# Experimental evidence for nature's hidden network of higher-order interactions

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**Abstract**

Transcending pairwise interactions in ecological networks remains a challenge. Higher-order interactions, the modulation of a pairwise interaction by a third species, have so far only been demonstrated in models or small isolated systems. Their ubiquity at a community level remains unknown. Using field experiments, we tested how multiple interactions within a network changed with species composition by reducing the densities of distinct species in a diverse arthropod community. We revealed an extensive hidden network of higher-order interactions modifying each other and the "visible" direct interactions. Most pairwise interactions were affected by the manipulation of a non-interacting taxonomic group. The pervasiveness of these interaction modifications challenges pairwise approaches to understanding interaction outcomes and could shift our thinking about the structure and resilience of ecological communities.

# Experimental evidence for nature's hidden network of higher-order interactions

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## Summary

Transcending pairwise interactions in ecological networks remains a challenge. Higher-order interactions, the modulation of a pairwise interaction by a third species, have so far only been demonstrated in models or small isolated systems. Their ubiquity and importance at a community level in the real world remains unknown. Using field experiments, we tested how multiple interactions within a diverse arthropod community were modified by the removal of distinct species. We revealed an extensive hidden network of higher-order interactions modifying each other and the "visible" direct interactions. Most pairwise interactions were affected by the manipulation of a non-interacting taxonomic group. The pervasiveness of these interaction modifications challenges pairwise approaches to understanding interaction outcomes and could shift our thinking about the structure and persistence of ecological communities.

**Key words:** ants**,** aphids, *Baccharis dracunculifolia,* Cerrado, experimental manipulation, galling insects, indirect interactions, interaction modification, parasitoid wasps, parasitism

**Introduction**

Ecologists have traditionally focused on networks of feeding relationships among species (direct trophic interactions) to understand community structure and dynamics (Dunne *et al.* 2002; Montoya *et al.* 2006; Rooney *et al.* 2006). Despite an increasing awareness of the importance of non-trophic and indirect interactions (mediated by a third species or via the environment) (Morris *et al.* 2004; Kéfi *et al.* 2012, 2015; Sanders *et al.* 2014; Barbosa *et al.* 2019), integrating the different types of interactions into a single network has proven extremely difficult and has not yet been done empirically, despite relatively recent efforts and developments (Ohgushi 2005; Fontaine *et al.* 2011; Kéfi *et al.* 2012, 2015; Golubski *et al.* 2016). This is because networks quickly become very complex, as the number of indirect interactions increases exponentially with the number of species involved (Patten 1983).

Amongst the non-trophic interactions, higher-order interactions (HOIs) or interaction modifications (the modulation of a pairwise interaction by a third species) (Wootton 1993), play a particularly important role in stabilising species coexistence (Hammill *et al.* 2015; Kelsic *et al.* 2015; Bairey *et al.* 2016; Grilli *et al.* 2017; Terry *et al.* 2019; Singh & Baruah 2021). For instance, they can attenuate negative interactions, such as when non-prey species increase the persistence of predation-susceptible species by modifying the ability of predators to detect prey (Hammill *et al.* 2015). This way, HOIs can promote diversity and the long-term persistence of ecological communities (Hammill *et al.* 2015). HOIs are also regulated by feedbacks, meaning that their influence on interactions changes over time with population densities (Sanders *et al.* 2014; Barbosa *et al.* 2019). This context-dependency of interactions makes it difficult to predict the effects of perturbations on ecosystems (e.g., species loss) (Barbosa *et al.* 2017), with consequences for conservation and management strategies. Thus, investigations of interaction modulation by HOIs are unveiling a new, and previously unaccounted for, higher level of

community structuring processes. However, HOIs cannot be observed from studying traditional pairwise networks, unlike other indirect effects (e.g., apparent competition) (Morris *et al.* 2004), because they often involve several other species or environmental factors beyond the interacting pair. The role of HOIs in shaping ecological communities has mostly been explored theoretically (Bairey *et al.* 2016; Grilli *et al.* 2017; Mayfield & Stouffer 2017; Terry *et al.* 2019; Singh & Baruah 2021; Kleinhesselink *et al.* 2022) or by experiments with particular subsets of communities and tractable laboratory model systems testing specific effects (Hammill *et al.* 2015; Kelsic *et al.* 2015; Barbosa *et al.* 2019). How pervasive and influential these effects are at a community level, remains largely unknown.

Here we hypothesised that a complex network of HOIs could be constantly modifying pairwise interactions and shaping ecological communities, and that consequently the outcome of pairwise interactions would be a product of many influences from distinct sources. We predicted that the manipulation of any species (or group of species) within the community – or even of one of their by-products (e.g. deadwood, leaf-litter, $O_2$, faeces) – would reverberate throughout the entire community and thus modify apparently unrelated interactions. We performed experimental manipulations of species living on the tropical shrub *Baccharis dracunculifolia* D.C. (Asteraceae), a highly self-contained system with a diverse arthropod fauna (for details about the study system see the Supplementary Material). In different treatments, we excluded all ant species or live or hatched insect galls of the dominant galler species. Each treatment, including a control with no species exclusion, consisted of 16 replicated plants of *B. dracunculifolia*. Over two months, every week we quantified the changes in densities of several other species or guilds (hereafter groups; ants, herbivores, predators, and aphids) as well as changes in direct interactions involving two gallers, such as gall induction (herbivory), parasitism by wasps, and inquilinism (sharing occupation of the gall for shelter and feeding) by aphids. Specifically, we

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combined direct observation and gall dissection data to quantify the effects of groups on each other's population densities or traits, including direct trophic and non-trophic interactions and the effect of the exclusion of a group on another group (hereafter, density effects; Fig. 1). We also investigated how the direct interactions changed in different contexts defined by the density or exclusion of a third group (for detailed methods see the Supplementary Material). This allowed the construction of a unique "effect network" based on multiple manipulations performed simultaneously on the same system, investigating the same direct interactions under different contexts. Links were categorised into two types: node modulation (node-to-node effects), which are pairwise trophic and non-trophic interactions or density effects; and link modulation (HOIs; node-to-link effects), which are three-way interactions (interaction modification), or four-way interactions (modification of an interaction modification).
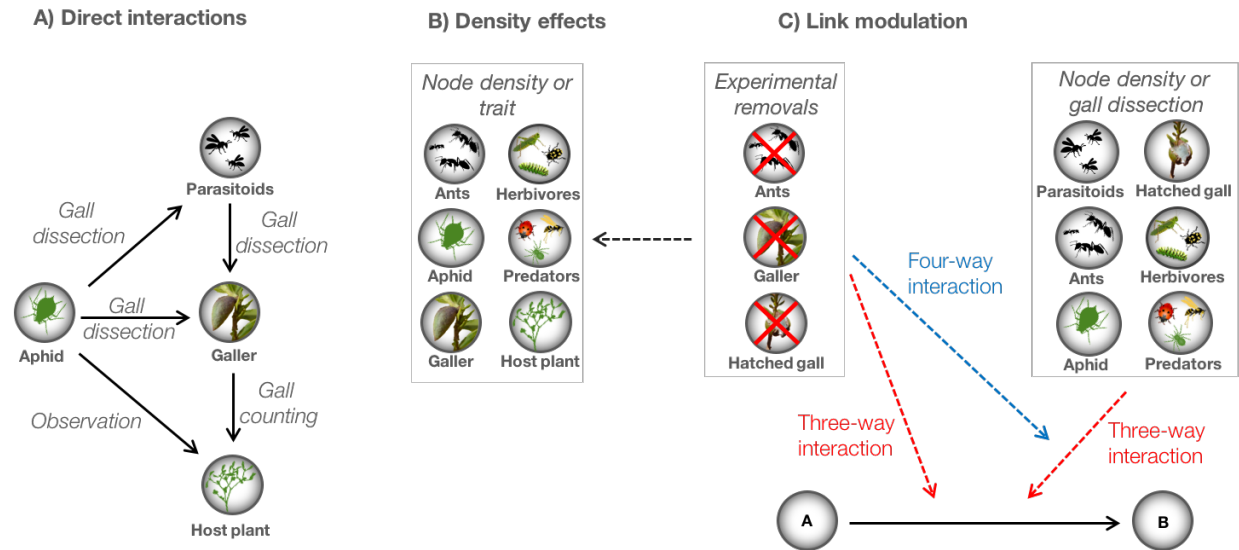


**Fig 1. Methods used to detect and quantify different effects in the network:** (**A**) Direct trophic and non-trophic interactions were measured through gall dissection or direct observation; (**B**) Density effects were effects on a node's density or trait following the exclusion of another node; (**C**) Three-way interactions were measured by how direct interactions changed in different contexts

defined by the exclusion or density of a third node; and four-way interactions were measured by how the dependence of an interaction on the densities of other nodes changes in each context of exclusion of a fourth node.

**Results**

Over two months we quantified 1,427 ants from 15 different species found on 988 branches, 1,109 predators from nine morphospecies (spider species were grouped as a single morphospecies), 629 free-feeding herbivore insects from 41 morphospecies, and 22,564 terminal buds occupied by aphids. We quantified 1,346 hatched galls, and 1,260 live galls of the galler *Baccharopelma dracunculifoliae* (Sternorrhyncha: Psyllidae), of which 522 live galls were collected and dissected. We also collected and dissected 92 live galls of the galler *Rachiptera limbata* Bigot (Diptera: Tephritidae). The effects identified are portrayed in an effect network showing pairwise trophic and non-trophic interactions, density effects, as well as HOIs (Table 1, Fig. 2). From a total of 29 links in our network (Table 1, Fig. 2) there were ten node modulation links - five trophic and two non-trophic direct interactions, and three density effects; 12 three-way links (interaction modification), and five four-way links (modification of an interaction modification). For the sake of simplicity, the results of the statistical tests for each link in the network are shown in Table 2.
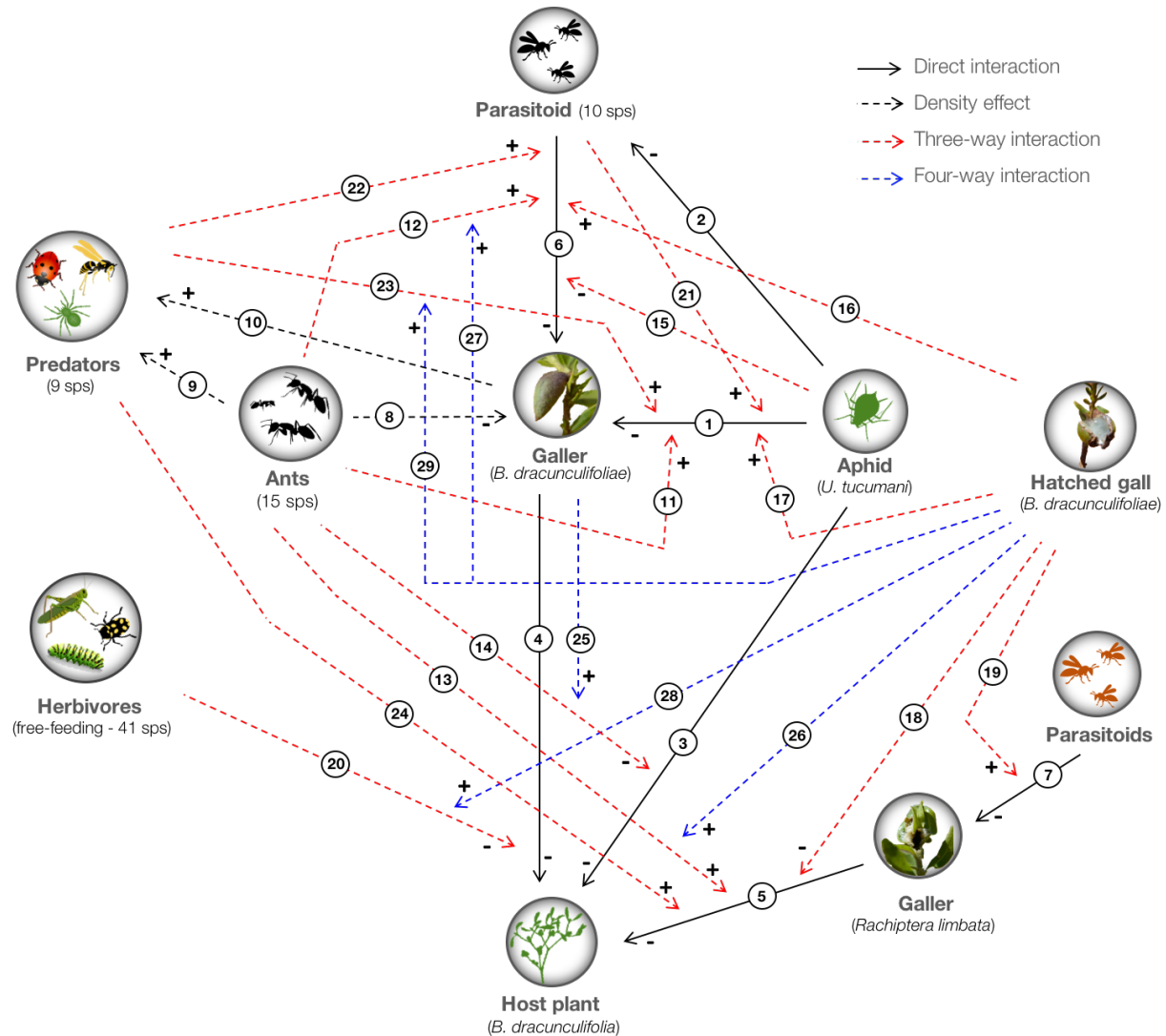
**Fig 2. Effect network on the host plant *Baccharis dracunculifolia* showing links categorised into two main types**: node modulation (black arrows), which are pairwise trophic and non-trophic interactions (solid arrows) or density effects (dashed arrows); and link modulation (higher-order interaction), which are three-way interactions (interaction modification; dashed red arrows), or four-way interactions (modification of an interaction modification; dashed blue arrows). The link codes (numbers) refer to interactions and corresponding statistical tests depicted in Tables 1 and 2. Positive and negative signs mean strengthening or weakening a link, respectively.

**Table 1. Interactions among groups of arthropods on the host plant *Baccharis dracunculifolia*.** The link codes refer to interactions depicted in Fig. 2, and the relevant statistical tests are presented in Table 2. G7 and G10 refer to the gallers *Baccharopelma dracunculifoliae* and *Rachiptera limbata,* respectively.

| Link code | Link type | Affecting | Effect* | Affected | Detection method | Statistics (see Table2) |
|---|---|---|---|---|---|---|
| *Node modulation* | | | | | | |
| 1 | Direct interaction | Aphid (inside galls) | Negative | Galler G7 | Gall dissection | lines 7, 8 |
| 2 | Direct interaction | Aphid (inside galls) | Negative | Parasitoids of galler G7 | Gall dissection | line 9 |
| 3 | Direct interaction | Aphids (on branches) | Negative | Host plant | Observation | NA (herbivory) |
| 4 | Direct interaction | Galler G7 | Negative | Host plant | Gall counting | NA (herbivory) |
| 5 | Direct interaction | Galler G10 | Negative | Host plant | Gall counting | NA (herbivory) |
| 6 | Direct interaction | Parasitoid | Negative | Galler G7 | Gall dissection | NA (parasitism) |
| 7 | Direct interaction | Parasitoid | Negative | Galler G10 | Gall dissection | NA (parasitism) |
| 8 | Density effect | Ants (exclusion) | Negative | Volume of Gall G7 x Nymph abundance | Node removal | line 6 |
| 9 | Density effect | Ants (exclusion) | Positive | Predator abundance | Node removal | line 2 |
| 10 | Density effect | Galler G7 (exclusion) | Positive | Predator abundance | Node removal | line 2 |
| *Link modulation* | | | | | | |
| 11 | Three-way interaction | Ants (exclusion) | Positive | Link Aphid x Galler G7 | Modifier removal | line 4 |
| 12 | Three-way interaction | Ants (on branches) | Positive | Link Parasitoid x Galler G7 | Modifier density | line 13 |
| 13 | Three-way interaction | Ants (on branches) | Positive | Link Galler G10 x Host Plant | Modifier density | line 16 |
| 14 | Three-way interaction | Ants (on branches) | Negative | Link Aphid x Host Plant | Modifier density | line 1 |
| 15 | Three-way interaction | Aphids (on branches) | Negative | Link Parasitoid x Galler G7 | Modifier density | line 15 |
| 16 | Three-way interaction | Hatched Gall G7 | Positive | Link Parasitoid x Galler G7 | Modifier removal | line 5 |
| 17 | Three-way interaction | Hatched Gall G7 | Positive | Link Aphid x Galler G7 | Modifier density | line 11 |
| 18 | Three-way interaction | Hatched Gall G7 | Negative | Link Galler G10 x Host Plant | Modifier density | line 17 |
| 19 | Three-way interaction | Hatched Gall G7 | Positive | Link Parasitoid x Galler G10 | Modifier density | line 19 |
| 20 | Three-way interaction | Herbivore abundance | Negative | Link Galler G7 x Host Plant | Modifier density | line 12 |
| 21 | Three-way interaction | Parasitoid | Positive | Link Aphid x Galler G7 | Gall dissection | line 3 |
| 22 | Three-way interaction | Predator abundance | Positive | Link Parasitoid x Galler G7 | Modifier density | line 14 |
| 23 | Three-way interaction | Predator abundance | Positive | Link Aphid x Galler G7 | Modifier density | line 10 |
| 24 | Three-way interaction | Predator abundance | Positive | Link Galler G10 x Host Plant | Modifier density | line 18 |
| 25 | Four-way interaction | Galler G7 | Positive | Link 14 | Modifier removal | line 1 |
| 26 | Four-way interaction | Hatched Gall G7 | Positive | Link 13 | Modifier removal | line 16 |
| 27 | Four-way interaction | Hatched Gall G7 | Positive | Link 12 | Modifier removal | line 13 |
| 28 | Four-way interaction | Hatched Gall G7 | Positive | Link 20 | Modifier removal | line 12 |
| 29 | Four-way interaction | Hatched Gall G7 | Positive | Link 23 | Modifier removal | line 10 |

*Positive and negative modulation respectively strengthens and weakens a link.

**Table 2**. **Summary of results of statistical tests**. The interaction numbers refer to corresponding interactions described in Table 1 and depicted in Fig. 2. Only values for variables with statistically significant results (P < 0.05) are reported. For a complete list of statistical tests performed see Tables S1 and S2 in the Supplementary material. G7 and G10 refer to the gallers *Baccharopelma dracunculifoliae and Rachiptera limbata,* respectively.

| Line | Dataset | Interaction | Response variable | Explanatory variable | χ2 | d.f. | p | Score / Post hoc comparison |
|---|---|---|---|---|---|---|---|---|
| 1 | *Observation* | 14, 25 | Aphid presence | Ant presence : Treatment | 22.454 | 2 | <0.001 | t = -1.745; Slopes comparison: Control < Live gall exclusion (χ2(1) = 7.844, p = 0.010) |
| 2 | | 9, 10 | Predator abundance | Treatment : Week | 40.055 | 15 | <0.001 | In week 2, Control > Ant exclusion (χ2(1) = 11.823, p = 0.020), and Live gall excluded (χ2(1) = 14.744, p = 0.008) |
| 3 | *Dissection* | 21 | Aphid per gall | G7 Parasitism (binary) | 4.897 | 1 | 0.026 | t = 2.325 |
| 4 | | 11 | | Treatment | 7.157 | 2 | 0.027 | Control > Ant exclusion (χ2(1) = 7.526, p = 0.018) |
| 5 | | 16 | G7 Parasitism | Treatment | 8.175 | 2 | 0.016 | Control > Hatched gall exclusion (χ2(1) = 7.972, p = 0.014) |
| 6 | | 8 | G7 volume | G7 nymphs per gall : Treatment | 10.317 | 2 | 0.005 | t = 2.061; Slopes comparison: Control < Ant exclusion (χ2(1) = 10.359, p = 0.003) |
| 7 | | 1 | | Aphid per gall | 8.162 | 1 | 0.004 | t = -2.872 |
| 8 | | 1 | G7 nymph mortality | Aphid per gall | 5.924 | 1 | 0.014 | t = 2.520 |
| 9 | | 2 | Parasitoid mortality | Inquilinism (binary) | 10.129 | 1 | 0.001 | t = 3.465 |
| 10 | *Observation /Dissection* | 23, 29 | Aphid per gall | Predator abundance : Treatment | 6.729 | 2 | 0.034 | t = 2.402; Slopes comparison: Control > Hatched exclusion (χ2(1) = 6.220, p = 0.037) |
| 11 | | 17 | | Hatched abundance | 4.963 | 1 | 0.025 | t = 1.255 |
| 12 | | 20, 28 | G7 abundance | Herbivore abundance : Treatment | 9.925 | 2 | 0.007 | t = -2.610; Slopes comparison: Control < Hatched exclusion (χ2(1) = 6.102, p = 0.040) |
| 13 | | 12, 27 | G7 Parasitism | Ant presence : Treatment | 8.137 | 1 | 0.004 | z = 2.687; Slopes comparison: Control > Hatched excluded (χ2(1) = 7.840, p = 0.005) |
| 14 | | 22 | | Predator abundance | 21.054 | 1 | <0.001 | z = 4.376 |
| 15 | | 15 | | Aphid presence | 8.014 | 1 | 0.004 | z = -2.811 |
| 16 | | 13, 26 | G10 abundance | Ant presence : Treatment | 4.770 | 1 | 0.028 | t = 2.668; Slopes comparison: Control > Hatched exclusion (χ2(1) = 4.654, p = 0.030) |
| 17 | | 18 | | Hatched abundance | 4.134 | 1 | 0.042 | t = -2.714 |
| 18 | | 24 | | Predator abundance | 6.452 | 1 | 0.011 | t = 2.609 |
| 19 | | 19 | G10 parasitism | Hatched abundance | 4.768 | 1 | 0.028 | z = 2.085 |

Note: p-Values were generated by likelihood ratio tests of the full model (generalised linear mixed effect model, generalised linear model) with and without the explanatory variables.

### Ant effects

Excluding ants reduced the abundance of predators (link 9 - all link codes described hereafter refer to Table 1 and Fig. 2). Also, in the Ant Exclusion treatment, the positive relationship between the number of galling nymphs and volume of *B. dracunculifoliae* galls was stronger (link 8). Ant presence on branches was positively correlated with parasitism of the galler *B. dracunculifoliae* (link 12), as well as with the abundance of the galler *R. limbata* on the plants (link 13), and negatively related to the presence of aphids on branches (link 14). Ant exclusion lowered the number of inquiline aphids inside *B. dracunculifoliae* galls (link 11) - ants seem to facilitate the aphid-galler interaction, apparently by leading or even carrying aphids inside galls.

### Aphid effects

Galls of *B. dracunculifoliae* with higher aphid inquilinism presented lower volume, and higher nymph mortality (link 1). Mortality of parasitised nymphs was also higher in galls with aphid inquilines (link 2). On plants with more branches occupied by aphids, parasitism of the galler *B. dracunculifoliae* was lower (link 15).

### Effect of hatched galls

Excluding hatched galls reduced parasitism of the galler *B. dracunculifoliae* (link 16). Plants with higher abundance of hatched galls also presented higher aphid inquilinism (link 17), higher abundance of the galler *R. limbata* (link 18), and higher parasitism of the galler *R. limbata* (link 19). In the Hatched Gall Exclusion treatment, the positive relationship between ant presence and the abundance of the galler *R. limbata* (link 13) was weakened (link 26). Excluding hatched galls also weakened (link 27) the positive relationship between ant presence and parasitism of the

galler *B. dracunculifoliae* (link 12); weakened (link 28) the negative relationship between herbivore abundance and the abundance of the galler *B. dracunculifoliae* (link 20); and also weakened (link 29) the positive relationship between predator abundance and aphid inquilinism (link 23).

### *Effect of the galler B. dracunculifoliae*

Excluding the galler *B. dracunculifoliae* reduced the abundance of predators, but only in the second week of the two-month study period (link 10); it also positively affected (link 25) the relationship between ant presence and aphid presence on branches (link 14) over the two months.

### *Parasitoid effects*

Aphid inquilinism (link 1) was higher in parasitised galls of the galler *B. dracunculifoliae* (link 21). Parasitoids appeared to facilitate the interaction between the aphid inquilines and the galler *B. dracunculifoliae*, possibly by changing gall shape and allowing aphids to enter through the longitudinal aperture of the gall.

### *Net effects*

We identified several three-way and four-way interactions, demonstrating how different effects combine to create net effects on species densities. For example, inquiline aphids can kill the nymphs of the galler *B. dracunculifoliae* (link 1), but because they preferentially occupy parasitised galls (link 21) and can kill parasitized nymphs (link 2), they can negatively affect parasitism and therefore also benefit the galler *B. dracunculifoliae* (link 15). As another example, aphid-tending ants, by increasing aphid inquilinism (link 11), possibly by leading aphids into galls, may positively affect parasitism on the galler *B. dracunculifoliae* (link 12) by reducing

aphids on branches (links 14) and therefore their negative effect on parasitism (link 15). On the other hand, by increasing aphid inquilinism, ants negatively affect parasitoids, since parasitoid mortality is higher in galls occupied by aphids (link 2). Furthermore, hatched galls increase parasitism on the galler *B. dracunculifoliae* (link 16), perhaps serving as a clue for parasitoids, but they also increase aphid inquilinism (link 17) and therefore can reduce parasitism on the galler *B. dracunculifoliae*. Finally, parasitism of the galler *B. dracunculifoliae* was also positively associated with ant presence on branches (link 12), but the relationship was dependent on the presence of hatched galls (link 27; four-way interaction).

Every direct interaction on the network was influenced by at least one of the other groups. In several cases, it was not possible to propose a mechanism behind the indirect links, even though the natural history of the system is fairly well-known.

**Discussion**

Whilst we already knew from studies on particular subsets of communities that non-trophic indirect effects must be important for understanding community structure and dynamics (Wootton 1993; Knight *et al.* 2005; Ohgushi 2005; Bukovinszky *et al.* 2008; Kéfi *et al.* 2012, 2015; Sanders *et al.* 2014; Hammill *et al.* 2015; Barbosa *et al.* 2019) this is thought to be the first truly empirical study to demonstrate, for an entire community, how numerous HOIs are acting at the same time. Here, we not only explored community-wide effects but experimentally tested each of the interaction modifications identified in situ in the field. By performing several manipulations concomitantly under natural field conditions, we were able to study the same interactions in different contexts and detect how multiple non-trophic interactions can interfere with or modify a single pairwise interaction. Thus, HOIs are not particular to certain sets of species but rather an

integral part of communities. The manipulations revealed a hidden network of HOIs modifying the direct interactions, as well as modifying each other, a level of complexity unexplored in empirical studies and seldom mentioned in theoretical studies. Whilst our study system focused on an insect community on a tropical shrub, a hidden network of HOIs will occur in all ecological communities, and therefore our results are of huge ecological relevance. The removal of different groups indirectly affected the interactions between several other pairs of groups with which the manipulated groups did not directly interact. That indicated that the species are indirectly connected and pairwise interactions are context-dependent. We acknowledge that the three density effect links in the network may represent species association and not true links (it would be difficult to differentiate between the two), which would mean that the groups do not actually affect one another directly and may be both affected by a third group. However, we include these links, first, because it would be difficult to clarify whether that is the case and, second, because the fact that a node changes in the absence of other groups shows that they are somehow connected, therefore there is potential for indirect interaction, albeit via additional intermediate groups. Also, the HOIs are represented by straight arrows in the network, but in reality, multiple steps may be involved in their effect propagation pathways. What the arrow represents is that the pairwise interaction is affected by the third group. This level of detail on indirect non-trophic interactions, and in particular for HOIs, for such a species-rich multi-trophic system, is truly unsurpassed.

It seems almost impossible to determine how a species affects another when we consider that all those non-trophic indirect effects may be taking place at the same time and interfering with each other. Besides, the links represented in the network may vary in magnitude through time, or may even be transient (Barbosa *et al.* 2019). In the community studied, population densities at a given

time are influenced by the sum of several indirect effects taking place at that time. If this is the case for natural systems in general, no inference can be made on the magnitude or direction of a given interaction in nature by studying it in isolation such as in an experimental setting or in computational models. Thus, depicting direct links between species in a network may be a misrepresentation of species effects or roles at the community level.

The existence of this hidden network suggests that, in natural conditions, it is not very likely that one species alone can determine the persistence of any other, such as in competitive exclusion. The role of competition in species coexistence has been widely demonstrated for focal species (Hiltunen *et al.* 2017; Maynard *et al.* 2017), but rarely in species-rich communities (Aschehoug & Callaway 2015; Brazeau & Schamp 2019; McClean *et al.* 2019). Here we show that HOIs can hugely increase the context-dependency of pairwise interactions, and by modifying interactions and offsetting or complementing each other, can allow a flexible modulation of species coexistence. Thus, the hidden network of HOIs very likely plays a crucial role in diversity maintenance in multispecies communities.

It is imperative to devise methods to harmonize pairwise interaction networks with the hidden network of HOIs. This would allow us to recognise fundamental mechanisms involving non-trophic interaction modulation that, for instance, allow the community as a whole to respond to a specific manipulation such as the removal of a species. Advancing our understanding of such mechanisms is likely to elucidate the underlying causes of stability and persistence of ecological communities (Hammill *et al.* 2015; Kelsic *et al.* 2015; Bairey *et al.* 2016; Grilli *et al.* 2017; Terry *et al.* 2019; Singh & Baruah 2021) and increase our ability to predict how they might respond to perturbations. Computational simulations that account for HOIs are helpful, but the results presented here suggest that empirical data will be key to our understanding because of the

numerous and often unpredictable opportunities for indirect effects via a variety of mechanisms and pathways.

Community ecology research rarely studies entire communities, in part due to the logistical problems involved in studying multispecies systems, but mostly due to the lack of tools to approach the complex intricacies of ecological communities. The results gathered here are unique, as the experimental exclusion of more than one group was performed concomitantly in the field in a self-contained but diverse system, and the densities of almost all groups of species were monitored. The challenges of replicating this study in a larger or less self-contained community are evident, but should not be a barrier to further empirical investigation into the generality of the findings presented here. We hope that this study will instigate new methodologies for more holistic approaches to studying ecological communities in general. The changes identified here could not be predicted from analyses of pairwise networks. Unlike pairwise interactions, HOIs cannot be predicted based on species functional traits or from previously recorded interactions (e.g., plant pollination, predation, parasitism). HOIs can be transient and involve effect propagation through several intermediaries. Thus, through HOIs, species have global effects on the community context. At the same time, these effects can vary in intensity and direction depending on the community context (species composition and abundances mainly) at each moment. This circular relationship between the structure of the global system and the local interactions among the components is typical of complex adaptive systems (Levin 1998). Thus, developing new methods of applying complexity theory to ecosystems (Levin 1998) could be one way forward.

**Competing interests:** Authors declare that they have no competing interests.

**Data accessibility statement:** should the manuscript be accepted, the data supporting the results will be archived in the Dryad Digital Repository and the data DOI will be included at the end of the article.

**Methods**

*Study Site*

This study was conducted at Serra do Cipó, in southeast Brazil, in the Cerrado biome. This region is characterised by quartzitic soils covered by rocky grasslands, with a predominance of herbs and shrubs (Fernandes 2016). It has a Cwb Köppen climate type, with dry winters and rainy summers (Fernandes 2016). The average annual rainfall is between 1250 and 1550 mm, and the average temperature ranges from 15.1 to 20.7ºC (Fernandes 2016). The study site (19°16'48"S - 43°35'20"W; 1170 m elevation) is undergoing restoration with native species since

2010, after serving as a source of soil for the paving of the MG-010 highway. The plant species *Baccharis dracunculifolia* D.C. (Asteraceae) is one of the species planted and is now dominant in the area.

*Study System*

The plant species *B. dracunculifolia* is a perennial, evergreen, dioecious shrub, 2-3 m in height, which is widely distributed across southern and central South America (Fernandes 2016). *B. dracunculifolia* has a key role in natural succession and regeneration and is, therefore, important in terms of biodiversity and ecosystem functioning (Fernandes 2016). The plant species hosts a diverse fauna of free-feeding herbivores, mostly Hemiptera, Coleoptera and Orthoptera, and also many predators including the orders Araneae, Coleoptera, Mantodea, and Hymenoptera. Among the 17 species of gall-inducing insects recorded on *B. dracunculifolia* in multiple locations, *Baccharopelma dracunculifoliae* (Sternorrhyncha: Psyllidae) is the most common (Fernandes *et al.* 2014; Barbosa *et al.* 2017). It induces a gall in the midrib of the leaf, which bends over itself until the borders are joined, forming an elliptical, green, glabrous, single-chambered gall that usually harbours up to four nymphs. The galls remain attached to the plant after dehiscence and gradually become dry and woody. Both hatched and non-hatched galls of *B. dracunculifoliae* are occupied by many inquiline invertebrates, such as ants, spiders, aphids, etc. (Espírito-Santo & Fernandes 2002). These hatched galls can trigger indirect effects that feedback to the galler modifying its interactions with other species (Barbosa *et al.* 2019). At least ten parasitoid species have been reared from galls of *B. dracunculifoliae* (Barbosa *et al.* 2017) and parasitism rates are around 45% (Espírito-Santo & Fernandes 2002). When the parasitoids emerge, they leave a characteristic exit hole on the gall wall (Espírito-Santo *et al.* 2004), facilitating aphid colonisation of live galls (MB *pers. obs.*).

The aphid *Uroleucon tucumani* (Sternorryncha: Aphididae) is by far the most frequent inquiline (a species that occupy a living space produced by another species, such as a gall) and can indirectly kill the nymphs of the gall maker (Fagundes *et al.* 2005) (MB *pers. obs*.). This aphid species also feeds and reproduces on the apical meristems of the host plant, forming dense colonies that produce honeydew (sugary secretions). *U. tucumani* attracts at least 15 species of ants, which tend and protect them in a trophobiotic relationship (Fagundes *et al.* 2005; Neves *et al.* 2011) (MB *pers. obs*). It has been found that the presence of ants reduces the number of *B. dracunculifoliae* nymphs per gall, and the presence of aphids reduces *B. dracunculifoliae* gall size because aphids compete with the galler for sap assimilates and young leaves in terminal buds (Fagundes *et al.* 2005). In addition, the presence of ants and aphids on *B. dracunculifolia* decreased the abundance of other free-feeding herbivores, and the presence of aphids decreased plant shoot growth (Neves *et al.* 2011). Ants tending aphids can have a direct negative impact on herbivores (Fernandes *et al.* 1999). However, the aphids on their own can also reduce the abundance of fluid-sucking and chewing insects due to exploitation competition or by altering the nutritional quality of the host plant (Fay *et al.* 1996; Larson & Whitham 1997).

### *Experimental design*

Sixty-four isolated individuals of the plant species *Baccharis dracunculifolia* D.C. (Asteraceae) of 1.5-2.0m in height, distant at least 5m from conspecific plant individuals but in the same area were randomly identified and marked in the field. The plants were randomly assigned to four treatment groups (16 plants each) in blocks at least 20m apart from each other. Thus, there was one plant individual for each treatment in each block, and 16 blocks altogether. Different manipulations were performed in each treatment:

(i) *Ant Exclusion* - Ants were excluded by applying a non-toxic resin (Tanglefoot®, Tanglefoot Company, Michigan, USA) to the basal stem of the plants. During the study period, the plant individuals were monitored twice a week to check the effectiveness of the treatment, which was repeated where necessary;

(ii) *Live Gall Exclusion – B. dracunculifoliae,* the commonest galling species found on *B. dracunculifolia* was removed by direct collection. Since new galls could be induced over the monitoring period, the treatments were maintained by excluding newly induced galls every week, which prevented any galls from reaching full development;

(iii) *Hatched Gall Exclusion* - All hatched galls of *B. dracunculifoliae* were excluded from each of 16 plant individuals. Weekly observations were performed to exclude newly hatched galls;

(iv) *Control –* To emulate plant response to mechanical damage, non-galled leaves were removed in an equal amount to the average number of galls collected in treatments (ii) Live Gall Exclusion and (iii) Hatched Gall Exclusion.

### *Monitoring and data collection*

During the study period (August - September 2015), we combined direct observation and gall dissection data to quantify densities of several arthropod groups in each treatment as well as the frequency of direct interactions, including parasitism and inquilinism.

*Observations –* we performed weekly observations on the plant individuals during the study period, starting a week after setting up the experiments. The species and abundance of arthropods on isolated plants were quantified by directly counting individuals and morphospecies for 10 minutes per week (between 9:00am and 3:00pm), totalling one hour per plant over the two

months. We quantified the number of individuals of each species (or morphospecies) of ants, predators (e.g., spiders, lady-birds, praying mantids) and free-feeding insect herbivores, as well as the number of branches occupied by ants (ant presence), and terminal buds occupied by aphids (aphid presence). During the study period, at least one individual of each morphospecies was collected for identification. Plant shoot growth was also measured using a tape measure for treatments Control and Live Gall Exclusion to test the effect of the galler on plant growth. On the final week of the eight-week study period, we quantified the abundances of *B. dracunculifoliae* galls, and hatched *B. dracunculifoliae* gall (except in the treatments in which they had been excluded). We counted all full-sized or close to full-sized live galls and all hatched galls of *B. dracunculifoliae* found in three half-meter branches haphazardly chosen around the crown of each individual of *B. dracunculifolia*. We also quantified the abundance of an apical gall induced by *Rachiptera limbata* Bigot (Diptera: Tephritidae) on the same branches. We chose to include the galler *R. limbata* in the study because in a previous experiment (Barbosa et al. 2017), there was a twofold increase in the frequency of one of the parasitoid species (Bracon sp2) attacking *R. limbata* after removing the galler *B. dracunculifoliae*.

*Gall dissection* – After counting the aforementioned galls, we collected 15 of each gall type, *B. dracunculifoliae* and *R. limbata* from the same three branches (five of each type per branch). They were stored individually and taken to the lab for dissection. To calculate parasitism rates we dissected the galls and quantified the proportion of parasitised and unparasitised nymphs per gall through the presence of "mummies" (parasitised galler nymphs). We also determined the mortality rate of parasitised and unparasitised galler nymphs, and aphid inquilinism (aphids per gall; not to be confused with "aphid presence" on branches) in the *B. dracunculifoliae* galls. Before dissection, galls were also measured for width and length to calculate their volumes - as

an indicator of performance - according to their shapes (Volume = 4/3π [1/2 Length] [1/2Width]², for ellipsoid galls), although parasitism is thought to increase gall size (Espírito-Santo & Fernandes 2002).

*Statistical analyses*

Besides the interactions directly detected and quantified through observation and gall dissection, we tested for density effects by comparing a node's frequency, abundance or trait among the exclusion treatments (Fig. 1). To test for link modulation by the density of other groups we contrasted the detected direct links with the abundance or frequency of other groups, entering treatment as a covariate with interaction, and week, block and plant individual as random effects depending on the dataset used. This allowed testing for the effect of node removal on interactions (three-way interaction) as well as on interaction modifications (four-way interaction) by comparing treatments with regard to the slope of the relationship between an affected link and the affecting node – e.g., how aphid presence (herbivory) varies with ant presence in each treatment (Fig. 1, Tables S1 and S2).

We used generalised linear mixed effect models (GLMMs) (Crawley 2013) or, when it was not possible to obtain a satisfactory model fit, we fitted linear mixed effect models (LMMs) after square-root or log-transforming the data when necessary to improve the homoscedasticity of residuals. The structures of the maximal models are shown in the Supplementary Text. We used the lme4 package in R (R Core Team 2021) to fit the models. We checked the GLMMs for over-dispersion of residuals using the function overdisp.glmer (RVAideMemoire Package). To correct for over-dispersion we refitted the models with negative binomial distribution using the function glmer.nb (MASS Package) instead of glmer. To determine the structure of the random effect in the models we compared models allowing for variation in intercept within random effect to those

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allowing for variation in intercept and slope and selected the ones with lower Akaike Information Criteria (AIC) score. We performed simplifications of the maximal models by removing non-significant fixed effects to obtain a minimum adequate model (Crawley 2013). P-values of fixed effects were generated by likelihood-ratio tests of the full model with and without the explanatory variables. We then refitted the minimum adequate model using Restricted Maximum Likelihood (REML) and visually checked the residual plots for deviations from homoscedasticity or normality. We used the testInteractions function (phia Package) to perform Wald chi-square test for *post hoc* comparisons between treatments and to perform the pairwise comparisons of adjusted slopes with respect to the response variable for contrasts of the factor treatment.

*Node modulation* - We used data from the weekly observations to compare treatments in terms of the species and abundance of predators and free-feeding insect herbivores, as well as the number of branches occupied by ants (ant presence), terminal buds occupied by aphids (aphid presence), and shoot growth. We entered treatment as a fixed effect, week as a covariate with interaction and block and plant individual as random effects. Using the dissection dataset, we tested the effect of the node exclusion treatments on the relationship between the volume of *B. dracunculifoliae* gall and nymphs per gall. We also tested the relationships between inquilinism (aphid/gall) and gall volume and nymph mortality of *B. dracunculifoliae* galls, as well as mortality of parasitized nymphs. We entered treatment as a covariate with interaction in all models. We used block and plant individual as random effects (Table S1).

*Link modulation* - To test for link modulation by the density of other groups we used the observation dataset to contrast aphid presence (aphid-plant interaction) with herbivore and predator abundance, and ant presence, entering treatment as a covariate with interaction, and week, block and plant individual as random effects (Table S2). Also, for the analyses of the

effect of node removal on interactions (three-way interaction) as well as on interaction modifications (four-way interaction) we looked for variation among treatments in the slope of the relationship between pairs of variables. With the dissection data we compared inquilinism (aphid/gall) between parasitized and unparasitised galls and also parasitism rates of *B. dracunculifoliae* and *R. limbata* galls among treatments. Combining the observation plus dissection datasets, we fit models for the response variables abundance of *B. dracunculifoliae* and *R. limbata* galls, and aphid inquilinism, against the explanatory variables (abundance of predators and free-feeding insect herbivores; ant presence; aphid presence; and abundance of hatched *B. dracunculifoliae* galls). To test the relationship between parasitism of gallers *B. dracunculifoliae* and *R. limbata* against those same explanatory variables, we were not able to fit a GLMMs, and instead we used general linear models (GLMs) with binomial distribution. All models had treatment as a covariate with interaction. We only entered block as random effect since in merging the observation and dissection datasets, the observation data had to be combined across weeks.

**Supplemental information**

Table S1 – S2

Model structures

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# Supplemental Information

## Experimental evidence for nature's hidden network of higher-order interactions

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**This file includes:**

Table S1 – S2

Supplementary text - model structures

| AFFECTED NODES | | Exclusion treatments | | | Observation data | | | | Dissection data | | | Other | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ant (exclusion) | Galler G7 (exclusion) | Hatched gall (exclusion) | Aphid (frequency on branches) | Ants (frequency on branches) | Herbivores (abundance) | Predators (abundance) | Aphid (inside galls) | Parasitoids of G7 | Parasitoids of G10 | Hatched gall G7 | Galler G7 | Galler G10 | Host Plant |
| | Ants (frequency on branches) | x | NS - p = 0.851 | NS - p = 0.851 | x | x | x | x | x | x | x | x | x | x | x |
| | Aphid (frequency on branches) | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| | Herbivores (richness and abundance) | NS - richness p = 0.264; abundance p = 0.123 | NS - richness p = 0.668; abundance p = 1.00 | NS - richness p = 0.264; abundance p = 1.00 | x | x | x | x | x | x | x | x | x | x | x |
| | Predators (richness and abundance) | **NS - richness p = 0.092; Positive on Predator abundance - line 2** | **NS - richness p = 1.00; Positive on Predator abundance - line 2** | NS - richness p = 1.00; abundance p = 0.944 | x | x | x | x | x | x | x | x | x | x | x |
| | Parasitoids of G7 | x | x | x | x | x | x | x | **Negative - increase mortality of parasitised nymphs - line 9** | x | x | x | x | x | x |
| | Parasitoids of G10* | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| | Hatched gall** | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| | Galler G7 | **Negative on Relationship Volume of Gall G7 x Nymph abundance - line 6** | x | NS (G7 volume and nymph mortality) - p = 0.166 | x | x | x | x | **Negative - decrease G7 volume, increase nymph mortality - lines 7 and 8** | x | x | x | x | x | x |
| | Galler G10* | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| | Host Plant | x | NS (Shoot growth) - p = 0.329 | x | x | x | x | x | x | x | x | x | x | x | x |

*Nodes upon which we could not test node modulation effects because we only measured their interactions.

** Hatched gall is not a species and its abundance is highly correlated with the abundance of live galls G7.

**Table S1.**

Statistical tests performed to detect node modulation effects among groups of arthropods on the host plant *Baccharis dracunculifolia*. For the significant tests (in bold), the detailed statistical results are provided on the indicated lines of Table 2. G7 and G10 refer to the gallers *Baccharopelma dracunculifoliae* and *Rachiptera limbata*, respectively. Empty cells are combinations for which it was not possible or applicable to test with the available data.

| AFFECTED LINKS | AFFECTING NODES | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Exclusion treatments | | | Observation data | | | | Dissection data | | | Other | | | |
| | Ant (exclusion) | Galler G7 (exclusion) | Hatched gall (exclusion) | Aphid (frequency on branches) | Ants (frequency on branches) | Herbivores (abundance) | Predators (abundance) | Aphid (inside galls)* | Parasitoid of G7 | Parasitoid of G10* | Hatched gall G7 | Galler G7* | Galler G10* | Host Plant* |
| Aphid-Host plant | NS - p = 0.939 | | | x | Negative; Control < Live gall exclusion - line 1 | NS - p = 0.614 | NS - p = 0.375 | x | x | x | NS - p = 0.519 | x | x | x |
| Ant-Galler G7 | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Ant-Predator | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Aphid-Galler G7 | Positive - line 4 | x | NS - p = 0.228 | x | NS - p = 0.811 | NS - p = 0.614 | Positive; Control > Hatched gall excluded - line 10 | x | Positive - line 3 | x | Positive - line 11 | x | x | x |
| Aphid-Parasitoid G7 | NS - p = 0.928 | x | NS - p = 0.928 | x | x | x | x | x | x | x | x | x | x | x |
| Galler G7-Predators | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Hatched gall-Ant | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Galler G7-Host plant | NS - p = 0.527 | x | NS - p = 0.527 | NS - p = 0.738 | NS - p = 0.828 | Negative; Control < Hatched gall excluded - line 12 | NS - p = 0.876 | x | x | x | x | x | x | x |
| Galler G10-Host plant | NS - p = 0.997 | | | NS - p = 0.878 | Positive; Hatched exclusion < Control - line 16 | NS - p = 0.802 | Positive - line 18 | x | x | x | Negative - line 17 | x | x | x |
| Parasitoid-Galler G7 | NS - p = 0.105 | x | Positive - line 5 | Negative - line 15 | positive; Control > Hatched excluded - line 13 | NS - p = 0.056 | Positive - line 14 | x | x | x | NS - p = 0.123 | x | x | x |
| Parasitoid-Galler G10 | NS - p = 0.532 | | | NS - p = 0.436 | NS - p = 0.234 | NS - p = 0.470 | NS - p = 0.190 | x | x | x | Positive - line 19 | x | x | x |

*Nodes whose interaction modulation effects we could not test because we only quantified their interactions (e.g., galls per plant).

**Table S2.**
Statistical tests performed to detect link modulation effects among groups of arthropods on the host plant *Baccharis dracunculifolia*. For the significant tests, the detailed statistical results are on the indicated lines of Table 2. G7 and G10 refer to the gallers *Baccharopelma dracunculifoliae* and *Rachiptera limbata*, respectively. Results in red and blue are three-way and four-way interactions, respectively. Empty cells are combinations for which it was not possible or applicable to test with the available data.

## Model Structures

Node modulation:

*A) Observation data:*

i) Model<- glmer (or lmer) (node ~ treatment : week + (1 | block/ individual))

Nodes:

Ants (frequency on branches)

Aphid (frequency on branches)

Herbivores (richness and abundance)

Predators (richness and abundance)

ii) Model<- lmer (Host plant (shoot growth) ~ treatment + (1 | block))

*B) Dissection data:*

i) Model<- glmer (or lmer) (node ~ aphid inside galls : treatment + (1 | block/individual))

Nodes:

Galler G7 (gall volume, nymph mortality)

Parasitoids of Galler G7 (mortality of parasitized nymphs)

ii) Model<- lmer (G7 gall volume ~ G7 nymph abundance : treatment + (1 | block/individual))

Link modulation:

*A) Observation data:*

i) Model<- lmer (affected link ~ affecting node : treatment + (1 | week) + (1 | block/individual))

Affected link:

Aphid-Host Plant (aphid frequency on branches)

*B) Dissection data:*

i) Model<- glmer (affected link ~ treatment + (1 | Block))

Affected links:

Parasitoid-Galler G7 (parasitism of the galler G7)

Parasitoid-Galler G10 (parasitism of the galler G10)

ii) Model<- lmer (aphid inquilinism ~ G7 parasitism (binary) : treatment + (1 | Block/individual))

*C) Observation + Dissection data:*

i) Model<- glmer (or lmer) (affected link ~ affecting node : treatment + (1 | Block))

Affected links:

Aphid-Galler G7 (aphid inquilinism)

Galler G7-Host plant (gall abundance)

Galler G10-Host plant (gall abundance)

ii) Model<- glm (affected link ~ affecting node : treatment)

Affected links:

Parasitoid-Galler G7 (parasitism of the galler G7)

Parasitoid-Galler G10 (parasitism of the galler G10)