



UNIVERSIDADE FEDERAL DE UBERLÂNDIA

INSTITUTO DE BIOLOGIA

Programa de Pós-Graduação em Ecologia Conservação e Biodiversidade

**Sexually dimorphic galls induced on leaflets of *Matayba guianensis*  
(Sapindaceae): a rare phenomenon occurring in Diptera (Cecidomyiidae)**

**Pedro Henrique Pereira Gonçalves**

Uberlândia

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

Área de concentração: Ecologia

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Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Denis Coelho de Oliveira apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

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Esta defesa faz parte dos requisitos necessários à obtenção do título de Mestre.

O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

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## Resumo

Galhas são formadas a partir de estímulos químicos e/ou mecânicos de diversos organismos, especialmente insetos que estabelecem relações extremamente específicas com suas plantas hospedeiras. Todavia, algumas espécies são capazes de induzir diferentes morfotipos de galhas na mesma planta hospedeira, com diferenças morfológicas, anatômicas, histoquímicas e fitoquímicas, podendo estas características estarem relacionada com o dimorfismo sexual do inseto galhador. Insetos galhadores são considerados verdadeiros manipuladores do fenótipo da planta hospedeira, alterando os tecidos hospedeiros e sua composição em benefício próprio. O objetivo deste trabalho foi observar e analisar as possíveis diferenças morfológicas, anatômicas e composicionais de dois morfotipos de galhas induzidas por *Lopesia mataybae* em folíolos de *Matayba guianensis*, por meio de análises anatômicas e histocitométricas. Os insetos galhadores adultos eclodiram de morfotipos diferentes, fêmeas eclodiram do morfotipo globoide e machos eclodiram do morfotipo cilíndrico. As galhas ocupadas por Fêmeas (altura média = 4.67 mm. S=0.43/largura média=4.59 mm. S= 0.70) se mostraram significativamente maiores que as galhas ocupadas por machos (altura média=2.95 mm. S=0.46/largura média=2.34 mm. S= 0.45). Ambos morfotipos, globoide e cilíndrico, são compostos por células alongadas no parênquima interno e células arredondadas no parênquima externo. Não houve diferença nos compostos histoquímicos detectados. Neste trabalho foi elucidado a importância morfológica e histológica entre galhas ocupadas por machos e fêmeas, possivelmente em consequência de diferentes requisitos nutricionais dos sexos de *Lopesia mataybae*. Portanto, a expressão do dimorfismo sexual em morfotipos de galhas pode estar associada à diferenciação das vias de desenvolvimento entre os sexos de *L. mataybae*, dentre elas, a estrutura e modificação dos compostos de parede celular e possíveis diferenças em quantidade de fitormônios, ao qual necessitam ser investigadas.

## Abstract

Galls are formed from chemical and/or mechanical stimuli from different organisms, especially insects that establish extremely specific relationships with their host plants. However, some species are capable of inducing different morphotypes of galls in the same host plant, with morphological, anatomical, histochemical and phytochemical differences, and these characteristics may be related to the sexual dimorphism of the galling insect. Galling insects are considered true manipulators of the host plant phenotype, altering the host tissues and their composition for their own benefit. The objective of this work was to investigate the possible morphological, anatomical and compositional differences of two morphotypes of galls induced by *Lopesia mataybae* on leaflets of *Matayba guianensis*, through anatomical and histocytometric analyses. Adult galling insects hatched from different morphotypes, females hatched from the globoid morphotype and males hatched from the cylindrical morphotype. Galls occupied by females (average height =4.67 mm. S=0.43/average width=4.59 mm. S= 0.70) were significantly larger than galls occupied by males (average height=2.95 mm. S=0.46/average width= 2.34 mm. S= 0.45). Both morphotypes, globoid and cylindrical, are composed of elongated cells in the inner parenchyma and rounded cells in the outer parenchyma. There was no difference in the histochemical compounds detected. In this work, the morphological and histological importance between galls occupied by males and females was elucidated, possibly as a result of different nutritional requirements of the sexes of *Lopesia mataybae*. Therefore, the expression of sexual dimorphism in gall morphotypes may be associated with the differentiation of developmental pathways between the sexes of *L. mataybae*, including the structure and modification of cell wall compounds and possible differences in the amount of phytohormones to which need to be investigated.

## **Introdução geral**

Galhas são órgãos vegetais neoformados desenvolvidos a partir de estímulos químicos e/ou mecânicos de diversos organismos externos a planta hospedeira, especialmente insetos. A interação desses insetos galhadores com uma planta hospedeira, culminando na formação da galha, é extremamente específica e considerada uma das estratégias mais eficientes de herbivoria (MANI, 1964; STONE & SCHÖNROGGE 2003; SHORTHOUSE *et al.* 2005). Estas alterações durante o desenvolvimento da galha passam, necessariamente, por rediferenciação e hipertrofia celular, além de hiperplasia tecidual (Mani 1964, Oliveira et al 2016). Assim, a estrutura da galha provê vantagens adaptativas aos insetos galhadores discutidas com base em três hipóteses funcionais: a hipótese nutricional onde galhas garantem recursos nutricionais para a dieta do galhador como resposta ao constante estímulo alimentar do galhador; a hipótese do inimigo natural na qual sugere que as alterações morfológicas causadas pelas atividades do galhador nos tecidos da hospedeira protegem contra a ação de inimigos naturais e a hipótese do microambiente com a proteção ao contato com condições abióticas desfavoráveis como altas temperaturas (PRICE *et al.* 1986; STONE & SCHONROGGE 2003).

Ainda, a indução de galhas ocorre por um vasto grupo de insetos e, em consequência dessa alta diversidade, as respostas celulares das plantas hospedeiras aos diferentes indutores de galhas levam à formação de inúmeros morfotipos de galhas, com variações em sua forma, tamanho, cor, graus de anisotropia, direção e tipos de expansão celular (ROHFRITSCH 1992; ISAIAS *et al.* 2013, 2014; BEDETTI *et al.* 2017). A formação de um morfotipo está relacionada com as mudanças morfogenéticas presentes na indução, determinando assim o grau de complexidade das galhas. Tais mudanças se relacionam com os estímulos químicos e mecânicos do hábito alimentar do inseto indutor, e apesar de galhas serem consideradas fenótipos estendidos de suas plantas hospedeiras, a interação é totalmente dependente à

resposta genômica da planta hospedeira aos estímulos, fator que determina os padrões de desenvolvimento e estabelecimento de novos tecidos (FERREIRA *et al.* 2019).

Comumente, cada espécie de inseto induz apenas um morfotipo de galha (ROHFRITSCH & SHORTHOUSE 1982). Entretanto, em alguns casos, uma espécie de inseto galhador pode induzir até três morfotipos de galha diferentes na mesma hospedeira, em órgãos diferentes ou em momentos distintos do ciclo de vida do inseto galhador, sendo tal aptidão caracterizada como polimorfismo (e.g. GONÇALVES *et al.* 2005; DORCHIN *et al.* 2009; KURZFELD- ZEXER *et al.* 2015; PFEFFER *et al.* 2018).

O polimorfismo aumenta indiretamente a variabilidade dos recursos vegetais que tais insetos galhadores podem acessar (TOKUDA 2012). Em alguns casos, morfotipos diferentes são induzidos como forma de sincronismo entre os ciclos de vida do inseto galhador (estágio de desenvolvimento) e sua planta hospedeira (e.g. PFEFFER *et al.* 2018). Entretanto, a formação de diferentes morfotipos de galhas pela mesma espécie de inseto galhador também pode estar relacionada diferentes respostas celulares e teciduais da hospedeira devido ao dimorfismo sexual da espécie galhadora (RHOMBERG 1980; DORCHIN *et al.* 2009; GONÇALVES *et al.* 2005; GULLAN *et al.* 2005). Em alguns grupos taxonômicos como os Hemiptera (Eriococcidae), o dimorfismo sexual em galhas induzidas por inseto de sexos diferentes, é relativamente comum (GULLAN *et al.* 2005, MAGALHÃES *et al.* 2015).

Em outros grupos, como os Cecidomyiidae o polimorfismo é relatado para galhadores que possuem a capacidade de indução de diferentes morfotipos, porém a causa destas diferenças morfológicas é pouco relatada e discutida na literatura. Por exemplo, Mishima & Yukawa (2007), determinaram a existência de um dimorfismo presente em morfotipos de galhas induzidas por *Pseudasphondylia neolitseae* (Diptera: Cecidomyiidae), porém os autores relatam que a existência desses diferentes morfotipos estaria relacionada a eventos de especiação, uma vez que a coexistência de ambos os tipos em uma única árvore

hospedeira era relativamente rara, e sempre que coexistiam em uma única árvore apareciam juntos na mesma folha. Em outro sistema de estudo, Lin et al. (2020) demonstra que o Cecidomyiidae *Bruggmanniella litseaea* é capaz de induzir galhas polimórficas, incluindo cinco subtipos, bem como formas intermediárias de galhas foliares em *Lonicera acuminata*, e discute tais resultados baseado em especiação em decorrência de pressões ambientais. Sendo assim, os estudos abrangentes do polimorfismo geralmente relatam que tais diferenças ocorrem em detrimento de processos evolutivos de surgimento de novas espécies, sem citação à possibilidade de diferenças por dimorfismo sexual.

Do ponto de vista morfológico, os Diptera: Cecidomyiidae são capazes de induzir galhas complexas, com a formação de diferentes compartimentos e tipos celulares (FERREIRA *et al.*, 2019). Em geral, as células e tecidos da hospedeira passam por um processo de rediferenciação, com mudanças estruturais e metabólicas e alterações na produção e no acúmulo de metabólitos primários e secundários para compor os tecidos das galhas (FORMIGA *et al.* 2011; COSTA *et al.* 2018, KUSTER *et al.* 2019). Dentre os compostos secundários, os fenólicos estão, geralmente, associados a mecanismos de defesa, porém, podem também estar associados ao desenvolvimento da galha, exercendo um papel chave no controle do estresse oxidativo e concentração do fitohormônio IAA (Auxina, Ácido indol-3-acético) nos sítios de hipertrofia e hiperplasia da galha (HARTLEY 1998; BEDETTI *et al.* 2014; SUZUKI *et al.* 2015, ISAIAS *et al.* 2015).

Apesar do polimorfismo geralmente não ser relacionado com o dimorfismo sexual em Cecidomyiidae, algumas observações em saídas de campo na Reserva Ecológica do Panga – Uberlândia/MG trouxeram evidências de um caso dessa peculiaridade. No sistema constituído pelo inseto galhador *Lopesia mataybae* (Diptera – Cecidomyiidae) e pela planta hospedeira *Matayba guianensis* (Sapindaceae) é possível distinguir-se dois morfotipos de

galhas induzidas pela mesma espécie nas folhas da planta hospedeira. Ao dissecar as galhas e analisar os estágios de desenvolvimento do inseto galhador, percebe-se que os mesmos estágios de desenvolvimento (larva e pupa) ocorrem em ambos, o que descarta a possibilidade dos morfotipos diferentes serem resultado de estágios distintos de desenvolvimento do inseto galhador. De acordo com a descrição da espécie do inseto galhador por Garcia & Urso Guimarães (2018), machos e fêmeas possuem diferenças em tamanho (macho 1.3mm; fêmea 1.6mm) e composição do ápice de seus abdomens (Ovipositor curto em fêmeas, Aedeago bilobado em machos).

Dito isto, este estudo objetivou avaliar se o sexo do galhador *Lopesia mataybae* (Diptera – Cecidomyiidae) determina diferentes formas da galha foliar em *Matayba guianensis* (Sapindaceae) e ainda, objetivamos (I) observar a eclosão de insetos galhadores machos e fêmeas, dos morfotipos pequeno e grande respectivamente, das galhas; (II) Analisar as diferenças morfométricas e anatômicas dos dois morfotipos de galhas induzidas por *L. mataybae* em folíolos de *M. guianensis*, e sua relação com o sexo do inseto galhador; (III) Investigar a composição nutricional dos tecidos vegetais dos morfotipos das galhas, e possíveis diferenças.

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# Sexually dimorphic galls induced on leaflets of *Matayba guianensis* (Sapindaceae): a rare phenomenon occurring in Diptera (Cecidomyiidae)

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## Abstract

In a general way, galling insects can induce only one gall morphotype on the host plant species. However, some taxa of galling insects such as Eriococcidae can induce sexually dimorphic galls on the same host. In the present study, *Lopesia mataybae* (Diptera: Cecidomyiidae) induced two gall morphotypes on the leaflets of *Matayba guianensis* (Sapindaceae), a rare evidence of sexual dimorphism in galls induced by Cecidomyiidae. We investigated the adult emergence of galling insects and the morphological, histological, and histochemical attributes of the gall morphotypes. Both gall morphotypes showed the galling insect in the larval or pupal stage, and the insect adult emergence from different morphotypes was sexually distinct. Galls occupied by females (Globoid) were significantly larger (average height = 4.67 mm.  $S = 0.43$ /average width = 4.59 mm.  $S = 0.70$ ) than galls occupied by males (cylindrical) (average height = 2.95 mm.  $S = 0.46$ /average width = 2.34 mm.  $S = 0.45$ ). Both gall types were composed of elongated cells in the inner cortex and rounded cells in the outer cortex of the vessel-like structure. The globoid galls showed 42 cell layers (14 of them cylindrical,  $S = 3.86$ ; 28 rounded,  $S = 5.89$ ) and cylindrical galls showed 29 layers. There were no differences between morphotypes in the histochemical compounds detected. We have shown here significant morphological and histological differences between male and female galls possibly due to the different nutritional requirements of *Lopesia mataybae* sexes. Therefore, the expression of sexual dimorphism in gall morphotypes may be associated with developmental pathway differentiation between the sexes of *L. mataybae*.

**Keywords** Plant interaction · Plant anatomy · Sexual dimorphism · Galling insects

## Introduction

Galls are new plant organs developed by chemical and/or mechanical stimuli from specialized organisms, especially insects (Mani 1964; Shorthouse et al. 2005). The host plant interaction with these specialized herbivores, leading to gall formation, is one of the most efficient strategies of herbivory

(Roskam 1992; Stone and Schönrogge 2003). The galling insects are truly phenotype manipulators, changing the host tissues for their own benefit and, in general, inducing cell hypertrophy and tissue hyperplasia during gall development (Oliveira et al. 2016). Regardless of the site of gall induction and development (e.g., leaf, stem, flower bud), the gall structure provides a favorable environment for the galling insect larvae as well as protection against natural enemies, and also provides nutritional resources (Bronner 1992; Hawkins et al. 1997; Hartley 1998; Shorthouse et al. 2005; Oliveira et al. 2011; Ferreira et al. 2017a). The development of gall tissues depends on the activity of a phenotype manipulator, the galling insect, that changes the plant tissues metabolism, but can be limited by host plant morphogenetic constraints (Mani 1964; Meyer and Maresquelle 1983; Rohfritsch 1992; Oliveira and Isaias 2010; Bartlett and Connor 2014; Oliveira et al. 2016; Amorim et al. 2017; Rezende et al. 2019).

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Some plant species can host different gall morphotypes induced by different galling species (e.g., Hayward and Stone 2005; Oliveira et al. 2008; Costa et al. 2010). Usually, each insect species induces only one gall morphotype in a host plant (Rohfritsch and Shorthouse 1982; Price 2005). In a rare report, the same insect species (*Pseudophacopteron longicaudatum*) was shown to induce morphologically similar galls on distinct host plant species (*Aspidosperma macrocarpon* and *Aspidosperma tomentosum*) (Malenovský et al. 2015; Martini et al. 2021). Therefore, a single species of galling insect can induce different gall morphotypes on the same host plant depending on its own life cycle or sex (e.g., Gonçalves et al. 2005; Dorchin et al. 2009; Kurzfeld-Zexer et al. 2015; Pfeffer et al. 2018; Silva et al. 2019).

Some galling insects (Cecidomyiidae) induce dimorphic and polymorphic galls on the same host plants, such as the two gall morphotypes of *Pseudasphondylia neolitsea* on the same leaves of *Neolitsea sericea* (Lauraceae) (Mishima and Yukawa 2007), the polymorphic axillary bud galls induced by *Rhopalomyia longitubifex* on *Artemisia princeps* and *Artemisia montana* (Asteraceae) (Ganaha et al. 2007), the thick and thin galls induced by *Masakimyia pustulae* on leaves of *Euonymus japonicus* (Celastraceae) (Fujii et al. 2014), and the five shapes of leaf galls induced by *Brugmanniella litseae* on *Litsea acuminata* (Lauraceae) (Lin et al. 2019). The induction of more than one gall morphotype by one species of galling insects on a single host seems to increase the range of resources this galling species can access, and this peculiarity may be related to sexual dimorphism (Rhomberg 1980; Wool and Burstein 1991; Miller 1998; Dorchin et al. 2009; Gonçalves et al. 2005). Sexual dimorphism is common in some taxa of galling insects such as the Eriococcidae gall systems (Gullan et al. 2005; Gonçalves et al. 2005; Magalhães et al. 2015) but, to the best of our knowledge, sexually dimorphic galls are a rare phenomenon for relatively specialized Cecidomyiidae (Diptera).

The galling insect *Lopesia mataybae* (Diptera—Cecidomyiidae) induces two gall morphotypes (Globoid and Cylindrical) on leaflets of *Matayba guianensis* (Sapindaceae). Although these two gall morphotypes are quite similar, they differ considerably in size and thickness. Herein, we assumed that females and males induce distinct gall morphotypes. Cecidomyiidae are known to have sexual dimorphism and females are usually bigger than males (Condrashoff 1962; Gagné 1994; Dorchin et al. 2007; Tabadkani et al. 2012). Thus, considering that gall tissue develops in response to constant insect feeding stimuli (e.g., Ferreira et al. 2017b; Rezende et al. 2019) and that the feeding stimulus by females may stimulate a stronger host plant response due to developmental requirements, female galls should be larger than male galls. In addition, galls occupied by females may show a different distribution of nutritional compounds in the tissues than galls occupied by males.

## Materials and methods

### Study area and sampling

The study was carried out at Panga Ecological Station (19°10′S, 48°24′W), Uberlândia municipality, Minas Gerais State, Brazil. We collected randomly a total of 210 galls of both morphotypes (globoid and cylindrical galls – sensu Isaias et al. 2014a), from 10 plant hosts belonging to one population to standardize the host plant features. The galls induced by *Lopesia mataybae* Garcia and Urso-Guimarães 2018 (Diptera—Cecidomyiidae) on leaflets of *Matayba guianensis* (Sapindaceae) were sampled from July 2019 to October 2019, for observation of the developmental stages and hatching of the galling insect and for morphological, histological, and histochemical characterization.

### Gall midge identification

To confirm gall midge identification, six males and seven females collected on October 12, 2020, and two male pupae, two female pupae, and one larva collected on September 27, 2019, were mounted on microscope slides according to the methods outlined by Gagné (1994). The specimens were deposited in the Entomological Collection of the Museu Nacional (MNRJ)/Universidade Federal do Rio de Janeiro (MNRJ) as voucher material: males (MNRJ Ent-166953, MNRJ Ent-166957, MNRJ Ent-166958, MNRJ Ent-166960, MNRJ Ent-166962, MNRJ Ent-166964), females (MNRJ Ent-166951, MNRJ Ent-166954–166,956, MNRJ Ent-166959, MNRJ Ent-166965, MNRJ Ent-166965), male pupae (MNRJ Ent-166952), female pupae (MNRJ Ent-166963), and larva (MNRJ Ent-166952).

### Evaluating the presence of sexual dimorphism

To rule out the possibility of the different morphotypes being stages of gall development, a total of 40 galls of both morphotypes from 4 different host plants were opened to find out if both morphotypes contained the same insect developmental stages (larvae and pupae). We evaluated and measured the morphology of 124 galls, half from each morphotype randomly selected in the field from 10 different host plants. To determine possible sexual dimorphism, i.e., the presence of *L. mataybae* female or male individuals, the galls of both morphotypes were placed separately in plastic microtubes until adult emergence. The pupae and emerged adults found were analyzed under a stereomicroscope in order to distinguish males from females according to the adult insect descriptions reported by Garcia and Urso-Guimarães (2018). The height and width of these galls were

measured with a digital caliper (Digimess®) to obtain the mean values for further statistical analysis. All above procedures were carried out in the laboratory of plant anatomy, development, and interactions (LADEVI) of the Federal University of Uberlândia.

### Histological and cyto-histometric analysis

For histological analysis, 10 samples from both gall morphotypes were fixed in FAA 50 (formalin, acetic acid, 50% ethanol, 1:1:18 v/v/v) for 48 h (Johansen, 1940), dehydrated in an ethanol series, embedded in 2-hydroxyethyl methacrylate (Historesin®, Leica® Instruments, Germany), and cut into 5- $\mu$ m thick sections with a rotary microtome (YD-315 model, China). The sections were stained with 1% toluidine blue, pH 4.0 (O'Brien et al. 1965), and mounted with Entellan®.

For cyto-histometric analysis, 16 galls were submitted to hand-free cuts ( $N=8$  of both morphotypes) and stained with astra blue-safranin, 8:2 (Bukatsch 1972, modified to 0.5%). Photomicrographs were obtained with a Leica DM500® camera coupled to an HD camera. Sixteen light microscopy images were used for counting cell layers in a wide transect from external to internal surfaces of the gall wall structure, and the area of 30 cells from each image was measured. All analyses were carried out using the ImageJ software. To avoid pseudo-replicas of data, a mean value was calculated for each image where the cell measurements were obtained, and 32 (16 of each morphotype) mean values of the cell measurements were used for statistical analysis.

### Statistical analysis

Normality of distribution and homogeneity of variance of the data were analyzed using the Shapiro test and graphic observation. The data followed a normal distribution; thus, there was no need for data transformation. To compare the height and width of the globoid galls to those of the cylindrical galls, we conducted a nested ANOVA analysis, using the function lmer from the “lme4” package (Bates et al. 2015). The *T*-test was then used to perform comparisons of the cell layers and the cell area measurements. All analyses were carried out using the R environment software version 4.0.3 (R Core Team 2020).

### Histochemical analysis

We performed histochemical tests using fresh samples cut into handmade sections with a razor blade and immersed in specific reagents in order to detect starch, reducing sugars, proteins, and lipids in gall tissues. The samples were submitted to Lugol solution (1% iodine-potassium iodide solution) (Johansen 1940) for starch detection and to Fehling's reagent

reaction (solution “A” 6.93% II copper sulfate w/v and “B” 34.6% sodium potassium tartrate and 12% sodium hydroxide w/v) for reducing sugar detection (Sass 1951). The samples were immersed in 0.1% bromophenol blue (Mazia et al. 1953) for protein detection and in a saturated solution of Sudan III (Sass 1951) for lipid analysis. Control tests were performed using blank sections.

## Results

### Morphological data of the gall midge

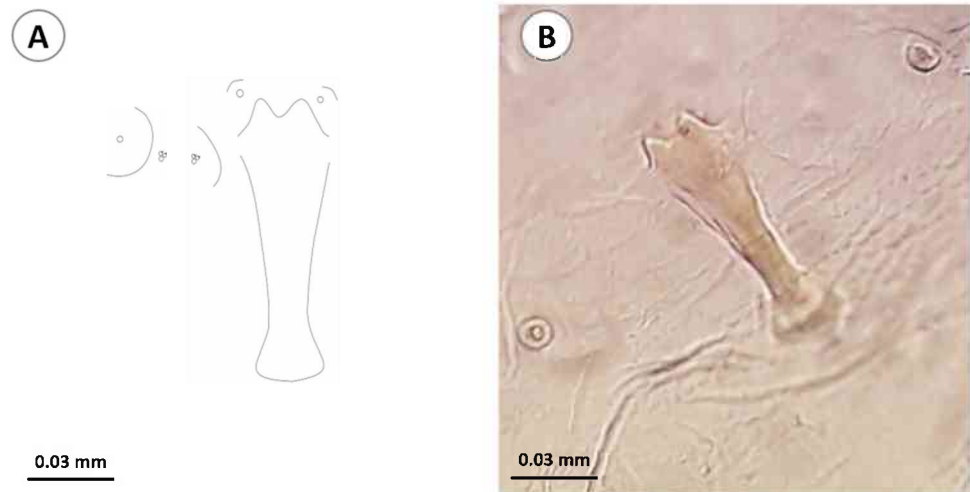
The specimens under study belong to *Lopesia mataybae*, a species characterized by setulose flagellomere necks, gynecoid male flagellomeres, 4-segmented palpi; wing with Rs joining R1 after its midlength, 1-toothed tarsal claws, narrow gonocoxites; bilobed male cerci with the outer lobe longer than the inner one; a short male hypoproct as long as the cercal lobe; aedeagus 1.5 times longer than the hypoproct; apex of the aedeagus with an irregular edge; pupa with a 0.07-mm-long antennal horn, 0.05-mm-long apical setae, and simple dorsal abdominal spines (Garcia and Urso-Guimarães 2018).

Although the larval stage was described by Garcia and Urso-Guimarães (2018), the prothoracic spatula and complement of lateral papillae remained unknown until today, since the cited authors stated that the anterior region was not visible on their slide mount. On our slide, the spatula and associated papillae were visible. *Lopesia mataybae* has a 0.09-mm long spatula ( $N=1$ ) with two apical pointed teeth and a 0.02 mm distance between tooth apices, no lateral subapical process, a well-developed 0.08-mm-long stalk constricted in the basal  $\frac{1}{4}$  and with a rounded base, subapical width of about 0.03 mm similar to the subbasal width; bare sternal papillae, and two groups of three lateral papillae, one with a seta and two bare in each group (Fig. 1 a, b), body length: 1.6 mm.

### Sexual dimorphism on galls induced by *L. mataybae*

The insects that occupied and emerged from both morphotypes were sexually distinct. Males occupied and emerged from small galls (cylindrical) and females emerged from larger galls (globoid). The globoid and cylindrical morphotypes (Fig. 2 a) were determined according to Isaias et al. (2014a). Among the 40 galls dissected, 9 *L. mataybae* female pupae (Fig. 2 b) and 11 larvae were found in the globoid galls, while 7 *L. mataybae* male pupae (Fig. 2 c) and 13 larvae were found in the cylindrical galls. We sampled 36 adult females (Fig. 2 d) hatched only from the globoid galls and 13 males (Fig. 2 e) hatched only from the cylindrical gall morphotypes. Two unidentified species

**Fig. 1** Illustration and photomicroscopic view of *L. mataybae* spatula. **a** Sketch of the spatula and associated papillae. **b** *Lopesia mataybae* spatula 0.09 mm long ( $N=1$ ), with two apical pointed teeth



of parasitoid wasps (Hymenoptera) were found interacting with the galls; adults of the first species emerged from both morphotypes (6 from globoid ones and 9 from cylindrical ones) and the second species had 2 adult individuals emerging from the cylindrical morphotype. Males and females started hatching together 3 days after collection in the field. Morphometrically, the female (globoid) galls were significantly larger and thicker (average height = 4.600 mm,  $S=0.43$ /average width = 4.519 mm,  $S=0.70$ ) than the male (cylindrical) galls (average height = 2.905 mm,  $S=0.46$ /average width = 2.308 mm,  $S=0.45$ ) (nested ANOVA height:  $F_{1, 121} = 633,9$   $p < 0.001$ ) (nested ANOVA width:  $F_{1, 121} = 505,7$   $p < 0.001$ ), respectively. Thus, analysis of the millimeter differences revealed a clear difference between the two morphotypes.

Although these galls differed significantly in size, some characteristics were similar to those usually induced on the abaxial leaflet surface (Fig. 3 a, b), which was covered with trichomes. Both gall morphotypes consisted of two main parts, an outer vessel-like structure (Fig. 3 c) that surrounded another innermost cocoon-like structure (Fig. 3 d), where the galling insect spent most of its life cycle. The insect emerges from the gall by pushing out of this cocoon-like structure and the wrapping part remains fixed to the leaf base.

### Anatomical features and cyto-histometric differences

Both globoid and cylindrical galls developed from cell hypertrophy and tissue hyperplasia of the abaxial epidermis and spongy parenchyma of leaflets. These sexually dimorphic galls showed similar histological profiles, as noted in the central cocoon-like structure with wall hardening due to intense lignification in both male and female galls (Fig. 4 a, b). At the base of both galls, we noted

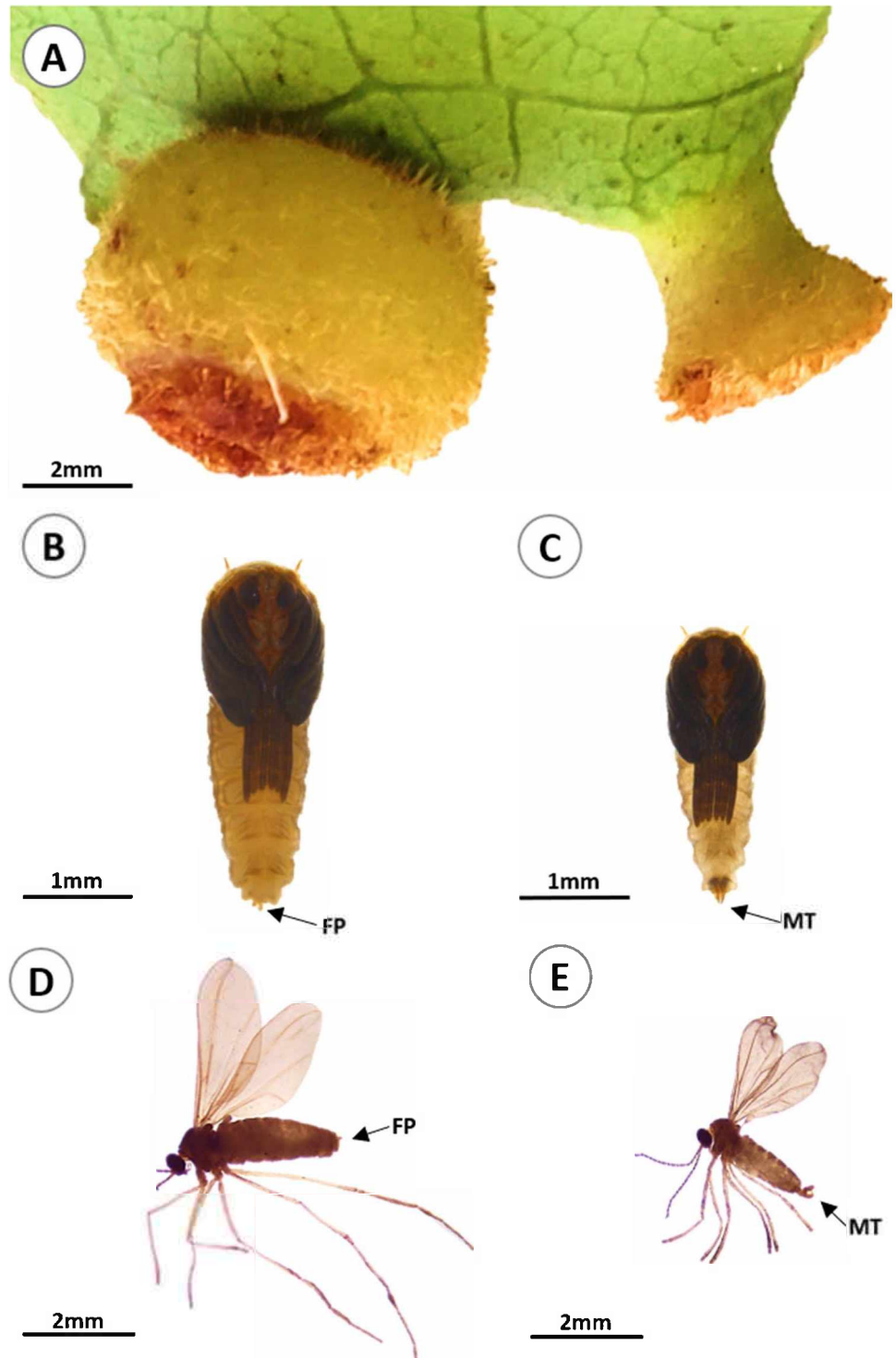
the development of neoformed vascular bundles and the presence of typical nutritive tissue at the point of insertion on the leaflet lamina, with both female and male galls maintaining only parts of the spongy parenchyma (Fig. 4 c, d). Gall tissue continued to expand by tissue hyperplasia with aleatory cell divisions and cell hypertrophy, and the gall epidermis continued from the abaxial leaf epidermis.

The differences between the globoid and cylindrical galls were in the outer vessel-like structure that remained active until gall senescence. Analysis of the cell composition of this structure revealed that both morphotypes had a heterogeneous parenchyma composed of two different cell shapes, i.e., elongated cells located in the inner cortex and rounded cells located in the outer cortex (Fig. 4 e, f). This wrap on globoid galls was smaller at the base and expanded in thickness in the region surrounding the cocoon-like structure, with a total average of 42 cell layers, 14 of which ( $S=3.86$ ) were composed of elongated cells and 28 ( $S=5.89$ ) of rounded cells. In male-occupied galls, the base attached to the leaflet was the largest part, but the vessel-like structure, in contrast to the globoid morphotype, did not grow larger and there was a total average of 29.5 cell layers, 17 ( $S=2.53$ ) of them consisting of elongated cells and 14.5 ( $S=1.77$ ) consisting of rounded cells.

On the vessel-like structure of the globoid morphotype, the elongated cell area was larger (average: 4.997 mm,  $S=0.72$ ) than that of the cylindrical morphotype (average: 3.587 mm,  $S=0.91$ ) ( $p < 0.001$ ,  $t = -4.3187$ ). In the cylindrical morphotype, the rounded cell area was larger (average: 4.947 mm,  $S=0.34$ ) than that of the globoid morphotype (average: 3.821 mm,  $S=0.67$ ). Accordingly, comparison by the Tukey test showed that the two morphotypes differed significantly in cell area ( $p < 0.001$ ,  $t = 4.4812$ ) (Fig. 5 a, b).



**Fig. 2** *L. mataybae* galls and galling insect stages. Globoid (female-occupied) and cylindrical (male-occupied) morphotypes induced by *L. mataybae* on leaflets of *Matayba guianensis* (a). Female pupa found on the globoid gall (b). Male pupa found on the cylindrical gall (c). Adult female hatched from the globoid morphotype (d). Adult male hatched from the cylindrical morphotype (e). PP pupae, FP female post abdomen, MT male terminalia

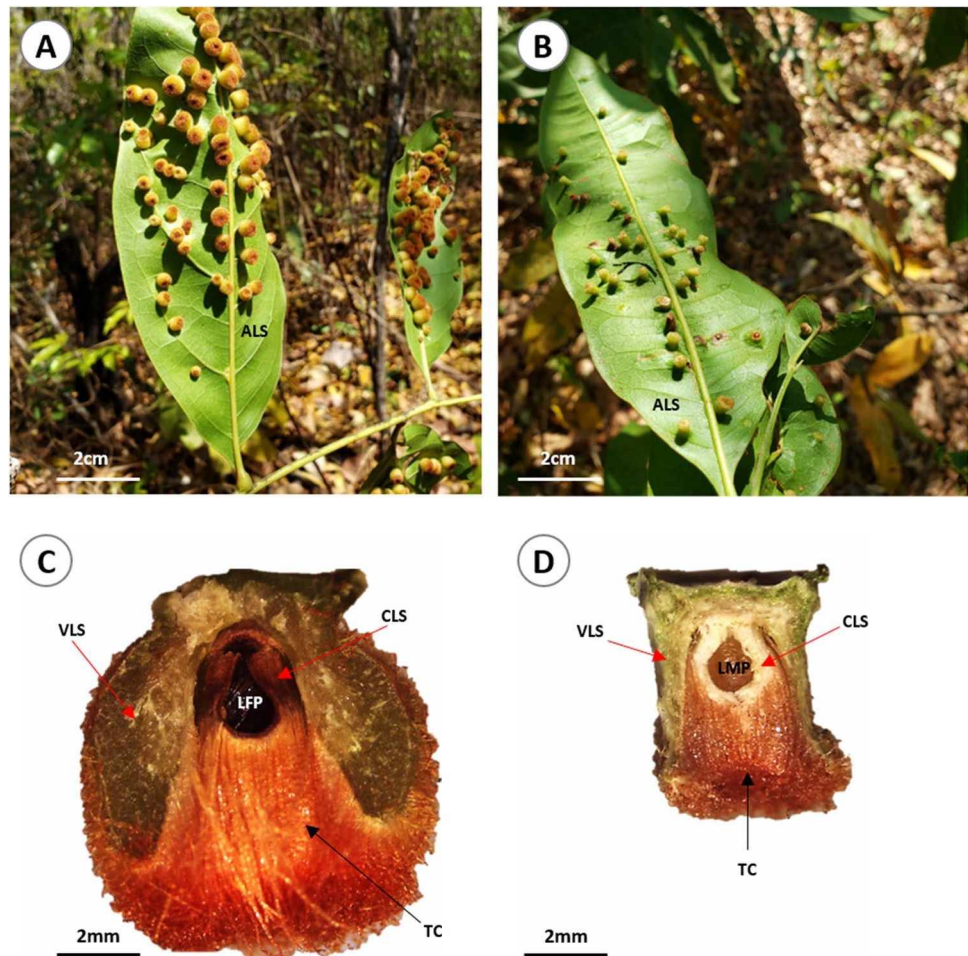


**Histochemical tests**

Histochemical tests revealed that there was no difference between the globoid galls and cylindrical galls of *L. mataybae*, both of which accumulated starch in the base of the cocoon-like structure and in the base of the lateral

regions in the vessel-like structure. Reducing sugars were detected in both galls, mainly next to the “cocoon-like structure” region, probably a true nutritive tissue. The tests for proteins and lipids were negative for both female and male galls.

**Fig. 3** *L. mataybae* gall morphotypes, positioning, and structures. Galls from both morphotypes usually induced on the abaxial leaf surface (a, b). Galls occupied by females (c) have a globoid shape and are larger and thicker laterally than those occupied by males, which have a cylindrical shape (d), despite having the same structure. ALS abaxial leaf surface, VLS vessel-like structure, CCL cocoon-like structure, TC trichomes, LFP *L. mataybae* female pupa and male pupa (LMP)



## Discussion

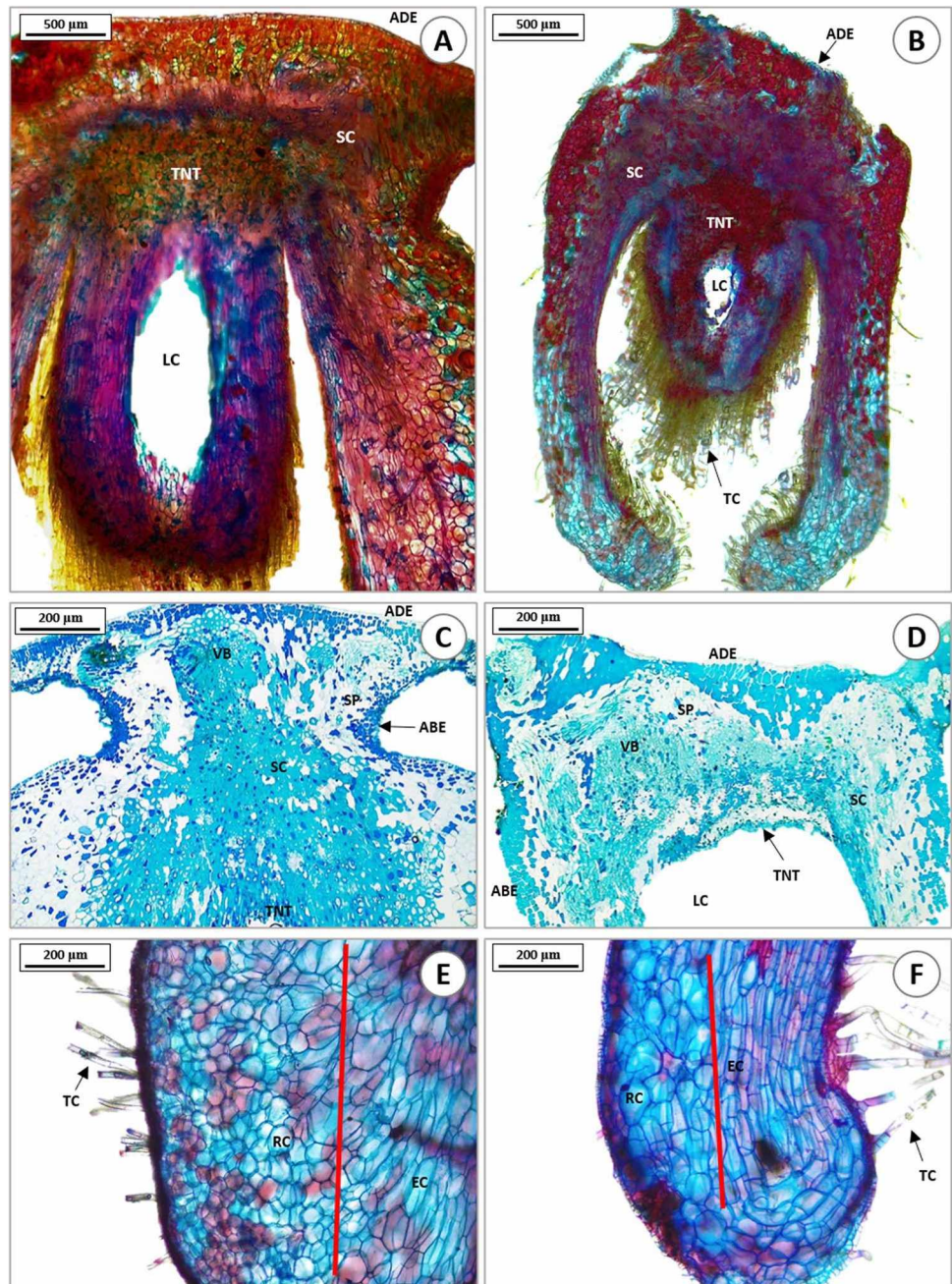
The gall morphotypes induced by *L. mataybae* on leaflets of *M. guianensis* are sexually dimorphic, as shown by the morphometric analysis and the significant differences in cell area between the vessel-like structures ( $P < 0.001$ ). Female insects are capable of inducing galls approximately twice the size in height and width as those induced by males. In addition, the tissue layers and cell sizes differed between morphotypes, explaining the distinct size and morphotype of male (cylindrical) and female (globoid) occupied galls. Thus, a possible explanation for the occurrence of sexually dimorphic galls, shown by Gonçalves et al. (2005) but not yet tested for *L. mataybae* galls, is that the nutritional requirements for development differ between sexes, inducing different rates of sink photosynthetic assimilate compounds at the gall formation site between morphotypes (Gonçalves et al. 2005).

When evaluating the induction of galls by *L. mataybae*, the available plant resources were found to be the same for both sexes since the galls are usually induced at the same site (abaxial leaflet surface). However, each sex explores

these resources differently, forming distinct gall morphotypes. The product of this insect/plant interaction could be determined by how the plasticity of *M. guianensis* leaf tissues reacts to the stimuli of *L. mataybae* sexes. The induction of different morphotypes by the same galling insect has already been reported for *Matayba guianensis*. On this host plant, *Bystracoccus mataybae* (Eriococcidae) (Hodgson et al. 2013) induces two different morphotypes depending on the developmental stage of the insect and the site of oviposition. The first instar nymph induces simple stem galls and the second instar nymph induces more complex leaflet galls (Pfeffer et al. 2018; Silva et al. 2019). Although induced in the same host plant species, the galls induced by *B. mataybae* show different morphology, anatomy, and histochemistry compared to those induced by *L. mataybae*. These findings indicate that the galling species, their development stage, and their sex have a direct influence on gall morphology when manipulating the plasticity of host plant tissues. For example, *B. mataybae* galls are glabrous (Pfeffer et al. 2018), while *L. mataybae* galls have trichomes. The mature leaves of *M. guianensis* are smooth, but the leaves in early developmental stages have trichomes (Silva et al.



**Fig. 4** Anatomical structure of galls induced by *L. mataybae*. **a, b** General overview of gall structures, with the presence of sclerenchyma, typical nutritive tissue, adaxial epidermis, larval chamber, and trichomes from the globoid and cylindrical morphotype, respectively. **c, e** Female gall. **c** Female's base smaller but expanding in thickness around the larval chamber. **e** Cortex cells resulting from hypertrophy and hyperplasia of the spongy parenchyma, with the presence of vascular bundles. **d, f** Male gall. **d** The male base is the larger part, containing more cell layers, and the regions around the larval chamber are thinner. **f** Vessel-like structure of the cylindrical morphotype, with rounded cells, elongated cells, and trichomes. SC sclerenchyma, TNT typical nutritive tissue, AEP adaxial epidermis, LC larval chamber, TC trichomes, VB vascular bundle, SP spongy parenchyma, ABE abaxial epidermis, RC rounded cells, EC elongated cells



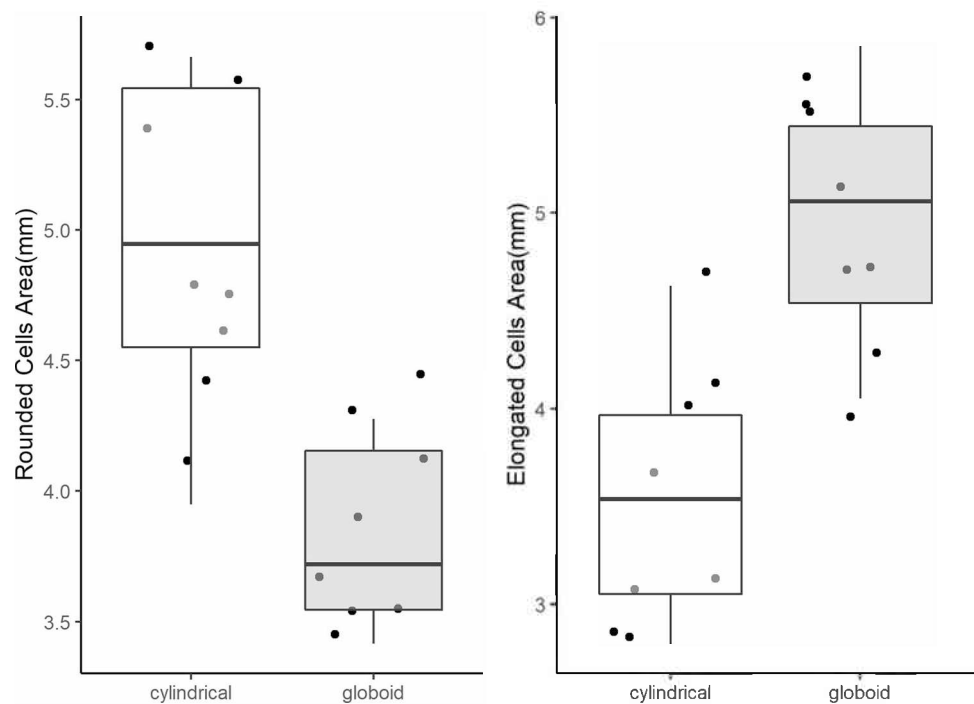
2019). Therefore, the galls induced by *L. mataybae* not only maintain this characteristic of young leaves but increase its expression, with the development of many trichomes mainly on the cocoon-like structure, where the galling insect larva lives.

The determination of gall shape depends on the feeding behavior of the galling insect, which stimulates the process of gall tissue development in a different manner (Rohfritsch and Anthony 1992; Oliveira et al. 2014; Ferreira et al. 2019). Therefore, gall tissue differentiation and growth depend on the constant specific galling feeding stimulus (Rezende et al. 2019). Here we believe that the differences expressed by

the gall structures occur due to the intensity of the feeding stimulus, that must differ between sexes. *L. mataybae* adult galling insects are especially different in body size, and sexually dimorphic gall structures can show differences in phytohormone concentrations with distinct rates of metabolic activity according to the galling insect sex, with females usually inducing stronger metabolic responses than males (Dorchin et al. 2009).

According to the nutritional hypothesis, the gall structure guarantees nutritional resources for the galling insect's diet which, depending on the rate, can induce the formation of a specialized tissue for its nutrition, i.e., the nutritive

**Fig. 5 a, b** Graphic representation of histometric and cytometric measurements. Elongated/globoid cell layers and elongated/globoid cell area of the globoid and cylindrical morphotypes



tissue (Stone and Schönrogge 2003). On the basis of this hypothesis, we can say that *L. mataybae* males and females already have advantages over free-living insects and yet they manipulate and influence the resources differently. Hence, the different interactions of male, female, and host plant can help us understand the sexually dimorphic galls. Based on their nutritional requirements for development, female gall-ing cecidomyiids stimulate more cell hypertrophy and tissue hyperplasia on the host tissues, and consequently develop larger and sexually dimorphic galls. The gall systems *Pseudotectococcus rolliniae* (Eriococcidae)—*Rollinia laurifolia* (Annonaceae) (Gonçalves et al. 2005) and *Trichilogaster acaciaelongifoliae* (Pteromalidae)—*Acacia longifolia* (Fabaceae) (Dorchin et al. 2009) are further examples of female-induced galls being larger than male-induced ones. In both systems, the galls are similar in general patterns; however, they differ in size according to galling insect sex. Here, we showed that the sex of the galling insect *L. mataybae* stimulates differently the processes of cell hypertrophy and tissue hyperplasia, leading to the formation of sexually dimorphic galls on *M. guianensis*.

Regarding the compositional differences of the vessel-like structure between morphotypes, the elongated cells occur where a more sclerified tissue is found, i.e., the innermost part of the parenchyma. On cylindrical galls, the elongated cells had more layers than the rounded cells, while the opposite occurred on globoid galls. Considering the composition of this structure, these tissue differences explain the occurrence of different morphotypes (Guedes et al. 2018; Costa et al. 2021). With more rounded cell layers on the outer

cortex and less elongated cell layers on the inner cortex, the globoid shape of female-occupied galls is formed, whereas with fewer rounded cell layers on the outer cortex and more elongated cell layers that are smaller than those occurring in the globoid shape, the cylindrical shape of male-occupied galls is formed. This development of different quantities of cell layers and of cell area size between the sexes of *L. mataybae* could be a consequence of differences in the nutritional requirements of the sexes influencing different phytohormone concentrations (Dorchin et al. 2009; Guedes et al. 2018). Thus, females induce larger and globoid-shaped galls because of their developmental needs, increasing in size and thus laying more eggs. However, this mechanism is still to be elucidated.

According to the natural enemy hypothesis, the thickness and the development of a mechanical zone (lignified cells) in galls can provide protection against parasitoids (Price and Pschorn-Walcher 1988). However, both female and male galls induced by *L. mataybae* are attacked by unidentified species of parasitoids. Thus, more studies about the biological cycle and taxonomy of parasitoids may provide information about differential protection against parasitoids by female and male galls. Since it was not possible yet to identify the two parasitoids found, we were not able to determine whether these parasitoids were early or late attackers in order to better describe their relations to the gall's developmental process (Fujii et al. 2014).

Galls are true sinks of photoassimilates from different plant parts (Jankiewicz et al. 1970; Larson and Whitham 1991; Castro et al. 2012; Oliveira et al. 2017) and these



resources are used to support gall development and metabolism, as well as the galling insect's diet (Hartley 1998; Oliveira et al. 2006; Isaias et al. 2014b). Thus, the differential investment in cell division and hypertrophy represents the differences in life cycles and how much the female or male insect can act as a sink of photoassimilates on the host tissue of *M. guianensis*. In addition to representing a resource for gall tissue development, nutritive substances accumulate in the storage tissues of galls (Bronner 1992). Usually, cecidomyiid-induced galls develop a typical nutritive tissue (Ferreira et al. 2019) with high sugar metabolism, and a storage tissue with starch, as detected in galls induced by *Eugeniamyia dispar* in *Eugenia uniflora* (Rezende et al. 2018) and by an undefined species of Cecidomyiidae in *Copaifera langsdorffii* (Oliveira et al. 2010). In both galls of *L. mataybae*, starch was detected in the storage tissue and reducing sugar was detected in the typical nutritive tissue. However, no protein was detected in the tissues of both galls, which is unusual for Cecidomyiidae galls.

We have shown here that galls occupied by different sexes of *L. mataybae* result in gall structures with significant differences in morphology. The globose galls (induced by female insects) were higher and wider than cylindrical galls (induced by male insects), with significant differences in cell layer composition and cell area observed by histometry, structurally explaining the development of these different morphotypes. Nevertheless, the two gall types were histochemically similar in terms of production of some primary metabolites such as starch and reducing sugars, but lacking protein and lipids. This indicates that the differences between the two gall morphotypes as an expression of the galling insect's sexual dimorphism should be due to the differentiation of developmental pathways and possible distinctions of feeding activity within the sexes.

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**Author contribution** Pedro Henrique Pereira Gonçalves collected and analyzed the material, searched the bibliography, and wrote the full text. Uíara Costa Rezende helped collect and analyze the material and write the text. Phabliny Martins Silva Bonfim analyzed the laboratory material and the statistical data. Valéria Cid Maia described the insect's spatula and the overall features of the developmental stages of *L. mataybae*. Denis Coelho de Oliveira was responsible for the analysis of the anatomical features and the revision of the text throughout the writing process.

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## Declarations

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## **Perspectivas e Considerações finais**

Logo no início do meu ano de ingresso no PPGECB, 2020, uma pandemia se instaurou, provocando *lockdowns* por todo o mundo durante um certo período de tempo. Tal fator atrasou o cronograma de desenvolvimento de minha pesquisa de tal forma que só pude começar a desenvolver partes das análises durante o ano de 2021. Sendo assim, me vi impossibilitado de ir para atividades de campo antes de termos a vacina por possuir pessoas grupo de risco residindo comigo.

Durante o primeiro semestre do ano de 2021 não foram encontradas galhas suficientes para realização das análises presentes no projeto de dissertação. Em setembro deste mesmo ano, a Reserva Ecológica do Panga foi devastada por uma queimada, culminada numa perda imensa da população de estudo que só encontrávamos nesta reserva. Desde então acompanhávamos pelo menos duas vezes no mês a recuperação da população de galhas do sistema de estudo, porém, as primeiras galhas foram encontradas somente a partir do fim do mês de maio, o que impossibilitou a realização das análises propostas à tempo de escrita e revisão para defesa junto com esta dissertação.

Foram propostas, também, análises de fitormônios, de composição de parede celular e citologia, para investigar as diferenças existentes entre os morfotipos distintos de galhas induzidas por *lopesia mataybae* em decorrência do dimorfismo sexual. Análises de quantificação de fitormônios necessitava coletas do material fresco em nitrogênio líquido. Já as análises de imunocitoquímica dos compostos de parede celular e caracterização dos tecidos nutritivos e de reserva eram necessárias serem coletadas em Karnovsky para posterior análises. Tais técnicas comporiam o segundo capítulo desta dissertação de mestrado, porém, não foi possível a realização devido aos impactos decorrentes da pandemia e das queimadas que dizimaram as populações de galhadores e hospedeiras nas áreas de coleta. Contudo, vale ressaltar que a população da hospedeira está se recuperando e amostras

de galhas já foram coletadas. Desta forma, já estamos preparando as amostras para novas análises, especialmente aquelas que dizem respeito à quantificação de fitormônios, composição de parede células e citologia de ambas as galhas induzidas por galhadores machos e fêmeas. Análises que serão realizadas durante o curso de Doutorado.

Ainda em relação a perspectivas futuras, continuarei compondo o corpo discente do Programa de Pós-Graduação em Ecologia Conservação e Biodiversidade a partir do mês de setembro, como doutorando, orientado pelo Professor Denis Coelho de Oliveira e coorientado pelo Professor Bruno Garcia Ferreira da UFRJ. Como projeto de Tese avaliaremos como os inimigos naturais são capazes de burlar as barreiras estruturais e químicas das galhas interagindo com os galhadores e causando ou não a morte da larva do inseto galhador. Esta interação pode reverberar efeitos diretos e indiretos na estrutura vegetal (galha), por interação direta com o inseto galhador (parasitoides) ou consumo do tecido vegetal (inquilinos). Esta proposta de doutorado pretende investigar os resultados da interação multitrófica estabelecida entre o inseto galhador, planta hospedeira e inimigos naturais, e os efeitos na estrutura e metabolismo dos tecidos vegetais das galhas. Para tal, análises fenológicas, anatômicas, citométricas e fisiológicas serão realizadas a fim de testar se a presença de inimigos naturais altera o padrão estrutural e fisiológicos das galhas beneficiando o próprio inimigo natural.