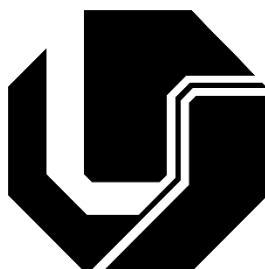


UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOLOGIA

**O PAPEL DAS GALHAS INTERMEDIANDO INTERAÇÕES EM UM SISTEMA
MULTITRÓFICO**

UIARA COSTA REZENDE

2018



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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O papel das galhas intermediando interações em um sistema multitrófico

Dissertação apresentada à Universidade Federal de Uberlândia, como parte das exigências para obtenção do título de Mestre em “Ecologia e Conservação de Recursos Naturais”.

Orientador

Prof. Dr. Denis Coelho de Oliveira

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ATA

Ata da defesa de DISSERTAÇÃO DE MESTRADO junto ao Programa de Pós-Graduação em Ecologia e Conservação de Recursos Naturais - Instituto de Biologia da Universidade Federal de Uberlândia.

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Às **09 horas** do dia **23 de fevereiro** do ano de **2018**, na **sala 14A do Bloco 2D** – Campus Umuarama, da Universidade Federal de Uberlândia reuniu-se a Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação em Ecologia e Conservação de Recursos Naturais, composta pelos Doutores: **Paulo Eugênio A. M. Oliveira (UFU)**, **Thiago Alves Magalhães (UFLA)** e **Denis Coelho de Oliveira (UFU)** orientador da candidata.

Iniciando os trabalhos, o Presidente da mesa, Dr. Denis Coelho de Oliveira apresentou a Comissão Examinadora e a candidata, agradecendo a presença do público e concedendo à Discente a palavra para a exposição do seu trabalho. A duração da apresentação da Discente, o tempo de arguição e resposta foram estabelecidos conforme as normas do Programa.

A seguir, o Senhor Presidente concedeu a palavra aos examinadores, que passaram a arguir a candidata. Finalizada a arguição, que ocorreu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais.

Em face do resultado obtido, a Banca Examinadora considerou a candidata Aprovada sem sugestão de novo título.

Esta defesa de Dissertação de Mestrado Acadêmico é parte dos requisitos necessários à obtenção do título de Mestre. O competente diploma será expedido após o cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar, foram encerrados os trabalhos às 11 horas e 10 minutos. Foi lavrada a presente ata que, após lida e aprovada, foi assinada pela Banca Examinadora.



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Resumo

O hábito galhador provavelmente foi selecionado através da obtenção de vantagens adaptativas, como alocação de substâncias específicas (hipótese nutricional), estabilidade microclimática e proteção contra o ataque de inimigos naturais (hipótese do inimigo). Insetos galhadores modificam o metabolismo e fisiologia dos tecidos da planta hospedeira e o desenvolvimento das galhas depende do estímulo específico do galhador. Assim, a entrada de inimigos naturais no sistema pode provocar profundas alterações estruturais e químicas na galha e, por consequência, diminuir o fitness do galhador. Nosso sistema de estudos são as galhas induzidas por *Palaeomystella oligophaga* (Lepidoptera) em *Macairea radula* (Melastomataceae), que são conspícuas, com parênquima espesso e rígido, recobertas por projeções com tricomas, e apresentam um gradiente de colorações do verde ao vermelho. Apesar destas características, estão inseridas em um contexto multitrófico, onde os galhadores são atacados direta ou indiretamente por diferentes espécies de insetos. Neste contexto, investigar a importância de características das galhas mediando estas interações, e como estes organismos afetam seu metabolismo, pode revelar o real papel da galha enquanto intermediadora de interações galhador-inimigos naturais. Este estudo objetivou demonstrar o papel das galhas intermediando as interações multitróficas: (i) investigando quais traços da galha influenciam o sucesso de sobrevivência de *P. oligophaga*, considerando e descrevendo todos os diferentes tipos de inimigos presentes na comunidade; (ii) quais alterações metabólicas os diferentes grupos de inimigos naturais promovem na composição química e estrutural dessas galhas, e por fim (iii) quanto os tecidos especializados dependem dos estímulos específicos constantes do galhador nos últimos estágios de desenvolvimento. Foram registradas duas espécies de parasitoides, uma espécie de cecidófago, herbívoro e predação. Dentre as características analisadas, a espessura do parênquima foi negativamente relacionada a probabilidade de ocorrência de alguns inimigos naturais do galhador, enquanto que a altura da galha

na planta foi positivamente relacionada. Apesar da complexidade estrutural apresentada pelas galhas, *P. oligophaga* é atacado por uma diversidade de organismos. As atividades dos inimigos naturais estudados sobre os tecidos das galhas resultaram no colapso metabólico e degradação generalizada, em processo semelhante a senescência natural destas estruturas. Assim, somente o galhador é capaz de manter o metabolismo da galha ativo, alocação e acúmulo de substâncias específicas identificadas no tecido nutritivo (lipídios e proteínas) e de reserva (amido). Os tecidos das galhas são, portanto, totalmente dependentes do estímulo causado pelo galhador mesmo em estágios maduros.

Abstract

The galling habit probably was selected by obtainment of adaptive advantages such as specific substance allocation (nutritional hypothesis), microclimatic stability and protection against natural enemy attack (enemy hypothesis). Galling insects modify the host tissues structure and physiology, and the gall development depends on the galling specific stimulus. Thus, the entry of natural enemies into the system can provoke structural and chemical changes in the gall and consequently, decrease the fitness of the galling. Our study system *Palaeomystella oligophaga* (Lepidoptera) - *Macairea radula* (Melastomataceae) showed conspicuous galls, with thick and rigid parenchyma, covered by projections with trichomes, and show a gradient of green to red colorations. Despite these characteristics, they are inserted in a multitrophic context, where the gallings are attacked directly or indirectly by different species of insects. Herein, we investigate the importance of gall traits mediating these interactions, and how these organisms affect their metabolism, may reveal the true role of the gall as an intermediary of galling-enemy interactions. The aims of this study was demonstrate the role of galls mediating multitrophic interactions: (i) investigating which traits influence the survival success of *P. oligophaga*, considering and describing all the different types of enemies present in the community; (ii) what metabolic changes the

different groups of natural enemies promote in the chemical and structural composition of these galls, and finally (iii) how specialized tissues depend on the specific stimuli of the galling in the latter stages of development. Among the analyzed characteristics, parenchyma thickness was negatively related to the probability of occurrence of some galling natural enemies, while the height of the gall on plants was positively related. Despite the structural complexity presented by these galls, *P. oligophaga* is attacked by a variety of organisms. The activities of the natural enemies studied on galls tissues resulted in metabolic collapse and generalized degradation, similar to natural senescence. Thus, only galling is capable of maintaining the metabolism of the gall, with allocation and accumulation of specific substances identified in the nutritive tissue (lipids and proteins) and storage (starch). The tissues of the galls are, therefore, totally dependent on the stimulus caused by the galling even in mature stages.

Introdução geral

Dentre as interações inseto-planta existentes, algumas evoluíram a casos tão peculiares ao ponto de promoverem transformações em tecidos vegetais resultando em órgãos neoformados, conhecidos como galhas (Shorthouse et al. 2005, Stone and Schonrogge 2003). Assim, o hábito galhador provavelmente foi selecionado pela obtenção de vantagens adaptativas, como a alocação de substâncias específicas diferentes ou em maiores concentrações que as porções não galhadas dos tecidos da hospedeira (Price et al. 1987, Hartley 1992, Oliveira et al. 2014). De tal modo, o inseto obtém em quantidade e qualidade os nutrientes de forma mais acessível e rápida do que herbívoros de vida livre (Mani 1964). Consideradas como micro-habitats, galhas podem proporcionar condições abióticas mais estáveis em relação ao ambiente externo, diminuindo variações de temperaturas, umidade, exposição a ventos ou radiação UV (Lill and Marquis 2007). Possivelmente ainda, estas interações surgiram como adaptações protetivas para os insetos contra a ação de inimigos naturais, segundo a hipótese do inimigo, o que pode ainda justificar a diversidade de estruturas e compostos secundários encontrados em diversos sistemas galhador-planta hospedeira (Price et al. 1987, Stone and Schönrogge 2003).

Mesmo que as galhas funcionem como uma barreira que protege os insetos galhadores, estas estruturas são alvo de múltiplos organismos que buscam recursos alimentares ou abrigo, estabelecendo, em alguns casos, interações tritróficas especialistas (Price and Pschorn-Walcher 1988, Waring, Van Hezewijk and Roland 2003, Cuevas-Reyes et al. 2007, Sugiura and Yamazaki 2009; Bailey et al. 2019). A forte pressão seletiva exercida por estas interações pode, inclusive, justificar a aparência bizarra de algumas galhas que então podem apresentar parênquimas espessos, tricomas e projeções, colorações conspícuas, e acúmulo de compostos secundários (Stone and Schonrogge 2003). Portanto, a diversidade estrutural das galhas pode, supostamente, estar relacionada à proteção que conferem aos galhadores contra o ataque de inimigos naturais (Stone e Schonrogge

2003). Considerando que o morfotipo da galha está diretamente relacionado ao grupo taxonômico ao qual o galhador pertence, ou ainda, particular à espécie (e.g. Oliveira et al. 2008, Isaias et al. 2014).

Insetos galhadores modificam o metabolismo e fisiologia do tecido galhado (Isaias et al 2015, Oliveira et al 2017) e o desenvolvimento das galhas depende do estímulo específico do galhador. Assim, a entrada de inimigos naturais no sistema pode provocar profundas alterações estruturais e químicas na galha e por consequência, diminuir o fitness do galhador. Determinadas espécies de parasitoides, por exemplo, paralisam seus hospedeiros quando o consumo começa (sensu Hanson and Nishida 2014), o que provavelmente cessa o estímulo do galhador sobre os tecidos vegetais. Deste modo, surge o questionamento: o que acontece com a ausência de estímulo, causado pela paralização do galhador por parasitoides, durante estágios avançados da galha? Também, o que acontece quando galhas são atacadas por organismos como cecidófagos, que se alimentam dos tecidos destas estruturas (Sanver e Hawkins 2000, Sugiura and Yamazaki 2006), onde o estímulo de alimentação continua? Existe a possibilidade de esses organismos manterem o metabolismo das galhas, ou o comportamento e substâncias inespecíficas resulta no colapso dos tecidos vegetais? A remoção mecânica dos galhadores pode gerar danos que influenciam resultados e conclusões. Portanto, os parasitoides e cecidófagos funcionam como um experimento natural, respectivamente, removendo o estímulo alimentar específico e fornecendo um inespecífico.

Nosso sistema de estudos são as galhas induzidas por *Palaeomystella oligophaga* (Lepidoptera) em *Macairea radula* (Melastomataceae) que são conspícuas, com parênquima espesso e rígido, recobertas por projeções com tricomas, e apresentam um gradiente de colorações do verde ao vermelho. Apesar destas características, estão inseridas em um contexto multitrófico, onde os galhadores são atacados direta ou indiretamente por diferentes espécies de insetos. Neste contexto, investigar a importância de características das galhas mediando estas interações, e como

estes organismos afetam seu metabolismo, pode revelar o real papel da galha enquanto intermediadora de interações galhador-inimigos. Este estudo objetivou investigar o papel das galhas intermediando as interações multitróficas: (i) investigando quais os traços da galha influenciam o sucesso de sobrevivência de *P. oligophaga*, considerando e descrevendo todos os diferentes tipos de inimigos presentes na comunidade; (ii) quais alterações metabólicas os diferentes grupos de inimigos naturais promovem na composição química e estrutural dessas galhas, e por fim (iii) quanto os tecidos especializados dependem dos estímulos específicos constantes do galhador nos últimos estágios de desenvolvimento.

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Multiple gall traits and galling survival in a multi-enemy context

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Abstract

Since galls establish a barrier between the galling and the outside organisms, their morphological characteristics may have specialized, mediating roles in interactions of the multitrophic system that they are inserted. The gall system *Palaeomystella oligophaga* (Lepidoptera) - *Macairea radula* (Melastomataceae) a conspicuous structure attacked by several types of insects. Considering the enemy hypothesis and the selection of secondary characteristics, we aimed to respond which gall traits influence galling survival success, considering and describing all the different enemy kinds present in the community. The occurrence probability of each enemy kind was analyzed accordingly with galls color, heights on plants, projections lengths and parenchyma thickness. We found only 68 galls out of 321 containing alive gallings (21.2 %) and 78,8% of mortality caused by cecidophages (N = 131; 40.8 %), large parasitoids (N = 61; 19 %), small parasitoids (N = 23; 7.2 %), herbivores (N = 21; 6.5 %) and predation (N = 17; 5.3 %). The occurrence probability of small parasitoids and predation increases with gall height in the plant. Occurrence of large parasitoids, herbivory and predation decreases as the average parenchyma thickness increases, and there were not effects of color or projections average. We demonstrate how at the same time

different traits can or not be effective against directly or indirectly galling multi enemies that caused massive top-down regulation, with different proportions. In this sense, whatever trait effective against whichever enemy may have positive population effects, especially over the generations.

Keywords: gall aposematism, multitrophic, parenchyma thickness, parenchyma projections, enemy hypothesis

Introduction

Some insects have adapted to control and redirect the growth, redifferentiation and physiology of plant organs to its own advantage to form galls (Mani 1964; Price et al 1987; Stone and Schönrogge 2003; Oliveira et al 2016). This kind of interaction may be considered the most complex plant-insect association, with the galling acting as sophisticated herbivore (Shorthouse et al. 2005). Several hypotheses have been postulated to explain the pervasive adaptive irradiance of galls (Price et al 1987). One of these proposes that the galling habit might evolve as an adaptation related to dissection pressure on insect larvae, once the mechanical structure can provide an ideal microclimatic habitat (Danks 2002; Lill et al. 2007). Another hypothesis suggests that the formation of galls can be related to the absence of foraging behavior, and the galling can achieve its nutrition inducing the formation of specialized cells (e.g. Mani 1984; Shorthouse 1986; Bronner 1992). Finally, the enemy hypothesis states that the plant host tissues surrounding the galling protects it against the attack of natural enemies and can yet be the explanation for the diversity of shapes, sizes, secondary structures and substances displayed by galls (Price and Pschorn-Walcher 1988; Stone and Schonrogge 2003; Bailey et al 2009).

Plant traits can mediate interactions between free-living herbivorous insects and their natural enemies (e.g. Heath et al 2013; Keasar et al. 2013), even through signaling (e.g. Unsicker et al. 2009; Schaefer and Ruxton 2011). Considering that galls establish a barrier between the galling and the outside organisms, their morphological characteristics may have specialized, mediating roles in interactions of the multitrophic systems they are inserted. Gallings sustain a variety of natural enemies such as pathogens, parasitoids and predators (carnivores or herbivores), both arthropods and vertebrates (e.g. Abrahamson et al. 1989; Zamora and Gómez 1993; Van Hezewijk and Roland 2003; Sugiura and Yamazaki 2009; Forbes et al. 2015, Katilmis and Azmaz 2015; Mete and Mergen 2017). This scenario raises the question of how much galls can protect their inductors and, more specifically, what characteristics the selective pressure of natural enemies can select.

Among the several traits suggested as galling defenses, conspicuous coloration of galls seems to be the most intriguing (Inbar et al. 2010a, b; White 2010; Lev-Yadun 2016a). Although many mechanisms that may justify the selection of this characteristic have been speculated in the literature (reviewed in Lev-Yadun 2016a), the aposematic hypothesis deserves special attention and should be tested for validation (Inbar et al. 2010a). Considering that red plant organs are usually less predated (e.g. De Wert et al. 2012; Lev-Yadun 2016b), and that red coloration in galls can indirectly be stimulated by galling and even change accordingly its interactions (e.g. Dias et al. 2013), is plausible to suggest that this trait may have some role in galling survival.

In addition, gall size is another characteristic that has already been tested as a galling defense against parasitoids (e.g. Price and Clancy 1986; Tschardtke 1994; Price 1988; Zwolfer and Arnold-Rinehart 1993; Sopow and Quiring 2001; van Hezewijk and Roland

2003; Zargaran et al 2011; Figueiredo et al. 2014). Nevertheless, parenchyma thickness was less considered and tested as a defense against parasitoids, even being the actual barrier protecting the galling, (but see Waring and Price 1989; Zargaran et al 2011). Trichomes and projections have also been suggested as structures involved on gallings protection (Bailey et al 2009). Considering that they act as plant defense against the attack of herbivores (Levin 1973; Alahakoon et al. 2016; Yamazaki and Lev-Yadun 2015; López-Carretero et al. 2016), it is intuitive to think that this protection extends to galls that developed these secondary traits. Thus, both trichomes and projections can prevent gall and galling from serving as food for some organisms. Finally, the position of the gall in plant and, therefore, in the environment, may determine different survival success (e.g. Leite et al. 2017). This may occur because galls may be subjected to different environmental conditions and/or different enemies.

Despite the importance of these topics, some studies consider only one gall trait when testing galling protection against enemies (e.g. Weis and Abrahamson 1985; Sopow and Quiring 2001; Egan et al. 2011; Lev-Yadun 2016a; Nicholls et al. 2017). Other studies have examined just one species or kind of natural enemy (e.g. Weis and Abrahamson 1985; Waring and Price 1989; Bailey et al 2009; Cooper and Rieske 2011; Zargaran et al 2011; Rostás et al. 2013), while it's know that gallings may cope with a wide variety of different organisms (e.g. Askew 1980; Cooper and Rieske 2010; Stone and Schonrogge 2003; Forbes et al. 2015). In this sense, theoretical ideas need to be adapted to galling interactions in the field, aiming understand how a variety of galls traits works against each of the several galling enemies. For instance, galls induced by *Palaeomystella oligophaga* (Lepidoptera) on *Macairea radula* (Melastomataceae) are conspicuous structures, varying in color, size,

projections length and the height they occur in plant. Regardless of that, several types of insects attack the galling. Considering the enemy hypothesis and the selection of secondary characteristics, we aimed to respond which gall traits influence galling survival success, considering and describing all the different enemy kinds present in the community.

Materials and methods

Study site and system

The study was carried out at Estação Ecológica do Panga (19° 10'S, 48°24'O) in Uberlândia municipality, Minas Gerais state, in an ecotone area between a 'wet grassland' and a 'cerrado sensu strictu' phytophysiognomies (Cardoso et al. 2009). Climate in the region is seasonal, with rainy (October – March) and dry seasons (April – September) (AW in the Köppen-Geiger scale) (Kottek et al. 2006). *Palaeomystella oligophaga* Becker and Adamski 2008 (Lepdoptera: Momphidae) is a microlepidoptera, remaining during larvae and pupae stage inside globoid-shaped (sensu Isaias et al. 2014) galls induced on axillary stem buds of *Macairea radula* Bonpl. (Melastomataceae) shrubs (Becker and Adamski 2008).

Procedures

Collections were carried at the end of rainy season (March), when all insects (including enemies) are found on pupae stage, enabling us to infer survival rates of the galling at the end of its life cycle. Each gall was removed from the same population, but from different plants, totaling 321 samples. We considered as galls defensive characteristics to be tested: the height of each gall in *M. radula* shrubs, gall volume, thickness of parenchyma around the larval chamber, projections length and color variation. The height of each gall in

the host plant was measured before removal. In the laboratory, galls were opened for the observation and collection of the occupant using a Stereomicroscope (Leica® 500). Insects were incubated in plastic microtubules at room temperature, until adult emergence and identification at the lower taxonomic level. Gall height and width were measured and used to calculate the volume based on an oblate spheroid. The thickness of parenchyma was calculated using the size of the gall (considering the point of insertion on the plant until the opposite surface), discounting the larval chamber height and dividing by 2. Projections length was measured obtaining the average of three projections present on gall surface. All measurements were performed using a digital caliper (Digimes® ZAAS-1.0004, 0.01 mm readability).

We used the concentration of anthocyanins as a proxy of color pattern, considering that these are the main pigments responsible for red coloration in plant organs and provide a conspicuous coloration when accumulated in galls (Connor et al. 2012). For this purpose, we removed the gall projections, mounted them in a single layer and exposed together to a handheld JAZ Spectrometer (Ocean Optics®). Afterwards, we calculated the Anthocyanin Reflectance Index (ARI) through the inverse reflectance at 550 nm (anthocyanin absorption peak) (sensu Gitelson et al. 2001). With this procedure, we obtained a scale of anthocyanin concentration. Values close to 0 and 1 refer respectively to greener and redder galls.

Statistical analysis

We applied a multinomial logistic model using the package *mlogit* (Croissant 2012) to investigate the factors influencing the presence of different natural enemies in *M. radula* galls, and therefore killing *P. oligophaga*. Thus, the presence of the galling was taken as reference level and the occurrence of each enemy considered as an alternative outcome. We

considered gall occurrence height in plant, color, projections average and parenchyma thickness as predictor variables. Gall volume was not used because it had a high correlation of 0.89 with parenchyma thickness. We considered multicollinearity to be no problem among the remaining explanatory variables (below 0.44 in all cases). The significance of the model was assessed through the likelihood ratio (LR) test. Analyses were carried out in R statistical environment version 3.4.3 (R Core Team 2017).

Results

We found a diversity of insects that interact directly or indirectly with *P. oligophaga* (Fig. 1a). Most insect enemies used not only the food resource, but also the shelter, forming pupae within the galls and emerging as adults. Two species of these feed on plant tissues, and indirectly causes galling death. One of them, the Diptera sp. (Fig. 1b) occurs only on the larval chamber, where it feeds on the inner cells. For further analysis, we classify it as “cecidophage”. The other is a Lepidoptera sp. (Fig. 1c), which forms tunnels while feeding on the parenchyma during larvae stage and remains inside the larval chamber during pupae stage. We classify this behavior of feeding on gall walls as “herbivory”.

Gallings were directly attacked by two hymenopteran parasitoids. One of them, a Pimplinae sp. (Ichneumonidae), we classified as “large parasitoid” (Fig. 1d), showing a bigger body size (Mean \pm SD: 9,4mm \pm 2,5mm, n = 10) and ovipositor (7.9mm \pm 2mm, n = 10). On the other hand, the Doryctinae sp. (Braconidae) had smaller body size (1.7mm \pm 0.9mm, n = 10) and ovipositor (2.3mm \pm 1.3mm, n = 10), and we called it as “small parasitoid” (Fig. 1e). Finally, in some galls *P. oligophaga* larvae were found dried, together

with single scars traversing the whole parenchyma until the larvae position. These signs were considered as an action of a “predation” (Fig. 1 f), probably caused by a stylet of an insect.

The proportions of enemy's occurrence varied among the groups of organisms. We found only 68 galls out of 321 containing alive gallings (21.2 %) (Fig 2). Cecidophages were two times more abundant than gallings (N = 131; 40.8 %) (Fig. 2). Large parasitoids occurred in a greater number (N = 61; 19 %) than small parasitoids (N = 23; 7.2 %) (Fig. 2). Herbivory occurred in 21 galls (6.5 %) and predation in 17 (5.3 %) (Fig. 2).

We found a significant relationship regarding *P. oligophaga* natural enemies and the different predictor variables ($\chi^2 = 50.03$; $p = 0.0002$). The probability of incidence of small parasitoids (Odds Ratio: 1.014), herbivory (OR: 1.012) and predation (OR: 1.011) increases with gall height in the plant (table 1, Fig. 3a). Galls with the galling presence (height mean \pm SD: 86.19 ± 56.26) were positioned respectively 42.6, 29.3 and 28.7% lower than with small parasitoids (122.91 ± 54.18), herbivory (111.48 ± 58.59) and predation (110.94 ± 47.59), on average. On the other hand, the probability of occurrence of large parasitoids (OR: 0.772), herbivory (OR: 0.489) and predation (OR: 0.654) decreases as the average parenchyma thickness increases (table 1, Fig 3b). Galls with alive gallings (parenchyma thickness mean \pm SD: 6.72 ± 2.07) showed 13.8, 29.5 and 22.6% thicker parenchyma than those with large parasitoids (5.79 ± 1.88), herbivory (4.74 ± 1.52) and predation (5.20 ± 2.0). There were not effects of color or projections average (table 1).

Discussion

Despite the structural complexity of galls induced by *Palaeomystella oligophaga* on *Macairea radula*, these insects were attacked by a diversity of organisms, totalizing 78,8% of enemy occurrence and therefore galling mortality. The survival rates of the galling moth (21,2%) were smaller than the amount of cecidophages (40,8%), and similar to larger parasitoids occurrence (19%). Although the protective barrier offered by gall can works at some level, is possible suggest that the morphological characteristics analyzed are not very effective if considered the minor galling survival. However, the larger parasitoids attack is apparently diffculted by parenchyma thickness, which was also negative related with occurrence of herbivores and predation. On the other hand, the occurrence of herbivores and predation as well as small parasitoids were positive related with the heights of gall on plants. Gall color and size of projections did not relate with the presence of any galling enemy. Therefore, for this multitrophic system, only the thickness of parenchyma can be justified by enemy hypotheses. Nevertheless, it is important to note that this result may represent an event of transient enemy-free space (Agrawal 2001; Stone and Schonrogge 2003; Singer and Stireman 2005; Abrahamson and Blair 2008; Bailey 2009).

Cecidophages seems to be better strategists, considering that they did not related with any of the studied characteristics and presented the highest incidence. Although these insects affected the galling indirectly by feeding on the gall cells, showed a greater threat than those that nourished directly from them (parasitoids and predators). The danger that the cecidophages represent to *P. oligophaga* populations reinforce the importance of consider all kinds of interacting organisms to tests galls related hypothesis. For instance, Sanver and Hawkins (2000) also showed that cecidophages as inquilines can cause galling's populations damages more representatives than by parasitoids and predators. If we add the herbivores

rates to cecidophages, the total indirect attack caused 47,3% of mortality, against 31,5% of those that consumed the galling directly. Thus, the gall neoformed tissues can promote *P. oligophaga* instead of protecting it in some cases. These results contradict the enemy hypothesis and showing that although the apparent defense offers by gall structure, gallings can support rich communities of enemies and suffer high mortality (e.g. Waring and Price 1989; Hawkins et al 1990; László and Tothmérész 2013).

Even with color variation of *M. radula* galls, we did not find any preferences by enemies for green or red ones. Possibly, the interacting insects do not recognize the conspicuity as those chewing herbivores considered for aposematic hypothesis as suggested by Inbar et al. (2010). *P. oligophaga* – *M. radula* galls arise in first stages of development with red colors, and do not change until and over senescence, which discard the influence of gall maturation stage (e.g. White 2010; Dias et al. 2013). The sunlight exposition influence (Wool 2004) is also unlikely because galls occur at the extremities of host plants, which indicates that they probably are subject to similar luminous regime. Herein, we do not support gall color trait as having an aposematic function, so the most probably is that this trait is a consequence of host plant identity influencing the process of gall formation. The length of projections associated to trichomes also did not relate with the presence of any studied enemies. Still, we do not discard the hypothesis that their presence selects the attack by generalist herbivores or parasitoids. Hairy cover may develop an important role in defense against parasitoids as discussed and corroborate in front of community with few species associated to *Diplolepis* sp. - *Rosa* sp. shrubs (Askew et al 2006). However, on these systems the parasitism was sensitive to gall size and thickness (László and Tothmérész 2013).

In the present system, only parenchyma thickness was considered a negative selector factor, protecting the galling against large parasitoids, herbivores and predation. Some parasitoids show no preference for any gall size (Rossi et al. 1992), but as demonstrated by Weis (1985), the chances of success on oviposition are lower for those that attack large galls. Controversially, in some systems, parasitoids have preferred the largest galls (van Hezewijk and Roland 2003; Figueiredo et al. 2014). The studied large parasitoids occurred less on galls with parenchyma thickness above the average length of ovipositors, so the negative relation seems to be a result of the preference for smaller sizes of galls, which may benefit the selection of larger galls against the attack of these organisms. The negative relation between herbivores and parenchyma thickness seems controversial, whereas they potentially make available more food and shelter. Probably, larger galls also have a higher superficial toughness, hindering the entry of enemies or occurs a greater accumulation of substances that avoid the consumption by these organisms (Sanver and Hawkins 2000). Concerning predators, we can suggest that they need to have an oral apparatus long enough to reach the larva in the gall center, and this can drive a selection for more thickness or predators that are more effective.

Finally, gall location influenced the chance of encountering by small parasitoids, herbivores and predator. However, it has no influence on cecidophages or large parasitoids, probably because these organisms can use clues provided by plant metabolism to find they targets insects (Hawkins 1988; Unsicker et al. 2009; Schaefer and Ruxton 2011). Data collection regarding the number of potential sites per height vs. the number of sites induced may answer if gallings prefer any stratum. However, it appears that *P. oligophaga* induce galls on any axillary stem buds available, which may occur at several heights. In this sense,

an induction made at a random height may determine the probability of exposure to risk against small parasitoids, herbivores and predators. Since these enemies accounted together for 19 % of galling deaths, galls positioned below on vegetation may present an advantage to gallings. These patterns found here may be related to natural history traits of enemies, with have higher flight heights or avoid lower strata in the vegetation. Using the *P. oligophaga* – *M. radula* system as model of study, we demonstrate how different traits can or not be effective against directly or indirectly galling multi enemies at the same time. Despite color and trichome are considered as galling protective in the literature, we did not find their effectiveness preventing attack of any found organisms. Nevertheless, this cannot reject that these traits occurred as a character molded by other enemy's selective pressure along evolution. *Palaeomystella oligophaga* have many enemies that explore them and gall structures as resources, causing massive top-down regulation, with different proportions for each enemy. In this sense, whatever trait effective against whichever enemy may have positive population effects, especially over the generations.

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Table and Figures captions

Table 1. Results of multinomial logistic regression demonstrating the relationships between the predictor variables and the occurrence of the different natural enemies taking

the presence of the galling *Paleomystella oligophaga* as reference level. Significant results at the level of 0.05 are expressed in bold.

Figure 1. Associated enemies and the gradient of colors showed by *Palaeomystella oligophaga* - *Macairea radula* galls. The gallings (a) are indirectly affected by cecidophages (b) and herbivores (c) that feed on gall tissues, which cause *P. oligophaga* death. Also, these insects are directly attacked by two species of parasitoids called here as ‘large parasitoids’ (d) and ‘small parasitoids’ (e), as also by an unidentified predator which the damage is recognized accordingly with the scar on gall parenchyma and the dried galling larva (f, arrow).

Figure 2. Occurrence proportions of the different groups of organisms and processes (predation) found in galls of *Macairea radula* induced by *Paleomystella oligophaga* moth.

Figure 3. Probability of occurrence of the different natural enemies according to plant height (a) and parenchyma thickness average (b) on *Palaeomystella oligophaga* galls. Circles indicate data distribution, in which galls with *P. oligophaga* galling are indicated by 0 (black circles) and those with the natural enemies by 1 (color circles). Lines represent predicted probability.

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Table 1

Predictor	Natural enemy	Error	t	p
Color	Cecidophage	0.610	0.549	0.583
	Large parasitoid	0.719	-0.249	0.803
	Small parasitoid	0.908	0.249	0.803
	Herbivory	1.082	-0.860	0.390
	Predation	1.062	-0.461	0.645
Height	Cecidophage	0.003	1.439	0.150
	Large parasitoid	0.004	-0.171	0.864
	Small parasitoid	0.005	2.861	0.004
	Herbivory	0.005	2.438	0.015
	Predation	0.005	2.000	0.046
Parenchyma	Cecidophage	0.090	-1.455	0.146
	Large parasitoid	0.110	-2.351	0.019
	Small parasitoid	0.152	-1.115	0.265
	Herbivory	0.172	-4.146	<0.0001
	Predation	0.178	-2.378	0.017
Projections	Cecidophage	0.065	-0.445	0.657
	Large parasitoid	0.078	-0.358	0.721
	Small parasitoid	0.130	-1.641	0.101
	Herbivory	0.111	0.594	0.553
	Predation	0.143	-0.962	0.336

Figure 1

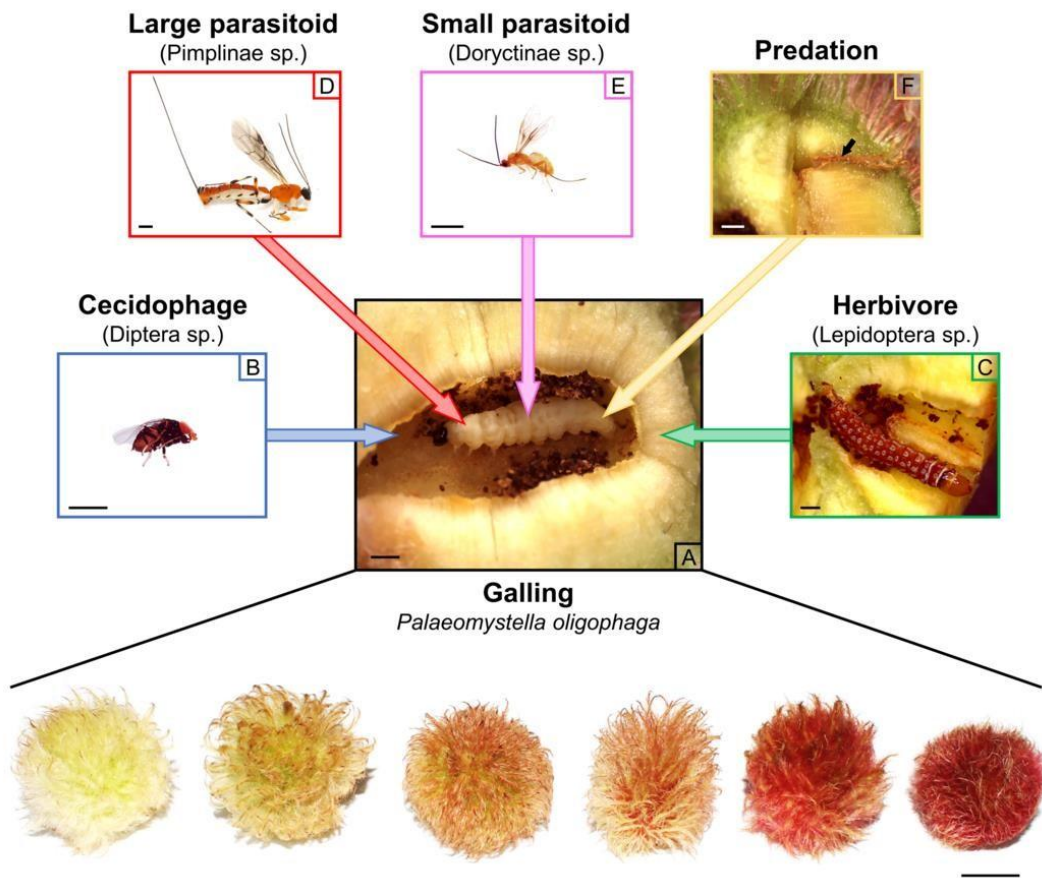


Figure 2

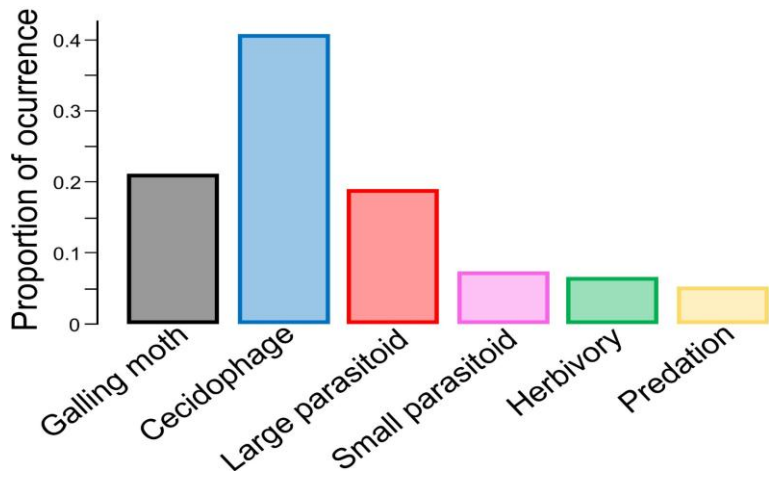
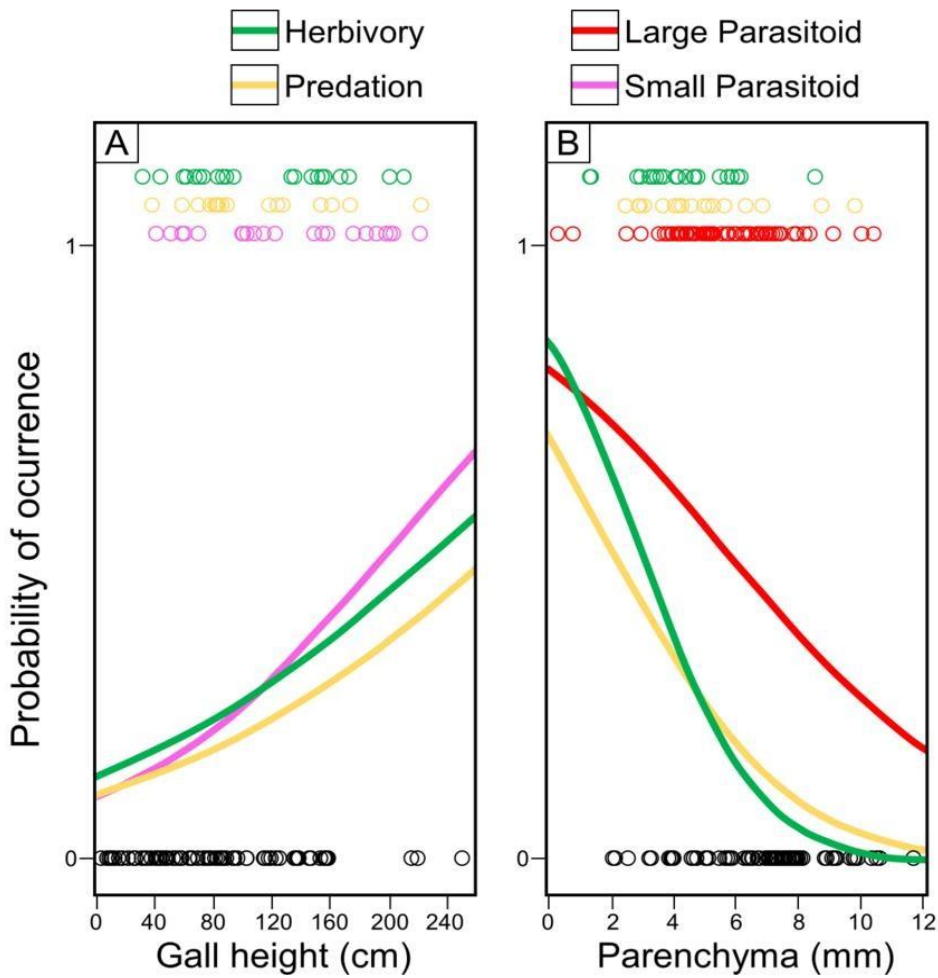


Figure 3



**How the activity of natural enemies changes the structure and metabolism of the nutritive tissue in galls? Evidences from the *Macairea radula* (Metastomataceae)-
Palaeomystella oligophaga (Lepidoptera) system**

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Abstract

Lepidopteran-induced galls develop nutritional cells, which they induce and consume directly, and any metabolic modification of those cells may reflect changes of the insect's own metabolism. The system *Palaeomystella oligophaga* (Lepidoptera) - *Macairea radula* (Melastomataceae) presents a series of natural enemies, including parasitoids and cecidophages which can function as a natural experiment, respectively removing the specific galling feeding stimulus and providing a nonspecific one. Considering that the process of induction and maintenance of gall tissues strictly depend on the constant specific stimulus of galling, we question: I - what kind of metabolic changes these different groups of natural enemies can promote in chemical and structural composition of this galls? II - How much the specialized tissues are dependent on the constant specific stimuli of galling in gall latter

stages of development? Galls without natural enemies, with parasitoids or cecidophages in larvae or pupae stages were analysed through histochemistry and cytological profiles compared to its natural senescence. Both parasitoids and cecidophages stopped the feeding activities of galling insect, which results in the paralysis of stimulus that maintain the metabolism of the gall tissues. The development and maintenance of a true nutritive tissue in these galls is totally dependent of the constant larval stimulus. Histochemical and cytological analysis demonstrate it by the accumulation of proteins and lipids in the typical nutritive tissue and starch on the storage tissue, as well the high integrity of cellular organelles and membrane systems.

Keywords: enemy hypothesis, plant-insect interaction, gall cytology, histochemistry, gall structure and metabolism

Introduction

As new plant organs (Shorthouse et al. 2005), galls show complex structural and metabolic profiles induced by a galling organism (Mani 1964; Hartley 1998; Schönrogge et al. 2000; Oliveira et al. 2014; Ferreira et al. 2016; Oliveira et al. 2017). These organisms, especially insects, build the gall through re-differentiation of the host cell, in response to its specific stimuli, developing a structure with new features and functions (Raman 2007; Stone and Schönrogge 2003; Oliveira et al. 2016; Giron et al. 2016; Carneiro et al. 2017). However, some galling insects such as cynipids, cecidomyiids and lepidopterans induce the development of nutritive cells, wherein they feed (Bronner 1992; Ferreira et al. 2017). For instance, lepidopteran-induced galls have nutritional cells filled with lipids, which larvae

consume directly through their scraper feeding habit (Bedetti et al. 2013; Ferreira and Isaias 2013; Vecchi et al. 2013). In this sense, the structure and nutritional metabolism of nutritive cells in galls may represent the diet of the galling (e.g. Bronner 1992; Rohfritsch 1992; Ferreira and Isaias 2013; Oliveira et al. 2016; Ferreira et al 2017), and any metabolic modification of those cells may reflect changes of the insect's own metabolism.

Galls are targeted by the attack of a diversity of natural enemies that establish different interaction types with the galling insect (e.g. Zamora and Gómez 1993; Van Hezewijk and Roland 2003; Sugiura et al. 2006, Sugiura and Yamazaki 2009; Katilmis and Azmaz 2015, Mete and Mergen 2017). For instance, parasitoids are among the most frequent enemies of galling insects, invading the gall structure, parasitizing the host larvae until the self-formation of the pupae, and remaining protected by the vegetal tissues until they outbreak (Wiebes-Rijks et al. 1992; Hanson and Nishida 2014). Although some parasitoids of free-living arthropods can perform behavioral manipulation in victims to increase their survival chances (e.g. Barrantes et al. 2017; Korenko et al. 2018), in galling insects they generally paralyze hosts when consume begins (Hanson and Nishida 2014). This phenomenon may be related to the protective structure already provided by the gall. That possibility leads us to ask: what happens during advanced gall stages in the absence of galling stimulus after paralyzation promoted by parasitoid? It is expected that galling feeding is important to maintain gall metabolism not only in the beginning, but also in latter stages of tissue development.

In addition to attack by parasitoids, cecidophages are other common enemies of gallings, with feed actively on gall tissues and not necessarily directly kill the gall maker (Sanver and Hawkins 2000; Sugiura and Yamazaki 2009). Therefore, they take advantage of

the galling ability to accumulate nutrients, benefiting by feeding directly from the nutritional tissue (Sugiura et al. 2006; Sugiura and Yamazaki 2009). Once the feeding stimulus continues inside the gall, another interesting question is whether these organisms can maintain gall metabolism, or if the nonspecific behaviour/substances would still lead the gall to collapse.

Since the gall is an enclosed structure, it is impossible to directly observe the galling behavior and/or enemy-induced behavior alterations. To cope with this, any long-term modification in the gall can be analyzed through the vegetal structure and its respective metabolism. In this sense, the histochemical and cytological profiles of the gall tissues during the attack of natural enemies of the galling larvae can be used as a proxy to respond which kind of metabolic manipulation these insects undergo. Galling mechanical removal can generate damages, influencing results and conclusions. Therefore, parasitoids and cecidophages function as a natural experiment, respectively removing the specific galling feeding stimulus and providing a nonspecific one. To test galling/gall metabolic changes under these two circumstances, the same system with both these natural enemies is required.

The system *Palaeomystella oligophaga* Becker & Adamski (Lepidoptera: Momphidae) - *Macairea radula* Bonpl. (Melastomataceae) presents a series of natural enemies, including parasitoids (Ichneumonidae: Pimplinae) and cecidophages (Diptera) as the most common. Considering that the process of induction and maintenance of gall tissues strictly depend on the constant specific stimulus of galling, we addressed the following questions: I - what kind of metabolic changes these different groups of natural enemies can promote in chemical and structural composition of these galls? and II - How much the

specialized tissues are dependent on the constant specific stimuli of galling in gall latter stages of development?

Material and methods

Study system

Macairea radula Bonpl. (Melastomataceae) occurs in an Cerrado ecotone, margin a 'wet grassland' at Estação Ecológica do Panga (19° 10'S, 48°24'O) in Uberlândia municipality, Minas Gerais state, Brazil. This plant species hosts globoid-shaped galls (sensu Isaias et al. 2014) (Fig. 1a) induced by *Palaeomystella oligophaga* Becker & Adamski, 2008 (Lepdoptera: Momphidae) (Fig. 1b) on the axillary stem bud. Gallings present a bivoltine life cycle, at rainy (March – April) and in dry seasons (August – September) (Rezende et al., unpublished data).

The galling insects are attacked by a diversity of natural enemies, including parasitoids (Fig. 1c) and cecidophages (Fig. 1d). Cecidophages (Diptera) represent the greatest threat to gallings, occurring in 40,8 % of galls (in 321 sampled) (Rezende et al. in prep). These enemies occur grouped within the gall in an average number of 3.7 (\pm 2.5 SD) larvae. They feed on small parts of the gall nutrition tissue until the pupae stage, which remains inside the gall until hatching (Rezende et al. in prep.). In the present study, we considered the *Pimplinae* sp. (Hymenoptera: Ichneumonidae) as the second most representative enemy, occurring in 19 %. They occur isolated, moored on paralysed galling larvae wherein they feed. When they finish the feeding behaviour, they turn into pupae stage inside the gall until outbreak (Rezende et al. *in prep*).

The samples were collected during rainy station (N = 200) and separated in one control group: I – with the galling *P. oligophaga* presence (in larval or pupal stage), and two treatments: II - with larvae or pupae of cecidophages; III- with larvae (feeding on the galling) or pupae of parasitoids. We contrasted gall tissues of these later groups with those of the natural senescence (galling pupae), expecting that they are different metabolic processes. All insects and internal parts of the gall were visualized in a Stereomicroscope (Leica® 500) and photographed with a U-photo system (Leica® ICC50HD) coupled.

Histological analysis

For anatomical description, galls without natural enemies were fixed in FAA (formalin, acetic acid, 50 % ethanol, 1:1:18 v/v/v) (Johansen 1940), dehydrated in an ethanol graded series (Kraus and Arduin, 1997), and embedded in 2-hydroxyethylmethacrylate (Historesin, Leica® Instruments). The sections were obtained with a rotatory microtome (Ancap®, YD-315) at 5 µm of thickness, stained with 0.05% toluidine blue - pH 4.7 (O'Brien et al. 1964), and mounted in Entelan®. The samples were observed under a light microscope (Leica® DM500) and photographed with a U-photo system (Leica® ICC50HD) coupled.

Histochemical analysis

Fragments containing all tissue gradient of the galls with gallings only were direct embedded with polyethylene glycol (PEG 6000) following Ferreira et al. (2014) and used for histochemical tests. The embedded was performed in solution of PEG 6000 and distilled water (1: 1) in a stove at 50 ° C until total water evaporation. Blocks with pure PEG 6000 were assembled and sectioned in rotary microtome (20 µm). The PEG 6000 were removed with water and glued with Haupt glycerinate gelatin (Kraus and Arduin, 1997) on the slides.

The samples were submitted to histochemical tests for the detection of total lipids with Sudan Red B (Brundett et al. 1991), total proteins with Bromophenol blue (Baker 1958) and for starch with Lugol reagent (Johansen 1940). All samples were visualized and photographed in a Microscope (Leica® DM500) coupled with a U-photo system (Leica® ICC50HD).

Cytological analysis

Samples of the inner cortex (around the larval chamber) and of the outer cortex were fixed in Karnovsky's solution (paraformaldehyde 4% and glutaraldehyde 2,5% in phosphate buffer - 0.1 M, pH 7.2) (Karnovsky 1965), dehydrated in acetone series and embedded in Epon resin EMBED-812 (Electron Microscopy Sciences, Industry Road, Hatfield, PA, United States) (Cotta-Pereira et al. 1976). The ultra-thin transverse sections obtained were stained with 2% uranyl acetate solution during 20 min, washed in double distilled water, immersed in 2% lead citrate solution during 6 min, and washed in double distilled water again. The samples were observed in transmission electron microscope (TEM) JEM 2100 (JEOL USA, Inc., Peabody, MA, United States), equipped with an Energy Dispersive X-ray Spectrometer (EDS) (Thermo Fisher Scientific Inc., Waltham, MA, United States).

Results

The gall induced by *P. oligophaga* on the axillar stem bud of *M. radula* are globoidshaped (Fig. 1a) (Isaias et al 2014) with a single larval chamber (Fig. 1b), and is covered by projections (Fig. 1a) that presents trichomes and emerge from parenchyma cells (Fig. 2a, b). These galls show three histological regions: (i) an outer cortex, with different shapes and sizes of hypertrophied cells, thin cell walls (Fig. 2a - c) and with intercellular

spaces (Fig. 2c). In the outer cortex, vascular bundles are randomly distributed (Fig. 2a, b). (ii) The transitory layer cells occur between the outer and inner cortex (Fig. 2a). These cells are smaller than those of outer cortex are and show more thickness cell walls. (iii) The inner cortex, surrounding the single larval chamber (Fig. 2a, d), do not show any evident intercellular spaces and are laterally compacted, which causes them a rectangular shape (Fig. 2 d - f). The cells adjacent the larval chamber have conspicuous protoplasm (Fig. 2f).

Histochemical profiles

Control (*P. oligophaga*) galls. There is a clear distinction between typical nutritive tissue (inner cortex) and storage tissue (outer cortex) (Table 1; Fig. 3a). Starch are detected in outer cortex of galls with larval and pupa stage of *P. oligophaga* (Fig. 3b; Table 1), and it is a storage tissue. The inner cortex presents the typical nutritive tissue with lipids and proteins being detected during galling larval stage (Fig. 3c, d; Table 1). However, these substances are not found in galls where the galling is in pupa stage (Table 1), or found in spots only, being relocated, clearly in a different disposition when compared to metabolically stimulated galls. The typical nutritive tissue is partially consumed by galling insect. No metabolic compounds are found in the common storage tissue during pupa stage (Table 1).

Parasitoids treatment. After observing immediately opened galls with parasitoid presence, we confirm that they paralyze the galling's body, feeding on its fluids (Fig. 1c). Once those parasitoids stop galling activity, there is a collapse of the gall function, with the nutrition substances being relocated and the tissue degraded. The typical nutritive tissue still maintains spots of lipids and proteins, but in a more dispersed form than in galls with only galling insect (Table 1). In addition, the proteins are relocated, appearing mainly within the vascular bundles in the cortex (Table 1). Cecidophages treatment. Galls occupied by

cecidophages (Fig. 1d) show negative results in all the histochemical tests (Table 1). They are not able to produce or maintain the substances tested in gall tissues, and apparently accelerate tissues senescence.

Cytological profiles

Control (*P. oligophaga*) galls. The cytological analyses of the storage tissue in galls occupied only by *P. oligophaga* in the larva stage revealed high number of mitochondria and some lipid droplets (Fig. 4a). This occurred especially in the cells of common storage tissue (outer cortex) on the limit transitory layers and with nutritive cells (inner cortex). Plastids with starch grains are also observed (Fig. 4b). In the typical nutritive tissue there are many lipid droplets in the cells. These are associated with endoplasmic reticulum, Golgi apparatus, many mitochondria, large nucleus and several fragmented vacuoles (Fig. 4c, d, e). The nucleus has lobed shape with conspicuous and condensed nucleolus (Fig. 4d). The chloroplast has well-structured thylakoids with plastoglobulus associated (Fig. 4e). In the storage tissue of galls, when the pupa stage is present, the nucleus is pyknotic starting to irreversible condensation of chromatin and the cells undergoing necrosis or apoptosis (Fig. 4f). Associated with pyknotic nuclei, autophagic bodies are found in the cell protoplast (Fig. 4f). Lipid droplets and many organelles are packed over the typical nutritive tissue, as well cytoplasm disorganization and membrane system ruptured (Fig. 4g). Parasitoids and cecidophages treatment. Galls with parasitoids (Fig. 5a, b) and cecidophages (Fig. 5c, d) showed complete degradation of nucleus as well as organelles and membrane systems (Fig. 5a, d). In galls with cecidophage larva, the typical nutritive tissue showed some lipid droplets and plastids with small thylakoids and reduced lamellation (Fig. 5c). However, autophagic

bodies and packing process are abundant, rupturing all organelles and membrane systems (Fig. 5d).

Discussion

When the larval stimulus of the galling *P. oligophaga* stopped and they come to pupal stage, the histochemical and cytological profile of these galls beginnings to rupture configuring initial senescence of gall tissues. In a natural experiment of stimulus nonspecificity/suspension, both cecidophages and parasitoids stopped the feeding activities of galling insect interrupting the stimulus that maintain the metabolism of the gall tissues. However, the degradation processes appear to occur more accelerate on galls with cecidophages than with parasitoids. The development and maintenance of a true nutritive tissue in galls induced by *P. oligophaga* on *M. radula* is totally dependent on the constant larval stimulus. Both histochemical and cytological analysis demonstrate it by the accumulation of proteins and lipids in the typical nutritive tissue and starch on the storage tissue, as well the high integrity of cellular organelles and membrane systems.

Histochemical profile: galling x natural enemies' stimulus

Larval stages of *P. oligophaga* stimulate the anatomical and chemical responses in the host plant tissues, related with its constant feeding behavior (Shorthouse and Rohfritsch 1992; Stone and Schonrogge 2003; Oliveira et al. 2016). Then, the development of a typical nutritive tissue, around the larval chamber is dependent on the galling taxa and feeding stimulus, as for chewing and scraping insects (Bronner et al 1992). In lepidopteran gallsystems, this pattern of nutritive tissue was found (Bedetti et al. 2013; Ferreira 2013;

Vecchi et al. 2013). Although some studies showed contradictory results, pointing out the absence of a typical nutritive tissue in galls induced by lepidopterans (Meyer and Maresquelle 1983; Shorthouse and Rohfritsch 1992). Herein we found a true nutritive tissue in the *P. oligophaga* - *M. radula* system and when the galling insect come to pupal stage, lipids and proteins degenerate and the typical nutritive tissue is ruptured. This is a clear indicative that only the larva of *P. oligophaga* can trigger and stimulate the nutritive cells formation.

We found that true nutritive tissue accumulates lipids and proteins. Similarly, galls induced by Lepidoptera on leaves of *Tibouchina pulchra* (Melastomataceae), *Marcetia taxiflora* (Melastomataceae) and *Bauhinia unguolata* (Fabaceae) induce the development of cells that storage especially lipids and proteins, in which galling larvae feed on (Bedetti et al 2013; Ferreira et al. 2013; Vecchi et al 2013). Lipids are a common energetic storage substance of plants (e.g. Buchanan 2000) that are associated to nutritive cells and diet of some galling cynipids (Bronner 1992) and lepidopterans. In addition to improve the insect's diet, proteins accumulation around the larval chamber are associated to the respiratory stress and high plant cell metabolism (Schönrogge et al. 2000; Oliveira et al. 2010; Oliveira and Isaias 2010). Herein, lipids are directly associated with larval feeding activity and proteins can also improve galling's diet as well as be a response from respiratory stress.

Plant galls are an excellent microlaboratory of biological assays. Then, our proposed natural experiment of specific stimulus suspension shows the key role of galling larvae in the determination of histochemical profile in galls. This conclusion comes from the degradation of lipids and proteins when the galling insects are parasitized with the parasitoids in the larval stage, and even more noticeable when these enemies were in pupal stage. Such results conflict with those found in the Lepidoptera - *Bauhinia unguolata* system, in which parasitized

galls were similar to non-parasitized, with nutritional tissue not consumed and intact in some regions of the inner gall (Bedetti, et al. 2013).

The activity of cecidophages in *M. radula* galls indirectly killed the galling insect and provoked complete histochemical rupture, and apparently, the process of tissue degradation is more pronounced and accelerated in these galls. This damage probably occurs due to the additional stress provoked by feeding activity and movement behavior of cecidophage larvae. It is possible that these organisms will benefit from the tissues senescence, feeding on the outflow of nutrients (White 1993, 2012; Steinbauer et al. 2014).

Cytological profile: galling x natural enemies' stimulus

The cytological features we found on cells of typical nutritive tissue of mature galls (cytoplasm-rich, noticeable nucleus and nucleoli and develop membrane systems) are also commonly found other different gall-systems (e.g. Bronner 1992; Oliveira et al. 2011; Vecchi et al. 2013; Ferreira et al. 2015; Oliveira et al. 2016; Ferreira et al. 2017). Other characteristics such as the large amounts of rough endoplasmic reticulum, smooth endoplasmic reticulum, free ribosomes and lipids droplets are related with the production of lipids and proteins on the typical nutritive cells. When the larva of the galling comes to pupal stage beginnings the processes of cell start to collapse: the nucleus becomes pyknotic and the membrane system is packaging. Pyknotic nucleus is a clear symptom of tissue necrosis (e.g. Lingua et al. 1999; Oliveira and Isaias 2010; Barraco et al. 2014), which indicates the end of galling feeding activity.

In the senescence processes of leaves, thylakoid membrane system, followed by packaged chloroplast are the first to be degraded (Kolodziejek et al. 2003). The same pattern

occurs in galls induced by *Pseudophacopteron aspidospermi* (Hemiptera: Phacopteronidae) and other unidentified Cecidomyiidae species on *Aspidosperma australe* (Apocynaceae) and *Copaifera langsdorffii* (Fabaceae) (Oliveira and Isaias 2010; Oliveira et al. 2011). In *M. radula* galls, symptoms are also detected when the galling insect is in the pupal stage. During plant senescence, the function of mitochondria is maintained, with a gradual breakdown (Gan et al. 2007). This process is essential to supply the cell energy demand during the senescence (Thomas and Stoddart 1980). We found that mitochondria can be observed even when the membrane system is packaging, and the nucleus becomes pyknotic.

Conclusions

The different kinds of natural enemies promote tissues degradation. However, it seems that the abruptly suspension of galling activities by the parasitoid is more similar to the effects of usual senescence of galls. Meanwhile, the directly nonspecific feeding activity of cecidophages on vegetal tissues was not capable of maintaining gall metabolism, which presented pronounced process of tissue degradation instead. One of the greatest curiosities involving gall formation is the hypothesis that plant tissues respond only to the specific galling stimulus, probably by the hormones released when they feed on vegetal tissues (Bartlett and Connor 2014; Zhang et al. 2017). Here we have shown that mature tissues maintenance is also still dependent on gallings feeding activity. Although another stimulus can initiate gall multiplication and hypertrophy, like oviposition fluid left by the female (e.g. Barnewall and Clerck-Floate 2012), only the galling larvae can give support from galls metabolism at maturity.

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Table and Figures captions

Table 1. Histochemical results of galls occupied by gallings (control - *P. oligophaga*), cecidophages and parasitoids treatment on larvae and pupae maturation stages. The positive (+) and negative (-) symbols indicate the presence or absence of starch, lipid or proteins on each tested tissue divided here as inner and outer cortex.

Figure 1. Galls of *Palaeomystella oligophaga* - *Macairea radula* system and natural enemies associated. a Globoid-shaped galls induced on the axillary stem buds, covered by projections. Gallling larvae occurs on a single larval chamber (b). They are attacked and paralyzed by parasitoid larvae, which feed of gallings body fluids (c). Cecidophages larvae feed of the inner cortex and remains in the larval chamber (d). LC - Larval chamber, LG - galling larva, LP - parasitoid larva, LCe - Cecidophage larva.

Figure 2. Cross-sections of a gall fragment, a showing all tissue divided in an outer cortex with vascular bundles distributed, transitory layer cells and an inner cortex limited externally by parenchyma emergences (projections) and internally by the larval chamber. Gall surface projections covered by trichomes (b). Outer cortex with different shapes and sizes of hypertrophied cells, thin cell walls and intercellular spaces (c). Inner cortex, surround

the single larval chamber (d), with any evident intercellular spaces and laterally compacted, which causes them a rectangular shape evidenced in (e, f). Cells adjacent the larval chamber with conspicuous protoplasm (f).

Figure 3. Gall macro-structure, a evidencing the single and central larval chamber (LC), recovered by nutritive tissue - NT followed by storage tissue - ST. The tests show protein accumulation (b) and lipids (c) defining the nutritive tissue on inner cortex. Starch grains found on the entire outer cortex determines this region as storage tissue (d).

Figure 4. Cells of galls containing only *Palaeomystella oligophaga* in transmission electron microscopy. a-e- larva stage; f-g- pupa stage; a, b, f- storage tissue; c, d, e, g- nutritive tissue; a- Cells with high numbers of mitochondrias; b- Cytoplasm in the parietal position with mitochondrias and plastids containing starch; c- lipidic droplets, mitochondrias, large nucleus, endoplasmic reticulum and fragmented vacuoles; d- Detail of large and lobed nucleus, as well fragmented vacuoles. e- Detail of lipidic droplets, endoplasmic reticulum and chloroplast with plastoglobules; f- Large nucleus with condensed nucleolus. Detail of the vesicles present around the nucleus. g- Packaging of organelles. Mi- mitochondria; St- starch; CW- cell wall; Va- vacuole; Nu- nucleus; ER- endoplasmic reticulum; LP- Lipidic droplets; Nuc- nucleolus; Cl- chloroplast; GA- Golgi apparatus; Pl- plastoglobules; Ve- vesicle; Pla- plastids; Pa- packaging.

Figure 5. Cells of nutritive tissue in galls induced by *Palaeomystella oligophaga*, with natural enemies, on axillary stem buds of *Macairea radula* in transmission electron microscopy. a, b- with parasitoid; c, d- with cecidophagus; a- Degrading of cytoplasmic content. Detail of the vesicles. b- Packaging and degrading of organelles; c- Lipidic droplets

in plastids; d- Packaging and degrading of organelles. CW- cell wall; Ve- vesicle; Pa- packaging; Pla- plastids; LP- lipidic droplets; Mi- mitochondria.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5