each gene all consisting of unlinked GTR+I+G models. A uniform clock was applied to each gene, with topology, branch lengths and relative mutation rates unlinked across loci, and ploidy set to haploid for cytb and diploid for the other two genes. Priors were set as: thetapr = invgamma(3,0.038), genemupr = uniform(0.025,1.975), poissonmean = 8, proptemp= 0.3. The beta value for the theta prior was 10x larger than the value estimated using the observed number of variable sites in the data (i.e. allowing larger population sizes) but this facilitated convergence in the model (see also Leaché 2009; Lee et al. 2012).

For the within-Disholcaspis analysis, a four-gene species tree analysis was conducted using the *BEAST module (Heled and Drummond 2010) within BEAST v1.6.2 (Drummond and Rambaut 2007). Independent substitution models for genes were determined following the simplification procedure in Nicholls et al. (2010), with final models being HKY+I, HKY and HKY+I for 1st, 2nd and 3rd codon positions respectively within cytb, HKY models for the intron and exon regions of opsin, HKY for D2, and GTR+I for ITS2. A birth-death species prior and constant population model were applied to the species tree, a strict clock was applied to each gene (calibrated to 2.3% divergence per million years for cytb, following Brower 1994), ploidy was haploid for cytb and diploid for the other genes, and topologies and branch lengths were estimated independently for each gene tree and the species tree.

Both the Cynipini-wide and Disholcaspis analyses were run twice for 100 million generations to confirm convergence, each time with 1 chain per run, and sampled every 12,500 generations. In both analyses, only samples taken from the final 15
million generations were used to generate posterior distributions of parameters and posterior sets of trees.

**Phylogenetic clustering analyses**

We tested for phylogenetic clustering of the nectar secretion trait in each dataset using the previously obtained sets of 2402 species trees from either the two BEST (Cynipini) or *BEAST* (*Disholcaspis*) runs using BaTS v2 (Parker et al. 2008). Taxa were coded categorically for the nectar character states (nectar; no nectar). BaTS calculates three statistics to test for clustering (PS - parsimony score, measuring the minimum number of state changes required to give the observed distribution across taxa; AI - association index, measuring the grouping of descendent taxa with same character state summed across nodes in the tree; MC - maximum monophyletic clade size where all clade members share the same character state), assessed against null distributions generated using 1000 character state randomisations across taxa.

**Character state evolution**

The presence or absence of nectar secretion through the *Disholcaspis* species tree was reconstructed using the MultiState module within BayesTraits v1.0 (Pagel et al. 2004). Character state reconstruction was restricted to nodes with posterior probability support greater than 0.6, using the MRCA algorithm. The input tree set was the posterior set of species trees generated by *BEAST*. A reversible jump MCMC (rjMCMC) method was used, allowing the analysis to move among different classes of models (in this case between reversible models with either different or symmetrical rates of character gain/loss, or unidirectional models where one of the rates of character gain or loss is set to zero). A hyperprior was used for seeding the
mean of an exponential distribution for the transition rate priors, with values taken from a uniform distribution on the interval (0,0.1). The ratedev parameter was set to 1.2, providing appropriate acceptance rates of approximately 21%. Two independent MCMC chains were run for every node, each for 100 million iterations with a burnin of 50 million iterations, subsequent to which each chain was sampled every 6,250 generations (providing 8001 samples per chain). The “fossil” command was also used in separate runs with the previously described settings to fix the character state at tested nodes to either of the two states of nectar present or absent, with support for one state over another then assessed using Bayes factors, estimated as twice the difference in the natural log of the harmonic mean of model likelihoods (2ΔlnHML, following Kass and Raftery 1995).

The Discrete module within BayesTraits v1.0 (Pagel and Meade 2006) was used to test for correlated evolution between nectar secretion and gall clustering. This technique uses Bayes factors to compare two models: an independent model where rates of change in one trait are independent of state of the second trait, and a dependent model where they are conditional upon the state of the second trait. The same input trees were used as for the MultiState analysis, but taxa were coded for two different traits: with/without nectar and clustered/solitary galls. A rjMCMC method was used, with a hyperprior (uniform on the interval (0,0.1)) used for seeding the exponential priors on transition rates. The ratedev was set to 0.2 for the independent model and 0.04 for the dependent model, providing acceptance rates of just over 20%. Two independent MCMC chains were run for each model, each for 100 million iterations with a burnin of 50 million iterations, subsequent to which each chain was sampled every 6,250 generations.
Results

Across the oak gallwasps, the well-resolved three-gene phylogeny showed no evidence for phylogenetic clustering of species inducing nectar-secreting galls (Figure 2; Table 1). Instead, with the exception of the genus Disholcaspis, the nectar-secreting trait was widely scattered across the oak gallwasp phylogeny. This implies that within the Cynipini, nectar secretion has independently evolved from a non-secreting ancestor at least eight times outside of the genus Disholcaspis (Figure 2).

The genus Disholcaspis contains a large proportion of the known gallwasp species inducing nectar-secreting galls (see Online Supplementary Table S1). In contrast to oak gallwasps in general, the well-resolved four gene phylogeny of Disholcaspis showed that several clades contain only nectar-secreting species (Figure 3). Moreover, there was significant phylogenetic clustering of nectar-secreting and non-secreting species (Table 1). In addition, no lineage was inferred to have lost nectar secretion once it had evolved.

The rjMCMC reconstruction of ancestral nectar secretion character states provided dominant support (99.4% of the 16002 posterior samples) for two models, with reconstructions under both models shown in Figure 3. The first model of character evolution (visited in 85.8% of the posterior samples) was a symmetric reversible model, with equal transition rates between the two character states. The second model (visited in 13.7% of posterior samples) was a unidirectional evolutionary model allowing only transitions from no nectar to nectar secretion, with the rate of reverse transition fixed at zero. Bayes factor comparison showed that the symmetric reversible model was marginally better supported over the unidirectional model ($2\Delta \ln HML = 2.10$). However, both models inferred similar patterns of character state evolution (Figure 3).
Multiple derivations of nectar secretion from a non-secreting ancestor were inferred under both models, and there was no evidence for a loss of nectar secretion once this phenotype had evolved. The two models did differ in the support for the ancestral character state inferred at certain nodes. Whilst the symmetric reversible model provided much higher support for one or other character state at most nodes (Figure 3, panel A), some nodes, in particular deeper nodes, had more equivocal character state reconstructions. In contrast, the unidirectional model gave 100% support for one or other state for all nodes in the Disholcaspis phylogeny (Figure 3, panel B), with a total of six independent derivations of nectar secretion inferred within the genus. The same conclusions were supported by Bayes factor comparisons of models where nodes were fixed to one or other state (Online Supplementary Table S3).

The evolution of nectar secretion was strongly correlated with the evolution of gall clustering, and hence aggregated oviposition, within the genus Disholcaspis. The dependent model, in which rates of change in one trait are conditional upon the state of the second trait, was supported over the model of independent evolution of the two traits (Bayes factor test, $2\Delta\ln\text{HML} = 5.14$). In Disholcaspis, nectar secretion was always associated with clustered galls, and species inducing solitary galls were always non-secretors (Figure 3).

Discussion

Our results clearly show multiple transitions from non-secreting to nectar-secreting galls in oak gallwasps - six within the genus Disholcaspis, and at least eight across the rest of the gallwasp tribe Cynipini. This pattern parallels that seen for other oak gall phenotypic traits (Stone and Cook 1998; Stone and Schönrogge 2003; Bailey
et al. 2009), suggesting an ongoing evolutionary arms race against natural enemies, with gallwasps constantly evolving different defensive gall phenotypes in order to find enemy-free space (sensu Bernays and Graham 1988). Although our sampling of nectar-secreting oak gallwasps is incomplete, this inference of multiple independent evolution of nectar secretion will not change with more sampling. However, it is possible that complete sampling could reveal additional phylogenetic clusters of nectar-secreting species within the Cynipini; for instance a group of potentially related North American Callirhytis acorn-galling species, of which only C. carmelensis and C. balanaspis were sampled in this study (see Online Supplementary Table S1; Figures 1E and 1F), may represent a nectar-secreting radiation similar to Disholcaspis.

The evolution of ant recruitment through gall nectar secretion is to our knowledge a trait unique to cynipids amongst all insect/plant galling interactions. Although this trait has protective benefits (Washburn 1984; Abe 1992; Seibert 1993; Fernandes et al. 1999; Inouye and Agrawal 2004), the rarity of nectar secretion amongst the full phylogenetic spectrum of insect-induced galls (an estimated 13,000 species in multiple insect orders: Stone and Schönrogge 2003) suggests that it is difficult to evolve. The physiological mechanisms of cynipid gall induction are poorly understood, with no indication yet of what controls among-species variation in gall defensive traits (Giron et al. 2016). An analogue of nectar secretion sometimes observed in oak gallwasp galls is ant tending of aphids feeding externally on developing galls (Figure 4). Although not a defensive phenotype under the control of the gallwasp (in that not all galls of a given type are associated with aphids), this ‘accidental’ association may confer some protection, as demonstrated for a fig/figwasp system involving ants and honeydew-secreting homopterans (Compton
and Robertson 1988). However, the presence of aphids feeding on the soft tissues of growing galls is rare and opportunistic, and unlikely to make galls consistently more attractive to either ants or enemies (aphids and oak galls are attacked by non-overlapping sets of parasitoids: Bailey et al. 2009). Hence any stable co-evolutionary interactions involving aphids, ant guards and oak galls are unlikely.

Patterns within *Disholcaspis* support our prediction that nectar secretion will be retained within lineages once it has evolved. This contrasts with the lability of ant associations in honeydew-secreting aphids (Shingleton and Stern 2003). This contrast may arise from the fact that while alternative aphid defenses (for example kicking, fleeing or falling from the host plant: Hartbauer 2010; Gish et al. 2011) are in principle compatible with honeydew secretion, many alternative gall defenses, such as coatings of spines, dense hairs, or sticky resins (Stone and Schönrogge 2003; Bailey et al. 2009), are morphologically incompatible with nectar secretion, which requires ant access to a living gall surface. Loss of nectar secretion without the concurrent evolution of an alternative defense would leave a gallwasp very vulnerable to parasitoid attack. This also has a parallel in aphids, in which species that are least able to escape enemies by employing alternative defensive strategies (such as fleeing) are most likely to evolve additional and more complex defenses such as trophobiotic associations with ant guards (Shingleton et al. 2005) or maternal care (Inbar 1998). Retention of nectar secretion by wholly immobile galler species could be considered a similar case.

Patterns within the genus *Disholcaspis* also support our prediction for strong co-evolution between the larval-controlled trait of nectar secretion, and the maternal behaviour of aggregated oviposition. Such maternal/larval trait co-evolution acting to enhance the effectiveness of defensive systems been reported for other insect taxa.
the rate of gain of nectar secretion when galls were not clustered was modelled as zero in 79% of the posterior sample, implying clustered galls are a prerequisite to the evolution of nectar secretion. In addition, the rate at which gall clustering was lost in the presence of nectar secretion was zero for 76% of the posterior sample, implying nectar secretion constrains maternal oviposition behaviour. The co-occurrence of nectar secretion and aggregated oviposition is also apparent in many other oak gallwasp species outside of *Disholcaspis* (Online Supplementary Table S1; Figures 1A, 1C and 1D). While some gallwasp species do induce solitary nectar-secreting galls, they also have specific maternal oviposition behaviours that potentially facilitate ant recruitment as their galls are found on developing acorns or axillary buds adjacent to the stems that ants typically walk along (see Figures 1E and 1F). In contrast, it is striking that no nectar-secreting galls are induced on leaves, despite the large number of gallwasp species inducing galls on leaves (Cook et al. 2002; Bailey et al. 2009) and the ability of many plant lineages to evolve extra-floral nectaries on leaves (Bronstein et al. 2006). Explanations for this pattern include a possible developmental constraint restricting the production of leaf gall-associated nectar, or simply that nectar secretion evolves sufficiently infrequently that it has not yet arisen in any leaf-galling oak gallwasp lineage.

There is potential for one further co-evolutionary interaction that may also be involved in the dynamics of oak gall nectar secretion, gall aggregation and ant guards. The host oak may potentially derive some indirect benefit from being galled, mediated though the protection provided against other herbivores by ants recruited to nectar-secreting galls, as seen in other plant-herbivore-ant systems (Styrsky and Eubanks 2007; Pringle et al. 2011). However, within the oak gall system the costs
and benefits of such indirect ecological effects require further investigation. Nectar-producing oak galls are located away from the new growth that may benefit most from ant-mediated protection, typically being present on or adjacent to previous years’ stem growth. In addition, acorn gallers can impose a direct reproductive cost to the plant by stopping the development of the acorn on which the gall is induced.

Aggregation of nectar-secreting galls may be beneficial as clusters provide a potentially larger and more predictable resource for ant guards that is more attractive than single galls (Washburn 1984; Abe 1992; Fernandes et al. 1999). Benefits of clustering are also seen in other insect systems, both those involving guard ant recruitment (e.g. Delabie 2001; Styrsky and Eubanks 2007), and other defensive traits (e.g. Riipi et al. 2001). However, further investigation is warranted to determine why some oak gallwasp species induce nectar-secreting galls that are never aggregated. Whilst aggregated galls attract more ants, they also offer a larger and more attractive resource to parasitoids; consistent with this idea, we observed that within

*Disholcaspis* most species inducing non-secreting galls also have solitary galls. Ant guarding does not effectively exclude all parasitoid enemies of oak galls (see Washburn 1984; Abe 1992; Seibert 1993; Fernandes et al. 1999; Inouye and Agrawal 2004), and parasitoids may exhibit specialised behaviours, such as attacking galls before they secrete enough nectar to recruit ants (Washburn 1984; Seibert 1993). Such ant-avoidance behaviours by parasitoids attacking concealed galling hosts are also known in other systems (e.g. *Idarnes* parasitoids of figwasps that attack through the fig wall: Jandér 2015). So a solitary gall that can attract enough ants to be guarded, but provide only a single host’s worth of resource to an enemy, might provide the best trait combination, although the relative rarity of such a trait combination suggests that a single gall cannot easily serve as such a suitable resource.
This points to future testing of hypotheses on the quantity and quality of nectar offered by different nectar-secreting oak gallwasp species. One hypothesis is that solitary nectar-secreting galls may produce more nectar per gall unit, or richer nectar, than galls in clusters. Anecdotal evidence may support this idea, as the solitary *Callirhytis* acorn gallers are frequently observed to produce copious amounts of nectar that form large droplets on the gall surface that can attract large-bodied insects such as bumblebees (Weld 1922; Larson 1999: pers. obs.), in contrast to clustered *Disholcaspis* galls that often have no obviously visible secretion. Alternatively, if solitary galls are harder for parasitoids to find, with correspondingly lower enemy attack rates, they may be able to afford to secrete less nectar per gall unit. A second hypothesis is that to minimise their window of vulnerability to parasitoids, secreting galls will produce nectar as early as they can in their development to avoid attack. One possibility is that relative to a solitary gall, aggregation allows a group of galls to secrete the minimal reward required to recruit ants at a smaller gall size, narrowing their window of vulnerability.

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Table 1. Test statistics describing the phylogenetic clustering of nectar secretion within the Cynipini and the genus *Disholcaspis*.

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<thead>
<tr>
<th>Taxon/Statistic</th>
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Note. - AI = association index, measuring the grouping of descendent taxa with same character state, summed across nodes in the tree; PS = parsimony score, measuring the minimum number of state changes required to give the observed distribution across taxa; MC = maximum number of taxa in a monophyletic clade where all clade members share the same character state.
Figure legends

Figure 1. Examples of cynipid galls examined in this study, some with attendant aggressive insects. A. *Dryocosmus cerriphilus*, a clustered nectar secretor, attracting ants (image György Csóka); B. *Disholcaspis spissa*, a clustered nectar secretor, attracting a vespid wasp; C. *Andricus hakonensis*, a clustered nectar secretor, attracting *Camponotus* sp. ants; D. *Callirhytis congregata*, a nectar secretor with aggregated galls fused into a single structure on catkins; E. *Callirhytis carmelensis*, a nectar secretor with solitary galls galling acorns; F. *Callirhytis balanaspis*, a nectar secretor with solitary galls galling acorns; G. *Disholcaspis quercusvirens*, a clustered nectar secretor; H. *Disholcaspis rubens*, a clustered non-secretor; and I. *Disholcaspis insulana*, a non-secretor with solitary galls.

Figure 2. Majority-rule consensus species tree of the Cynipini based upon three genes (cytochrome *b*, opsin and the D2 region of 28S rRNA). Grey squares and bold species names indicate taxa whose galls secrete nectar; white squares indicate non-secretors. Black stars at nodes indicate ≥95% posterior probability support, white stars indicate 70-94% posterior probability support. Grey diamonds indicate points at which nectar secretion may have evolved within the Cynipini (excluding *Disholcaspis*).

Figure 3. Majority-rule consensus species tree of the gallwasp genus *Disholcaspis* based upon four genes (cytochrome *b*, opsin, the D2 region of 28S rRNA and the ribosomal internal transcribed spacer 2). Branch thickness is proportional to posterior probability support (branch leading to *D. quercusvirens* and *D. quercussuccinipes* =
1.00). Nectar secretion and clustering character states for extant species are indicated by two columns of squares: first column: grey squares - galls secrete nectar, white squares - no nectar secretion; second column: grey squares - clustered galls, white squares - solitary galls. Pie charts adjacent to nodes indicate the corresponding posterior probability of alternative ancestral character states (grey - nectar-secreting galls, white - galls without nectar) estimated under two different models: panel A - symmetric reversible model, with equal rates of gain and loss of nectar secretion; panel B - model with rate of change from nectar to no nectar constrained to be zero. Grey bars across branches indicate points at which nectar secretion may have evolved.

Figure 4. Ants tending aphids feeding from the surface tissues of oak cynipid galls. A. ants on *Andricus kollari* galls in Spain (image Jose-Luis Nieves-Aldrey); B. *Lasius* sp. ants tending aphids on galls of *Andricus infectorius* in Hungary; C. *Crematogaster scutellaris* ants tending aphids on a gall of *Andricus polycerus* in Hungary; and D. ants tending aphids on *Andricus sieboldi* galls in Spain - galls of this species also actively secrete nectar (image Jose-Luis Nieves-Aldrey).
Supplementary material

Online Supplementary Table S1. Cynipid species inducing galls known to secrete nectar, with information on their galls.

Online Supplementary Table S2. Specimen collection and sequencing details, including GenBank accession numbers. Species names in bold are nectar secretors; individual codes in italics indicate Disholcaspis species included in the Cynipini-wide analyses. Four species currently classified as belonging to the genus Disholcaspis (D. chrysolepidis, D. corallina, D. plumbella and D. sulcata) were excluded from the Disholcaspis study as sequence data (see Figure 2) and morphology (see Melika and Abrahamson 2002) both indicate they are not true Disholcaspis.

Online Supplementary Table S3. Posterior probabilities of nectar secretion or no nectar secretion at ancestral nodes within the Disholcaspis phylogeny (nodes numbered in insert). Posterior probabilities of ancestral character states are presented for the full set of models visited in the rjMCMC run (columns 2 & 3), those under a symmetric reversible model (columns 4 & 5) and under a model where the rate of change of nectar to no nectar was fixed at zero (columns 6 & 7). The final set of columns indicate likelihoods of models run with the respective node fixed to one or other character state, and the result of a Bayes factor test comparing the two models.