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MIRMECOFILIA EM *PARRHASIUS POLIBETES* (LEPIDOPTERA: LYCAENIDAE): HISTÓRIA NATURAL, CUSTOS, SELEÇÃO DE PLANTA HOSPEDEIRA E BENEFÍCIOS DA CO-OCORRÊNCIA COM HEMÍPTEROS MIRMECÓFILOS

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Lucas Augusto Kaminski
e aprovada pela Comissão Julgadora.

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"Uma formiguinha atravessa, em diagonal, a página ainda em branco.

... Mas ele, naquela noite, não escreveu nada. Para quê? Se por ali já havia passado
o frêmito e o mistério da vida..."
Mario Quintana (Nova Antologia Poética, 1966)

Dedicado aos meus irmãos Martin, Fernanda, Moises, Sarah,
Leon, Lenon & Isadora, todos partes de um *patchwork* bem quiltado...

ÍNDICE

ABSTRACT

Ants are one of the most prominent groups of terrestrial organisms in terms of diversity, relative abundance and biomass. Their importance is due primarily to eusocial behavior combined with complex communication systems. Tropical foliage is rich in renewable feeding sources that promote ant foraging. As some of the most important predators on plants, ants strongly affect the herbivorous insects. The presence of ants on foliages may affect herbivores by two ways: (1) decreasing herbivore individual numbers due to antagonistic interactions (e.g., aggressiveness, predation); (2) providing an enemy-free space for myrmecophilous herbivores (i.e. those living in close associations with ants). The symbiotic interaction between Lepidoptera and ants is widespread but only among two butterfly families (Lycaenidae and Riodinidae). Due to the great importance of myrmecophily for the morphology and biology of these butterflies, it is supposed that much of the evolutionary history of organisms, including diversification, would be explained by their interactions with ants. However, most of the knowledge about the evolutionary ecology of lycaenids is based on studies of well known Palaearctic, Oriental, and Australian species while little is known about the rich Neotropical fauna, which contains nearly 1,200 species. Larvae of *Parrhasius polibetes* (Stoll) (Lepidoptera: Lycaenidae) co-occur spatially and temporally with honeydew-producing hemipterans on the host plant *Schefflera vinosa* (Araliaceae). This study describes new aspects of morphology and natural history of immature stages of *P. polibetes*, including costs of myrmecophily, host plant selection, and benefits of co-occurrence with hemipteran trophobionts. The development cycle from egg to adult is approximately 36 days, and includes four larval instars. The eggs are laid exclusively on reproductive tissues (flower buds) of the host plants. The larvae are polyphagous, and have already been recorded on 28 plant species from 16 families. Most of the observed host plants of *P. polibetes* present some kind of liquid reward potentially used by ants (78.57%), either honeydew-producing hemipterans and/or extrafloral nectaries. From the third instar on, the larvae are facultatively tended by more than fifteen ants species in three subfamilies (Formicinae, Myrmicinae, and Ectatomminae), especially ants of the genus *Camponotus* Mayr. As in other Lycaenidae, interactions between larvae and ants are mediated by a specialized gland (dorsal nectar organ) on the seventh abdominal segment, which produces caloric liquid rewards for ants. Therefore it is expected that the production of these secretions entail costs for the larvae. For *P. polibetes*, it is shown that *Camponotus crassus* and *Camponotus melanoticus* ants differ in the intensity of tending levels to larvae, with *C. melanoticus* presenting increased tending rates compared to *C. crassus*. This difference can lead to different costs for the larvae. For instance when tended by *C. melanoticus*, larvae take longer to pupate. However, the pupal weight and size of adults are not affected by ant tending, suggesting that *P. polibetes* has compensatory mechanisms to minimize the costs of myrmecophily. This is the first demonstration that specific differences in ant tending may affect performance parameters in an insect trophobiont. In the field, experiments involving the manipulation of ant-treehopper associations on host plants demonstrated that the spatial co-occurrence between *P. polibetes* caterpillars and honeydew-producing hemipterans is caused by two factors: 1) females are able to detect ant-treehopper associations on foliage before oviposition, and lay eggs in their vicinity; 2) larvae that develop near ant-tended treehoppers survive better than larvae on plants without such association. This effect occurs because the presence of ant-treehopper associations reduces the abundance of potential natural enemies (spiders and parasitoid wasps) of the caterpillars. Moreover, the larvae are more easily found by prospective tending ants that are recruited to nearby honeydew-producing treehoppers. That is, the presence of ant-treehopper associations creates an "enemy-free space" on the host plant, which is exploited by *P. polibetes*. These results show that a traditional pairwise approach is obviously inappropriate to assess the selective pressures operating within such multi-species systems.

RESUMO

Formigas constituem um dos mais proeminentes grupos de organismos terrestres em termos de diversidade, abundância relativa e biomassa animal. Sua importância se deve principalmente ao comportamento eusocial aliado a complexos sistemas de comunicação. A vegetação de áreas tropicais é rica em fontes de alimentos renováveis que induzem a visitação freqüente de formigas às plantas. Sobre a vegetação, as formigas podem atuar como predadoras e acarretar um forte efeito sobre a comunidade de insetos herbívoros. A presença de formigas sobre plantas pode afetar insetos herbívoros basicamente de duas formas: (1) limitando sua ocorrência na folhagem através de interações antagônicas (ex. agressão, predação) ou (2) propiciando espaços livres de inimigos naturais para herbívoros mirmecófilos (que mantêm associações simbióticas com formigas). Em Lepidoptera, a mirmecofilia é amplamente difundida em apenas duas famílias de borboletas (Lycaenidae e Riodinidae). Devido a grande importância da interação com formigas para a morfologia e biologia destas borboletas, acredita-se que grande parte da história evolutiva desses organismos, incluindo eventos de diversificação seja explicada pela mirmecofilia. No entanto, a maior parte da informação sobre borboletas mirmecófilas é baseada no conhecido para espécies das faunas Paleártica, Oriental e Australiana. Enquanto que a rica fauna de borboletas mirmecófilas Neotropicais permanece praticamente desconhecida. Dentre as cerca de 1.200 espécies de Lycaenidae Neotropicais, *Parrhasius polibetes* (Stoll) (Lepidoptera: Lycaenidae) foi reportada recentemente co-ocorrendo espaço-temporalmente com hemípteros mirmecófilos em *Schefflera vinosa* (Araliaceae). Neste trabalho são descritos novos aspectos relacionados à morfologia e história natural dos estágios imaturos de *P. polibetes*, incluindo custos da mirmecofilia, seleção de planta hospedeira, e benefícios da co-ocorrência com hemípteros trofobiontes. O ciclo de desenvolvimento de ovo a adulto é de aproximadamente 36 dias, e o estágio larval compreende quatro instares. Os ovos são depositados exclusivamente em tecidos reprodutivos (botões florais) das plantas hospedeiras. As larvas são polífagas, sendo registradas em 28 espécies em 16 famílias de plantas. A maioria da plantas hospedeiras de *P. polibetes* (78.57%) apresenta algum tipo de fonte de alimento líquido que promovem a visitação por formigas, sejam nectários extraflorais e/ou hemípteros produtores de exudatos. A partir do terceiro instar, as larvas são atendidas facultativamente por mais de quinze espécies de formigas em três subfamílias (Formicinae, Myrmicinae e Ectatomminae), principalmente formigas do gênero *Camponotus* Mayr. Assim como em outros Lycaenidae, as interações entre larvas e formigas são mediadas principalmente por uma glândula especializada (*dorsal nectar organ*) no sétimo segmento

abdominal que produz recompensas calóricas para as formigas. Nesse sentido, é esperado que a produção dessas secreções acarrete em custos para as larvas. Para *P. polibetes*, é demonstrado que as formigas *Camponotus crassus* e *Camponotus melanoticus* apresentam diferentes intensidades de atendimento. *C. melanoticus* atende mais intensamente as larvas que *C. crassus* em condições de laboratório. Por sua vez, essa diferença pode acarretar em diferentes custos para as larvas. Por exemplo, quando atendidas por *C. melanoticus* demoram mais tempo para empupar. No entanto, o peso pupal e o tamanho dos adultos não são afetados pela diferença de atendimento, sugerindo que *P. polibetes* possui mecanismos compensatórios para minimizar os custos da mirmecofilia. Esta é a primeira demonstração de que diferenças específicas de intensidade de atendimento podem afetar parâmetros de desempenho de um inseto trofobionte. Em campo, é demonstrado através de experimentos pareados que o padrão previamente detectado de co-ocorrência espacial entre larvas de *P. polibetes* e hemípteros mirmecófilos é provocado por dois fatores: 1) fêmeas são capazes de detectar e ovipositar em plantas com associação membracídeos-formigas; 2) larvas que se desenvolvem perto da associação membracídeos-formigas sobrevivem melhor que larvas em plantas sem associação. Tal efeito ocorre porque a presença da interação entre membracídeos e formigas reduz a abundância de potenciais inimigos naturais das larvas (aranhas e vespas parasitóides). Além disso, as larvas são mais facilmente encontradas e atendidas pelas formigas que são recrutadas pelos membracídeos. Ou seja, a presença da associação membracídeos-formigas gera um “espaço livre de inimigos” sobre a planta hospedeira, que é explorado por *P. polibetes*. Esses resultados mostram que o enfoque tradicional no estudo de mutualismo, baseado em pares de espécies, é inapropriado para entender as pressões seletivas operando em sistemas multitróficos.

INTRODUÇÃO GERAL

O Cerrado cobria originalmente uma área de mais de dois milhões de km², ao longo do Brasil central, correspondendo a aproximadamente 21% do território brasileiro (ver revisão em Oliveira & Marquis 2002), sendo a mais extensa formação savânica da América do Sul, apresentando uma grande riqueza de espécies, um alto grau de endemismo, e considerado um *hotspot* de diversidade (Mittermeier *et al.* 2005). A vegetação é composta por um mosaico de diferentes fitofisionomias, incluindo campo limpo, campo sujo, campos de murundus, cerrado *sensu stricto*, cerradão, matas de galerias e matas secas (Oliveira-Filho & Ratter 2002). O cerrado *sensu stricto* é caracterizado por apresentar árvores baixas, tortuosas, com ramificações irregulares e retorcidas, com casca grossa e geralmente com evidências de queimadas.

Nos últimos 30 anos, a importância biológica do Cerrado tem sido reconhecida, e o número de pesquisas acadêmicas concernentes a este bioma tem aumentado consideravelmente, principalmente nas disciplinas de Botânica, Zoologia e Ecologia (Oliveira & Marquis 2002). Uma característica emergente do cerrado que surge da conexão entre estas três áreas do conhecimento é a riqueza de interações entre plantas, formigas e herbívoros (ver Oliveira *et al.* 2002; Del-Claro 2004; Oliveira & Freitas, 2004). Segundo Oliveira *et al.* (2002), os principais fatores envolvidos neste padrão são a riqueza de formigas associadas às fontes de alimento líquido (nectários extraflorais, excreções de hemípteros e/ou secreções de lepidópteros mirmecófilos), que promovem a visitação de formigas sobre a vegetação. Nesse sentido, Brown & Gifford (2002) sugerem que a grande proporção de endemismo em alguns grupos de borboletas mirmecófilas em relação à não mirmecófilos no cerrado seria resultado desta diversidade de interações.

Tal riqueza de interações é o pano de fundo desta Tese de Doutorado, que se aprofunda no sistema que envolve o hemíptero mirmecófilo *Guayaquila xiphias* Fabr. (Membracidae) e sua planta hospedeira *Schefflera vinosa* March. (=*Didymopanax vinosum*) (Araliaceae). Esse sistema vem sendo estudado há muitos anos, tendo sido tema de teses de Mestrado e Doutorado em Ecologia na Unicamp (*e.g.* Del-Claro 1995, Quental 2002, Silveira 2008). A história deste sistema começa com uma Tese de Mestrado (Lopes 1984), que estudou aspectos da ecologia de membracídeos em vegetação de cerrado do estado de São Paulo. Nesta tese, foi observado que a espécie *G. xiphias* era relativamente abundante e ocorria quase exclusivamente sobre *S. vinosa* (ver também Lopes 1995). Posteriormente, Dansa & Rocha (1992) estudaram aspectos deste membracídeo, tais como uso e frequência sobre as plantas hospedeiras e correlações com herbivoria. O grande avanço no conhecimento sobre esse sistema veio com a Tese de Doutorado de Del-Claro (1995), que investigou várias questões relativas à interação entre *G. xiphias* e formigas atendentes (Del-Claro & Oliveira 1993, 1996, 1999, 2000). Foi demonstrado que as agregações desse membracídeos são atendidas “fielmente” dia/noite por várias espécies de formigas (Del-Claro & Oliveira 1993, 1999). As formigas localizam as agregações através de gotas de exsudação que caem no solo (Del-Claro & Oliveira 1996). A presença de formigas atendentes afeta positivamente a sobrevivência e fecundidade dos membracídeos, através de transferência de cuidado parental e redução da abundância de inimigos naturais (moscas Syrphidae, vespas parasitóides e aranhas Salticidae) (Del-Claro & Oliveira 2000). Similar a outros sistemas mutualistas facultativos (ver Bronstein 1994), o benefício da interação entre formigas e membracídeos é condicional (Del-Claro & Oliveira 2000, Quental *et al.* 2005), mas estável em uma escala de tempo maior. Ainda neste sistema, foi demonstrado recentemente que ninfas e adultos de *G. xiphias* possuem camuflagem química que impede a detecção e ataque por formigas que as atendem (Silveira *et al.* 2010).

Um dos aspectos mais relevantes deste sistema, é que a constante atividade de formigas sobre a planta hospedeira promove uma série de efeitos diretos e indiretos no contexto de comunidade (Fig. 1). Foi demonstrado que a presença de *G. xiphias* em *S. vinosa* aumenta o patrulhamento por formigas e diminui a herbivoria por lepidópteros minadores, besouros, e tripes sugadores (Oliveira & Del-Claro 2005). Por outro lado, foi notado um aumento na abundância de larvas da borboleta mirmecófila *Parrhasius polibetes* (Stoll) (Lycaenidae) nas inflorescências de *S. vinosa* que apresentavam associação *G. xiphias* e formigas (Oliveira & Del-Claro 2005). Em outras palavras, foi encontrado um claro padrão de co-ocorrência espaço-temporal entre larvas de *P. polibetes* e *G. xiphias*, que poderia ser explicado tanto por seleção de planta hospedeira mediada pela presença da associação, quanto por sobrevivência diferencial das larvas na presença de formigas (ver Kaminski 2008).

O projeto da presente Tese de Doutorado foi concebido e motivado pelas questões geradas nos estudos citados acima, e também pela oportunidade de estudar um sistema borboleta-formiga que fosse abundante e manipulável experimentalmente. Em adição, o sistema oferece uma oportunidade rara de estudar inter-relações entre sistemas mutualistas, no caso um sistema borboleta-formiga e outro membracídeo-formiga. Além disso, com exceção de poucos registros esparsos de planta hospedeira e o padrão de co-ocorrência com *G. xiphias*, nada era conhecido sobre a biologia e morfologia de *P. polibetes*. Assim, este estudo buscou investigar aspectos da

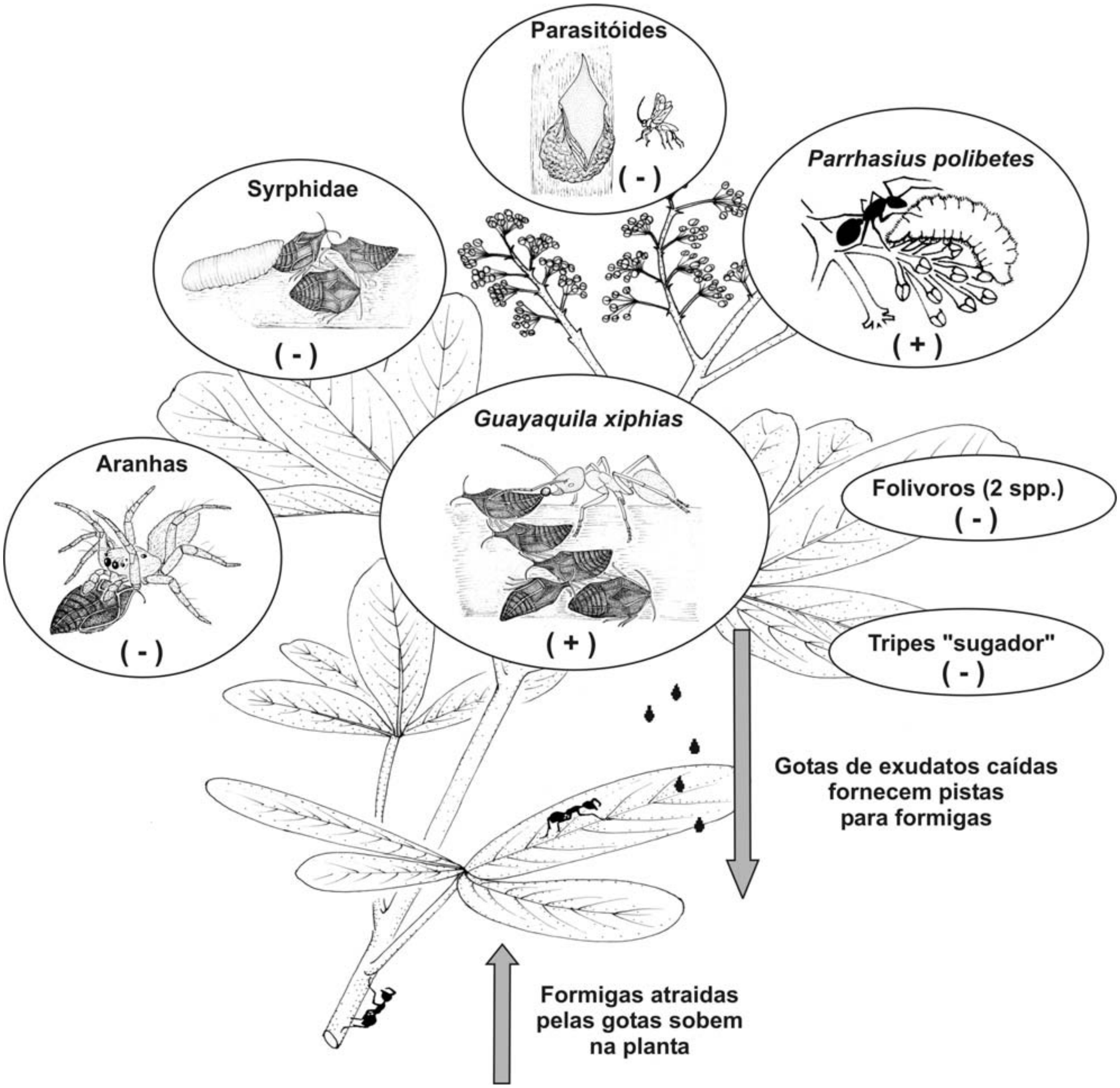

Figura 1. Representação esquemática do sistema multitrófico que envolve o membracídeo *Guayaquila xiphias* e formigas atendentes sobre a planta *Schefflera vinosa* em áreas de cerrado. As formigas são atraídas por gotas de exudatos dos membracídeos que caem no substrato. Formigas afetam negativamente os inimigos naturais dos membracídeos (aranhas, moscas sirfídeas e parasitóides) e herbívoros não mirmecófilos (folívoros e tripes). Por outro lado, os herbívoros mirmecófilos (*G. xiphias* e *Parrhasius polibetes*) são afetados positivamente pela presença de formigas. Sinal entre parênteses indica o efeito da formiga sobre o participante do sistema. Modificado de Oliveira & Del-Claro (2005).

morfologia, história natural e ecologia para melhor compreender a evolução e manutenção da mirmecofilia em *P. polibetes* e as conseqüências na seleção da planta hospedeira em um sistema de interações multitróficas.

Objetivos gerais:

1) Investigar aspectos da história natural de *P. polibetes* em ambiente de cerrado, tais como, plantas hospedeiras, formigas atendentes, potenciais inimigos naturais e co-ocorrência com hemípteros mirmecófilos.
2) Descrever a morfologia dos estágios imaturos de *P. polibetes* com ênfase nos órgãos associados à mirmecofilia.
3) Identificar os custos da interação com formigas em laboratório frente a duas espécies diferentes de formigas que comumente atendem as larvas.
4) Investigar experimentalmente o papel da presença de interações membracídeos-formigas na seleção de plantas hospedeiras.
5) Analisar o efeito da presença de associações membracídeos-formigas na sobrevivência larval de *P. polibetes* em campo.
6) Avaliar os efeitos da ocorrência de agregações de membracídeos e formigas atendentes sobre a abundância de potenciais inimigos naturais das larvas.

A tese esta dividida em quatro capítulos. O **Capítulo 1** apresenta uma revisão em língua portuguesa a respeito do papel das formigas sobre a vegetação na evolução da morfologia e comportamento de larvas de Lepidoptera. O **Capítulo 2** apresenta informações sobre a história natural e morfologia das larvas de *P. polibetes*. Tais informações serviram de base para o entendimento do sistema de estudo e desenvolvimento dos experimentos nos dois capítulos subseqüentes. Além disso, são discutidos possíveis aspectos relacionados à evolução da polifagia e florivoria nessa borboleta (ver também Rodrigues *et al.* 2010). O **Capítulo 3** avalia em laboratório os custos da mirmecofilia em *P. polibetes* frente ao atendimento por duas espécies de formigas. Os custos são acessados e comparados através de parâmetros de história de vida e são relacionados com diferenças comportamentais entre as duas espécies de formigas. Finalmente, o **Capítulo 4** se aprofunda nos fatores relacionados ao padrão de co-ocorrência entre larvas de *P. polibetes* e membracídeos em *S. vinosa*. Por meio de experimentos pareados foram avaliadas as pistas utilizadas na seleção de planta hospedeira pelas fêmeas e o efeito da presença da associação membracídeos-formigas na sobrevivência larval e abundância de potenciais inimigos naturais. Nos **Anexos** são apresentados dois estudos paralelos que foram desenvolvidos ao longo da Tese. Nestes trabalhos são descritos os estágios imaturos de duas espécies de Lycaenidae e são discutidos temas que estão diretamente conectados com a Tese. Como por exemplo, a relação entre a morfologia das larvas de licenídeos e a mirmecofilia, bem como, o papel da fenologia das plantas hospedeiras na evolução da polifagia e/ou oligofagia em Eumaeini.

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CAPÍTULO I

ECOLOGIA COMPORTAMENTAL NA INTERFACE FORMIGA-PLANTA-HERBÍVORO: INTERAÇÕES ENTRE FORMIGAS E LEPIDÓPTEROS*

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RESUMO

Formigas constituem um dos mais proeminentes grupos de organismos terrestres em termos de diversidade, abundância relativa e biomassa animal. Sua importância se deve principalmente ao comportamento eusocial aliado a complexos sistemas de comunicação, que permitem às formigas recrutar companheiras e defender recursos com grande eficiência. A vegetação de áreas tropicais é rica em fontes de alimentos renováveis que induzem a visitação freqüente de formigas às plantas. Sobre a vegetação, as formigas podem atuar como predadoras e acarretar um forte efeito sobre a comunidade de insetos herbívoros, estruturando redes tróficas e promovendo efeitos em cascata. A presença de formigas sobre plantas pode afetar insetos herbívoros basicamente de duas formas: (1) limitando sua ocorrência na folhagem através de interações antagônicas (ex. agressão, predação) ou (2) propiciando espaços livres de inimigos naturais para herbívoros mirmecófilos (que mantêm associações com formigas). Neste artigo revisamos e discutimos os cenários ecológicos onde estas interações podem ocorrer, com especial atenção aos potenciais efeitos de formigas sobre a biologia e o comportamento de larvas de Lepidoptera.

Palavras-chave: Espaço livre de inimigos, Formicidae, interações multitróficas, efeitos indiretos, Lycaenidae, mirmecofilia, Nymphalidae, Riodinidae.

ABSTRACT

BEHAVIORAL ECOLOGY AT THE ANT-PLANT-HERBIVORE INTERFACE: INTERACTIONS BETWEEN ANTS AND LEPIDOPTERANS. Ants are one of the most prominent groups of terrestrial organisms in terms of diversity, relative abundance, and biomass. Their importance is due primarily to eusociality combined with complex communication systems, which enable them to recruit nestmates to capture prey and/or protect profitable resources. Tropical foliage is rich in renewable food sources that promote visitation by ants. Because they are the principle predators among foliage, ants can strongly affect the communities of herbivorous insects and promote trophic cascades with relevant consequences to plants. The presence of ants on foliage can affect herbivores in two ways: (1) ant foragers can decrease the number of herbivores on plants through antagonistic interactions (e.g. aggressiveness, predation), (2) ants can create an enemy-free space for myrmecophilous herbivores (*i.e.* those living in close association with ants). Here, we discuss the ecological scenario in which these interactions occur, and examine the effects of foliage-dwelling ants on the biology and behavior of lepidopteran larvae.

Keywords: Enemy-free space, Formicidae, indirect effects, Lycaenidae, multitrophic interactions, Nymphalidae, myrmecophily, Riodinidae.

RESUMEN

ECOLOGIA DEL COMPORTAMIENTO EN LA INTERFACE HORMIGA-PLANTA-HERBÍVORO: INTERACCIONES ENTRE HORMIGAS Y LEPIDÓPTEROS. Las hormigas constituyen uno de los grupos de organismos terrestres más relevantes en términos de diversidad, abundancia relativa y biomasa animal. Su importancia se debe, principalmente, al comportamiento eusocial asociado a complejos sistemas de comunicación que permiten a las hormigas reclutar individuos y defender recursos con gran eficiencia. La vegetación de las regiones tropicales es rica en fuentes de alimento renovables, los cuales inducen la visita frecuente de hormigas a las plantas. En la vegetación, las hormigas pueden actuar como depredadores, generando un fuerte efecto sobre las comunidades de insectos herbívoros, estructurando las redes tróficas y promoviendo efectos en cascada. La presencia de hormigas sobre las plantas puede afectar a los insectos herbívoros, básicamente de 2 maneras: (1) limitando su presencia en el follaje a través de interacciones antagónicas (e.g. agresión, depredación) o (2) propiciando espacios libres de enemigos naturales en el caso de los herbívoros mirmecófilos (los cuales mantienen asociaciones con hormigas). En este trabajo, revisamos y discutimos los escenarios ecológicos donde estas interacciones pueden ocurrir, en especial, sobre los efectos potenciales de las hormigas en la biología y el comportamiento de larvas de Lepidoptera.

Palabras clave: Espacio libre de enemigos, Formicidae, interacciones multitróficas, efectos indirectos, Lycaenidae, mirmecofilia, Nymphalidae, Riodinidae.

FORMIGAS EM ECOSSISTEMAS E SUA RELAÇÃO COM A VEGETAÇÃO

Formigas são um dos grupos mais proeminentes de organismos da Terra e estão presentes em todos os ecossistemas terrestres, exceto em regiões polares, algumas ilhas oceânicas e grandes altitudes (Ward 2006). Em termos de diversidade, abundância relativa, e impactos ecológicos, as formigas desempenham um papel relevante em muitas comunidades, exibindo várias funções como detritívoros, predadores, granívoros e herbívoros. Em ecossistemas tropicais as formigas são um componente notável, constituindo mais de 15% da biomassa animal total (Beattie & Hughes 2002). Várias características têm sido propostas como responsáveis por essa preponderância ecológica, tais como o comportamento eusocial com operárias sem asas e a presença de uma grande variedade de mecanismos intra-específicos de reconhecimento e comunicação química (Hölldobler & Wilson 1990).

Uma fração significativa das atividades de forrageamento das formigas é realizada utilizando as plantas como substrato (Rico-Gray & Oliveira 2007). A biomassa e abundância de formigas sobre a folhagem em ecossistemas tropicais são especialmente altas quando comparada com outros habitats (Kaspari 2003), chegando a 50-94% da abundância e 70-86% da biomassa de artrópodes (Majer 1990, Tobin 1991, Dejean *et al.* 2000).

INTERAÇÕES PLANTA-FORMIGA E SEUS EFEITOS SOBRE HERBÍVOROS

A extraordinária abundância de formigas sobre a vegetação tem sido explicada pela predominância de espécies que funcionalmente atuam como herbívoros, devido a sua íntima associação com recursos líquidos derivados de plantas (Tobin 1991, Davidson 1997, Davidson *et al.* 2003). A vegetação nos trópicos é rica em fontes de alimentos renováveis que podem potencializar a visitação por formigas (Figura 1; revisado por Rico-Gray & Oliveira 2007). A mais conhecida destas fontes são as glândulas produtoras de néctar, não relacionadas diretamente à polinização, chamadas coletivamente de nectários extraflorais (NEFs) (Figura 1a) (Koptur 1992). Estas estruturas são amplamente distribuídas nas floras de diversos tipos de vegetações, incluindo florestas tropicais (Schupp & Feener 1991, Bluthgen & Reifenrath 2003) e savanas (Oliveira & Leitão-Filho 1987, Machado *et al.* 2008). Os NEFs são registrados para mais de 66 famílias de angiospermas e pteridófitas (Rico-Gray & Oliveira 2007), sendo encontrados em 18 a 53% das espécies lenhosas em fitofisionomias na Amazônia (Morellato & Oliveira 1991), e de 15,4 a 25,5% em áreas de cerrado (Oliveira & Leitão-Filho 1987). Além dos NEFs, um outro tipo de recurso alimentar oferecido por plantas para atrair formigas são os chamados corpúsculos alimentares (“*food bodies*”) (Figura 1b), presentes principalmente em espécies mirmecófitas (plantas que possuem órgãos especializados para abrigar colônias de formigas; ver Beattie 1985). Frutos também eventualmente podem agir como atrativos para formigas (Machado & Freitas 2001, Dutra *et al.* 2006). Outros tipos importantes de alimentos líquidos para formigas são fornecidos por herbívoros mirmecófilos (que mantêm associações com formigas), tais como exsudatos de hemípteros (Figura 1c) (ver Buckley 1987, Del-Claro & Oliveira 1999, Stadler & Dixon 2005), e secreções de larvas de lepidópteros (Figura 1d) (Fiedler 1991, Pierce *et al.* 2002).

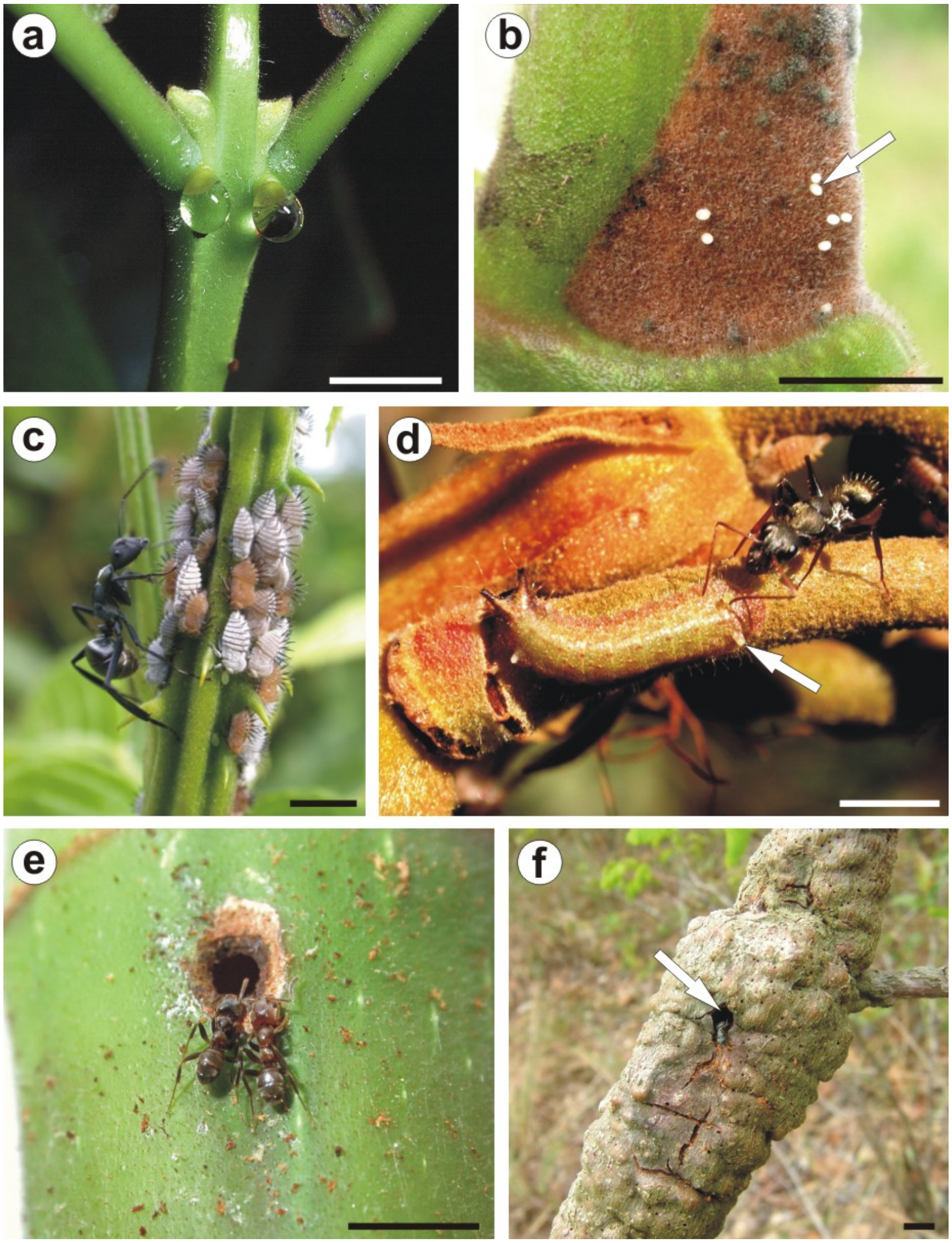

Figura 1. Exemplos de recursos sobre a vegetação que potencializam a visitação por formigas. (a) Nectário extrafloral em *Qualea grandiflora* (Vochysiaceae); (b) corpúsculos alimentares ("food bodies") em *Cecropia pachystachya* (Cecropiaceae); (c) *Camponotus sericeiventris* atendendo agregação de membracídeos; (d) *Camponotus* sp. atendendo uma larva de *Synargis* sp. (Riodinidae); note as glândulas nectaríferas evertidas (seta); (e) Operárias de *Azteca* sp. adentrando um internó de *Cecropia pachystachya*, onde reside a colônia; (f) *Camponotus* sp. utilizando uma galha abandonada em *Caryocar brasiliense* (Caryocaraceae) para nidificação. Escalas = 0,4 cm.

Além de recursos alimentares, um atributo importante para o aumento da incidência de formigas sobre a vegetação é a presença de sítios adequados para nidificação, como em inúmeras espécies de mirmecófitas que possuem estruturas especializadas que permitem a colonização por formigas (Figura 1e) (Hölldobler & Wilson 1990). Além disso, as formigas são oportunistas com relação à utilização de espaços em plantas gerados pela atividade de outros insetos, e qualquer cavidade pode servir como local de nidificação, como túneis criados em galhos por besouros brocadores (Oliveira & Freitas 2004), ou até mesmo galhas abandonadas (Figura 1f) (Araújo *et al.* 1995).

Apesar dos recursos fornecidos pelas plantas constituírem o principal item alimentar de muitas formigas arborícolas, muitas espécies podem também se comportar como predadoras oportunistas (Floren *et al.* 2002, Davidson *et al.* 2003), ou mesmo com alto grau de especialização (Morais 1994). Considerando essa dominância sobre a folhagem, é razoável pensar que as formigas devam exercer um forte impacto sobre a biologia de insetos herbívoros. Embora as interações específicas entre plantas e formigas sejam raras e restritas, interações facultativas e/ou oportunistas podem ser determinantes em ecossistemas tropicais, promovendo a estruturação de redes tróficas e efeitos em cascata (Dyer & Letourneau 1999, Heil & Mckey 2003, Rico-Gray & Oliveira 2007, e referências incluídas).

A atividade de formigas arbóreas pode influenciar a composição da fauna de artrópodes sobre as árvores em florestas tropicais, afetando fortemente seus efeitos sobre as plantas (Floren *et al.* 2002). Existem evidências de que o comportamento agressivo e predatório das formigas que utilizam recursos fornecidos por plantas pode reduzir efetivamente a abundância e a atividade alimentar de herbívoros, e em muitos casos este efeito aumenta o sucesso reprodutivo das plantas visitadas (Oliveira 1997, Oliveira *et al.* 1999, Sobrinho *et al.* 2002; para mais exemplos ver Rico-Gray & Oliveira 2007). Desta forma, é possível entender a interação entre planta e formiga como um tipo de defesa biótica indireta, comparável com outros tipos de defesas de plantas, como as morfológicas e químicas (Coley & Barone 1996, Agrawal & Rutter 1998, Gianoli *et al.* 2008, Heil 2008).

Este cenário de interação formiga-planta tem sido tratado como um processo coevolutivo a partir do qual pode se explicar o surgimento de estruturas atrativas para formigas em plantas mirmecófilas facultativas, bem como a existência de mirmecófitas especializadas (Janzen 1966). No entanto, alguns estudos têm mostrado que estas associações são frágeis e suscetíveis a espécies "trapaceiras" ("*cheaters*") que desviam os benefícios das interações apenas para um dos lados (Yu & Pierce 1998, Izzo & Vasconcelos 2002).

Os resultados das interações em sistemas multitróficos (planta, formiga e herbívoros), em especial o efeito sobre o sucesso reprodutivo das plantas, pode variar bastante no tempo e no espaço (Rico-Gray & Oliveira 2007). Estas variações frequentemente são dependentes de características das formigas associadas, como comportamento e densidade (Barton 1986, Heil & Mckey 2003, Ness 2003, Mody & Linsenmair 2004). Além disso, a eficiência da defesa dos herbívoros à presença de formigas e a interação com outros organismos pode ser determinante (Price *et al.* 1980, Heads & Lawton 1985, Oliveira *et al.* 2002, Oliveira & Freitas 2004, Mody & Linsenmair 2004). Tais fatores podem explicar a ausência de benefícios para plantas em alguns sistemas estudados que envolvem plantas com nectários extraflorais e formigas (O'Dowd & Catchpole 1983, Mackay & Whalen 1998, Mody & Linsenmair 2004).

Como predadoras generalistas, as formigas podem ser consideradas um dos principais fatores de pressão seletiva sobre insetos herbívoros. Conseqüentemente, elas podem afetar o padrão de utilização de plantas hospedeiras pelos herbívoros, incluindo o grau de especialização, bem como as estratégias de defesa contra predadores (Dyer 1995, Stamp 2001, Singer & Stireman 2003, Coley *et al.* 2006). Basicamente, existem duas conseqüências para insetos herbívoros inseridos em sistemas formiga-planta (Figura 2): (1) a alta freqüência de formigas sobre a folhagem exerce um efeito negativo sobre os herbívoros (através de agressão e/ou predação) e limita a existência de espaços seguros, livre de inimigos naturais (Novotny *et al.* 1999, Floren *et al.* 2002, Oliveira *et al.* 2002); (2) espécies de herbívoros mirmecófilos têm acesso a um espaço livre de inimigos na planta hospedeira por se beneficiarem da proteção oferecida pelas formigas associadas (Atsatt 1981a, Pierce *et al.* 2002). Neste artigo revisamos e discutimos os cenários ecológicos onde podem ocorrer interações antagônicas ou simbióticas entre formigas e herbívoros, com especial atenção aos potenciais efeitos de formigas sobre a biologia e o comportamento de larvas de Lepidoptera. Tendo em vista a dificuldade de se demonstrar o benefício para as formigas na interação com larvas (ver Fiedler & Saam 1995), o termo mutualismo é evitado aqui, e estas interações serão tratadas coletivamente como casos de simbiose ou mirmecofilia.

INTERAÇÕES ANTAGÔNICAS

Numa interação interespecífica antagônica, o efeito positivo no sucesso reprodutivo de uma das espécies participantes resulta num efeito negativo para a outra espécie (ver Bronstein 1994). A

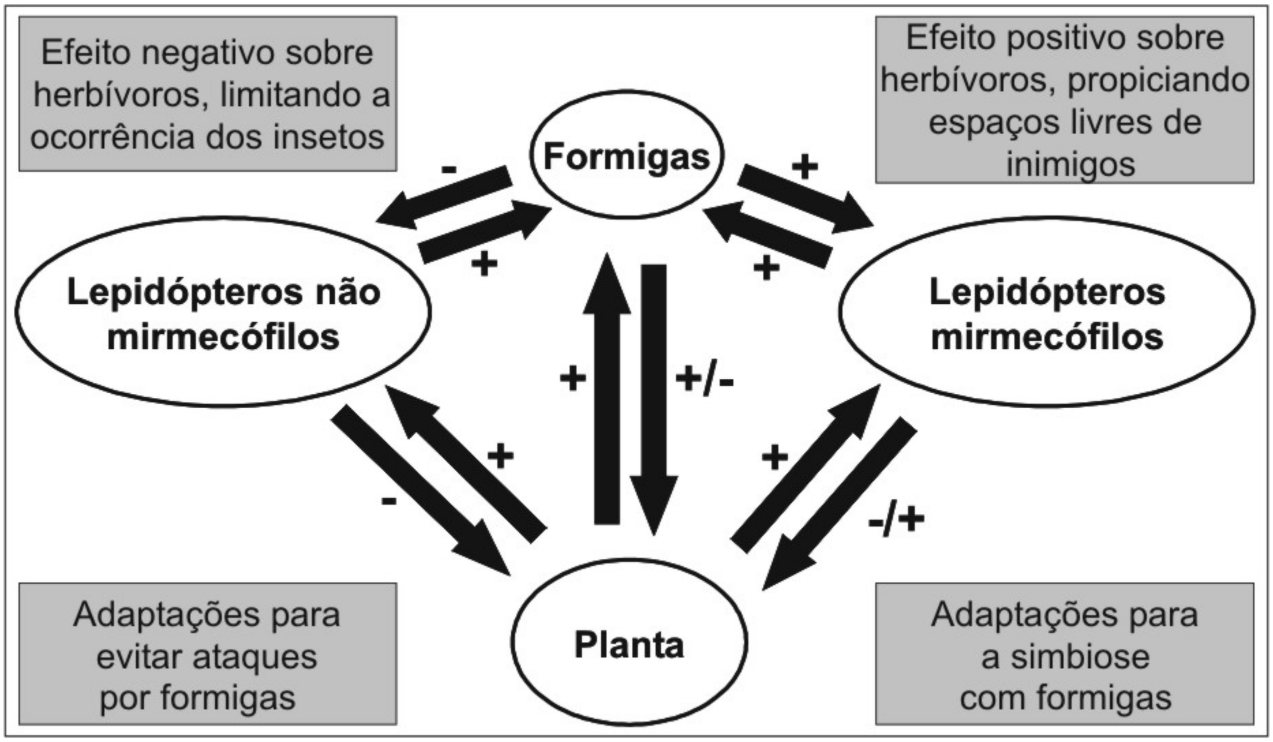

Figura 2. Esquema mostrando o cenário ecológico-evolutivo das interações entre plantas, formigas e lepidópteros. As setas indicam a direção das interações, e os sinais indicam o efeito destas (positivo ou negativo) para o participante afetado. Note que as interações com ambos os sinais indicam que o efeito é variável e pode estar condicionado à influência de outros fatores.

predação claramente se enquadra nesse tipo de interação, e constitui normalmente a maior parte das interações entre formigas e lepidópteros. De fato, juntamente com os pássaros, as formigas são consideradas os principais predadores de larvas de lepidópteros (Scoble 1995, Salazar & Whitman 2001). O efeito predatório das formigas ocorre quase exclusivamente nos estágios imaturos, durante o estabelecimento da lagarta sobre a planta hospedeira (Smiley 1985, Mega & Araújo 2008). Existem estimativas de que uma única colônia de *Formica rufa* (Formicinae) pode predar mais de 400.000 lagartas por ano (Adlung 1966).

A predação é um dois maiores problemas para a sobrevivência de larvas de Lepidoptera, sendo uma das principais causas de mortalidade (Salazar & Whitman 2001, Gentry & Dyer 2002). Estudos experimentais envolvendo a exclusão de formigas têm mostrado uma maior mortalidade larval em plantas visitadas por formigas do que em plantas sem formigas (ex., Sato & Higashi 1987, Freitas & Oliveira 1996, Dutra *et al.* 2006). Por exemplo, de 59 estudos compilados por Rico-Gray & Oliveira (2007) que abordam o efeito de formigas sobre plantas com nectários extraflorais, 25 envolvem efeitos negativos das formigas sobre lepidópteros. Além disso, interações antagônicas entre formigas e lepidópteros podem ser amplificadas pelo efeito de interações indiretas com outros herbívoros (Fukui 2001, Oliveira & Del-Claro 2005, Ando & Ohgushi 2008).

É evidente que se alimentar de espécies de plantas visitadas por formigas pode ser perigoso para insetos herbívoros. Nesse sentido, larvas de lepidópteros desenvolveram uma série de defesas para utilizar plantas deste tipo (Figura 3). Salazar & Whitman (2001) fizeram uma completa revisão de possíveis estratégias de larvas de Lepidoptera contra predadores. Estas defesas podem ser divididas em duas classes: defesas primárias previnem o encontro entre predador e larva, e defesas secundárias previnem o ataque após a detecção da larva por um predador potencial (Gross 1993).

A construção de abrigos é uma estratégia comum em larvas e está presente em pelo menos 18 famílias de Lepidoptera (Gaston *et al.* 1991, Scoble 1995, Lill *et al.* 2007). Diversos trabalhos têm demonstrado a eficiência destes abrigos aumentando a sobrevivência larval na presença de formigas (Heads & Lawton 1985, Bernays & Cornelius 1989, Vasconcelos 1991, Loeffler 1996, Jones *et al.* 2002, Mega & Araújo 2008), inclusive em mirmecófitas (Eubanks *et al.* 1997). Além de prevenir a detecção da larva por formigas predadoras, os abrigos podem também limitar o acesso das formigas quando as larvas são detectadas (Jones *et al.* 2002). Os abrigos (Figura 3a-b)

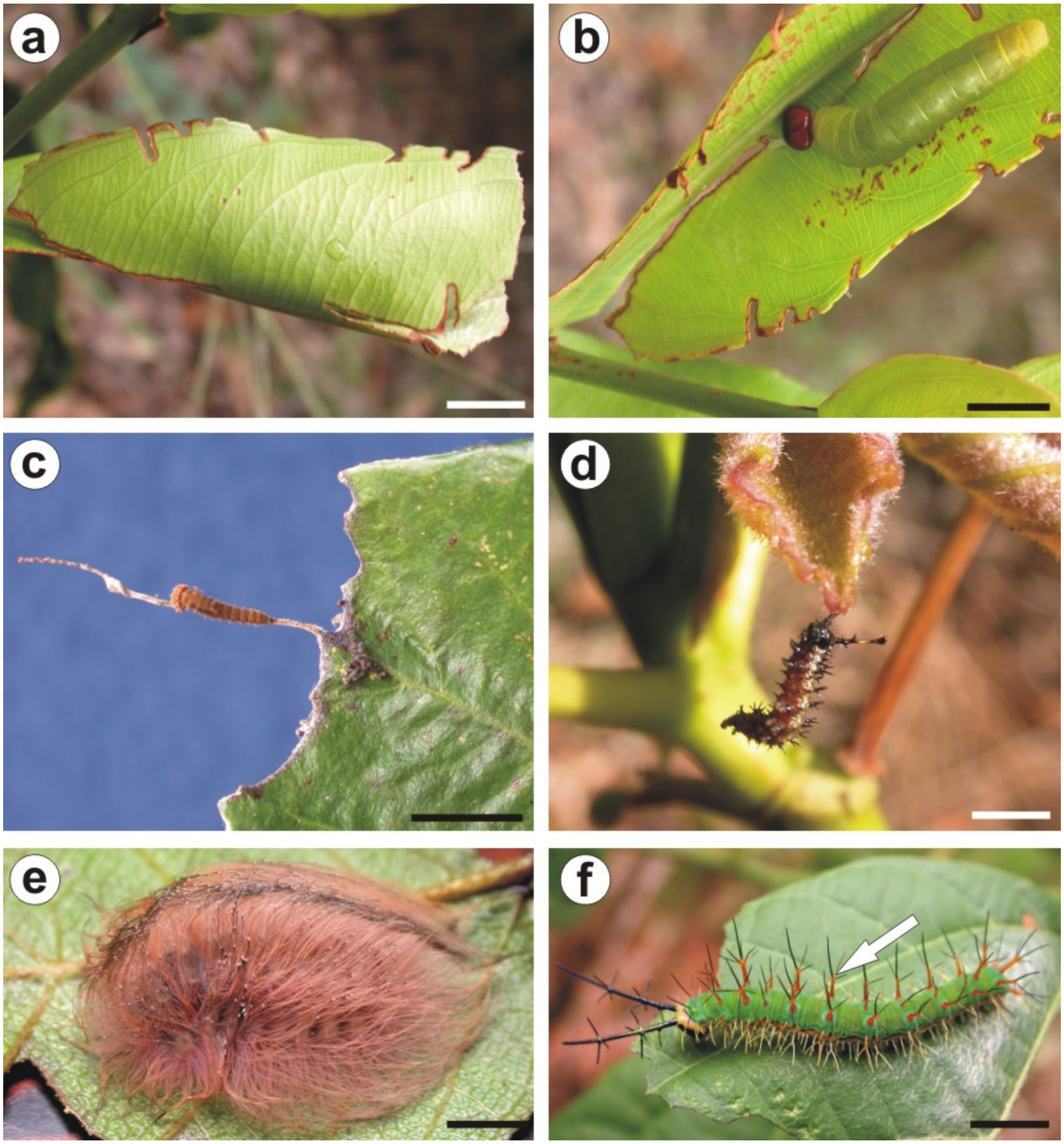

Figura 3. Exemplos de defesas em larvas de Lepidoptera. (a-b) Abrigo em forma de canudo construído por larva de *Udranomia spitzi* (Hesperiidae), e folha aberta evidenciando a presença da larva; (c) ponte de fezes contruída por larva de *Adelpha lycorias* (Nymphalidae) a partir da nervura central da folha; (d) larva de *Eunica bechina* (Nymphalidae) pendurada em folha (comportamento de "dropping") após ataque por formigas na superfície foliar; (e) larva de Megalopigydae ilustrando a grande quantidade de cerdas urticantes; (f) larva de *Catonephele acontius* (Nymphalidae) ilustrando escolos desenvolvidos (seta). Escalas = 0,4 cm.

variam quanto ao grau de complexidade, sendo construídos basicamente com material vegetal, fezes ou fragmentos diversos conectados com fios de seda (Salazar & Whitman 2001, Lill *et al.* 2007).

A construção de abrigos pode implicar no acúmulo de produtos do metabolismo da larva, em especial fezes. Estes produtos podem limitar o espaço disponível, propiciar a proliferação de patógenos, ou atrair inimigos naturais (Weiss 2003, 2006). Como solução, diversas espécies desenvolveram estratégias para limpeza, como expelir as fezes a longas distâncias ou simplesmente remover os dejetos com a mandíbula (Caveney *et al.* 1998, Weiss 2006). Formigas podem responder negativamente às fezes de suas presas. Por outro lado, as fezes acumuladas próximo ao abrigo podem também aumentar a eficiência deste como uma barreira mecânica contra formigas em alguns casos (Vasconcelos 1991).

Em algumas larvas de borboletas é comum a construção de "pontes" formadas por fezes e seda nas margens das folhas, às vezes deixando o final da nervura da folha intacta (Figura 3c) (Freitas & Oliveira 1992, Freitas 1999, Machado & Freitas 2001). A larva nos ínstares iniciais permanece sobre esta ponte enquanto não está se alimentando. Para *Eunica bechina* e *Smyrna blomfildia* (Nymphalidae) tem sido demonstrado que estas pontes diminuem a probabilidade de encontro da larva pela formiga, constituindo um efetivo mecanismo de defesa contra predação ou ataques por formigas (Freitas & Oliveira 1996, Machado & Freitas 2001).

Existem vários tipos de respostas comportamentais que as larvas podem exibir quando detectadas e que podem permitir sua sobrevivência após um encontro com o predador. Isto inclui morder o predador potencial, debater-se, regurgitar, e/ou atirar-se da folha e permanecer pendurada por um fio de seda (Salazar & Whitman 2001). Este último tipo de comportamento pode ser uma resposta efetiva ao ataque por formigas (Heads & Lawton 1985, Freitas & Oliveira 1992, Sugiura & Yamazaki 2006). A regurgitação é considerada um dos modos mais simples de defesa química quando a larva é perturbada (Salazar & Whitman 2001), e em muitos casos tem sido demonstrado que esta pode ter um efeito repelente para formigas (Freitas & Oliveira 1992, Smedley *et al.* 1993, Gentry & Dyer 2002). Muitas dessas defesas comportamentais são mais efetivas quando utilizadas conjuntamente. Por exemplo, o comportamento de se debater violentamente quando combinado com mordidas e regurgitação pode aumentar a possibilidade de sobrevivência da larva quando atacada por formigas (Dyer 1995).

A combinação de determinadas propriedades químicas do corpo da larva também pode ser uma característica determinante da probabilidade desta ser predada. Dyer (1995) comparou experimentalmente a importância relativa de diferentes tipos de defesa, constatando que a composição química da larva é a característica mais importante como previsora da rejeição por formigas. Isto é importante se consideramos que grande parte das características químicas das larvas de Lepidoptera é resultado do seqüestro de substâncias do metabolismo secundário das plantas hospedeiras (Dyer & Bowers 1996), ou mesmo sintetizadas dentro do corpo das larvas a partir de precursores obtidos das plantas (Trigo 2000). Desta forma, características químicas das plantas hospedeiras podem ser determinantes na susceptibilidade das larvas à predação por formigas (Coley *et al.* 2006). As propriedades químicas externas das larvas também podem ser importantes na detecção das larvas pelas formigas. Recentemente, foi demonstrado que os hidrocarbonetos cuticulares das larvas de *Mechanitis polymnia* (Nymphalidae) apresentam um padrão muito similar ao da sua planta hospedeira (Portugal & Trigo 2005). Esta similaridade pode ser considerada como uma forma de camuflagem química, uma vez que as larvas se tornam indetectáveis pelas formigas devido a sua semelhança com o substrato.

Muitas larvas de lepidópteros apresentam especializações epidérmicas relacionadas à defesa. As adaptações variam desde cerdas simples a estruturas mais complexas em forma de espinho (escolos), ou até mesmo glândulas especializadas que secretam substâncias nocivas aos seus potenciais predadores e parasitóides (ver Stehr 1987, Salazar & Withman 2001). Para avaliar a natureza destas defesas (se químicas ou mecânicas) e contra quem elas são realmente eficientes (se contra vertebrados ou invertebrados) são necessários estudos morfológicos, anatômicos, e bioensaios comportamentais. Entretanto, poucos estudos têm mostrado a relevância efetiva destas defesas contra formigas (mas veja Honda 1983, Osborn & Jaffé 1998, Shiojiri & Takabayashi 2005). Uma boa pista para entender o papel destas estruturas epidérmicas na defesa contra formigas pode ser obtida através da comparação entre larvas que apresentem interações simbióticas com formigas (mirmecófilas) e espécies aparentadas que não apresentem tal simbiose (Figura 4) (Kaminski 2008b). Neste último caso, as larvas não mirmecófilas possuem cerdas longas e plumosas ou escolos sobre o corpo (Figura 4a, c), que muitas vezes reagem a estímulos mecânicos e são evitados por formigas (DeVries 1991a, Kaminski 2008b). Larvas mirmecófilas, entretanto, geralmente apresentam tegumento liso com cerdas curtas (Figura 4b, d).

Um padrão comumente observado para muitos grupos de insetos fitófagos, e particularmente em Lepidoptera, é uma tendência à especialização em determinados grupos de plantas (Ehrlich & Raven 1964, Bernays & Graham 1988). Alguns trabalhos têm sugerido que a pressão dos inimigos

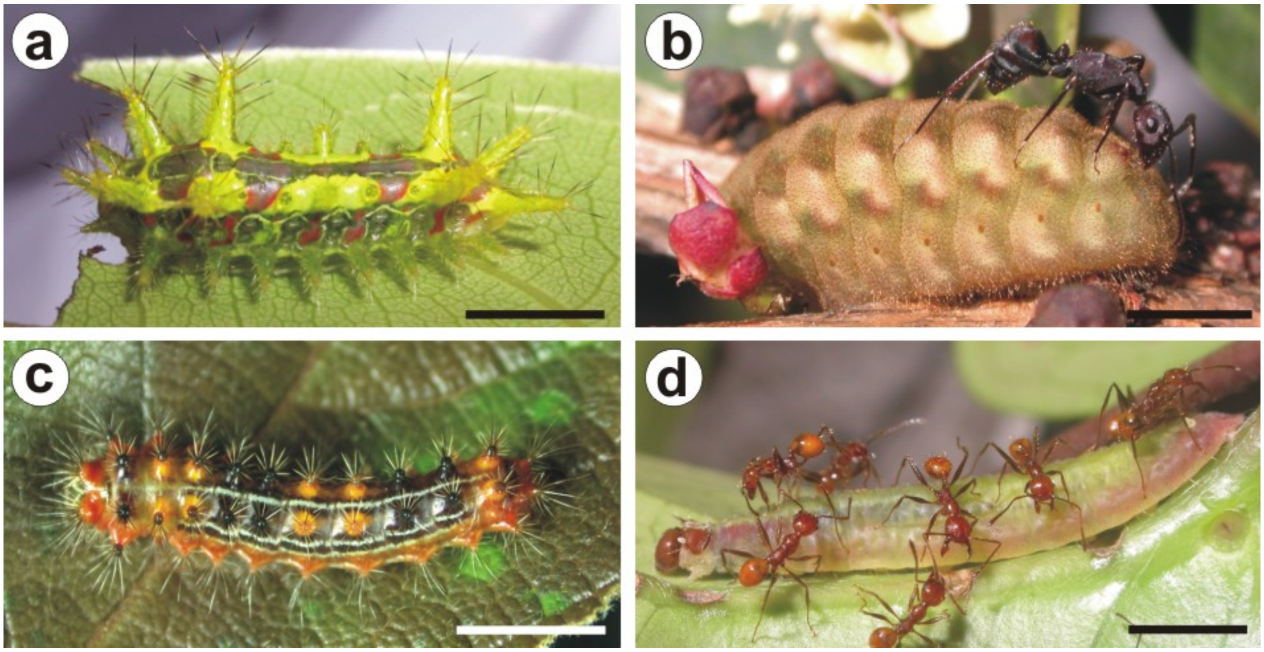

Figura 4. Larvas de Lycaenidae (a-b) e Riodinidae (c-d), ilustrando as diferenças entre espécies não mirmecófilas e mirmecófilas para ambas as famílias. Note as cerdas e escolos bem desenvolvidos nas larvas das espécies não mirmecófilas, (a) *Kolana* sp. e (c) *Emesis* sp.; (b) *Parrhasius polibetes* sendo atendida por operária de *Camponotus leydigi*; (d) *Nymphidium* sp. sendo atendida por um grupo de *Pheidole* sp. Escalas = 0,4cm.

naturais seja um fator chave na evolução do uso de plantas hospedeiras (Bernays & Cornelius 1989, Stamp 2001, Singer & Stireman 2003). Por exemplo, Jolivet (1991) observou que a assembléia de herbívoros associados à mirmecófitas tende a ser mais especializada do que assembléias encontradas em plantas que não apresentam interações com formigas. Uma possibilidade para explicar esse padrão é que as formigas são mais eficientes na captura de insetos generalistas, possivelmente devido à presença de defesas mais eficientes nos insetos especialistas (Heads & Lawton 1985, Dyer 1997).

O desempenho e sobrevivência das larvas podem ser influenciados pela probabilidade de encontrar inimigos sobre a planta hospedeira (Thompson 1988). Além disso, a hierarquia de preferência na seleção de planta hospedeira pela fêmea pode ser determinada em parte pela existência de espaços livre de inimigos (Ohsaki & Sato 1994, Oppenheim & Gould 2002). Em Lepidoptera a seleção da planta hospedeira ocorre no momento da oviposição, e a suscetibilidade dos ovos e larvas à predação por formigas pode ser fortemente influenciada pelo lugar onde a fêmea depositou seus ovos (Rashbrook *et al.* 1992, Nylin & Janz 1999). Nesse sentido, tem sido observado que a presença de formigas pode mediar a seleção da planta hospedeira, e no caso de lepidópteros não mirmecófilos tem sido observado um efeito inibidor de formigas na oviposição (Freitas & Oliveira 1996, Sendoya *et al*. 2009).

INTERAÇÕES SIMBIÓTICAS

Diversos grupos de organismos conseguem conviver com formigas como simbiontes, sendo defendidos ou até mesmo alimentados como um membro da colônia – essa relação é denominada mirmecofilia (Hölldobler & Wilson 1990). A mirmecofilia ocorre devido à habilidade desses simbiontes em mimetizar sinais químicos, morfológicos, e/ou comportamentais utilizados na comunicação intraespecífica pelas formigas (Hölldobler & Wilson 1990). Estas associações variam de facultativas a obrigatórias, e do mutualismo ao parasitismo (Hölldobler & Wilson 1990, Pierce *et al.* 2002, Hojo *et al.* 2008). Independente do tipo de associação, os simbiontes obtêm uma série de benefícios ao coexistirem com formigas, e várias adaptações foram desenvolvidas para manter estas interações. Nesse sentido, a história evolutiva destes organismos, incluindo eventos de especiação e diversificação são amplamente explicados por suas interações com formigas (Atsatt 1981a, Pierce 1984, Eastwood *et al.* 2006).

Existem vários registros de interações simbióticas entre larvas de Lepidoptera e formigas (ver revisão em Hölldobler & Wilson 1990), mas essas interações são bem documentadas e conhecidas em apenas duas famílias de borboletas: Lycaenidae e Riodinidae (Figura 4b, d). Por esta razão, trataremos aqui apenas o que é conhecido para estes dois grupos. Estas interações podem ser divididas em dois tipos: facultativa ou obrigatória (ver Fiedler 1991, Pierce *et al.* 2002). Nas interações facultativas não existe especificidade com relação à formiga, e as larvas podem sobreviver com ou sem formigas atendentes. Por outro lado, nas interações obrigatórias existe especificidade com relação à formiga atendente, as larvas sempre são encontradas com formigas, e existe uma dependência da interação para a sobrevivência das larvas no campo. Apesar de existirem muitos casos de interações obrigatórias entre borboletas e formigas, a maior parte das interações é facultativa e o balanço entre custo e beneficio das interações pode variar com diversos fatores, tais como a qualidade nutricional da planta, co-ocorrência com outros simbiontes, bem como a espécie de formiga envolvida (Pierce *et al.* 1991, Robbins 1991, Fiedler & Hölldobler 1992, Fraser *et al.* 2001, L.A.K. dados não publicados).

A natureza destas interações é tida como mutualística, uma vez que as formigas recebem secreções nutritivas produzidas por glândulas especializadas (Newcomer 1912, DeVries & Baker 1989, Daniels *et al.* 2005), e em contrapartida as larvas recebem proteção contra predadores e parasitóides (Pierce & Mead 1981, DeVries 1991b). O beneficio da interação já foi demonstrado para algumas espécies de Lycaenidae e Riodinidae (Pierce & Mead 1981, DeVries 1991b). Do ponto de vista das larvas, o benefício pode ser facilmente evidenciado a partir de experimentos no campo com larvas criadas na presença ou ausência de formigas. Em geral, estes experimentos mostram que larvas atendidas por formigas sobrevivem melhor devido à proteção contra parasitóides e vespas predadoras (Pierce & Mead 1981, Pierce *et al.* 1987, DeVries 1991b). Do ponto de vista da formiga, o benefício foi demonstrado para apenas uma espécie com interação obrigatória, ou foi estimado indiretamente (ver Pierce *et al.* 1987, Fiedler & Saam 1995). Isto se deve à dificuldade logística de se avaliar o ganho da interação para a colônia, principalmente no campo.

Existem duas hipóteses que tentam explicar a evolução da mirmecofilia e a natureza destas interações em borboletas (ver Malicky 1970). A primeira, denominada “mutualística”, sugere que as adaptações à mirmecofilia teriam surgido com o intuito de manter relações mutualísticas (Thomann 1901). A segunda, chamada “apaziguadora”, propõe que as primeiras adaptações surgiram com o intuito de apaziguar o comportamento agressivo das formigas (Lenz 1917). Diversos autores têm discutido sobre a contribuição do mutualismo e do apaziguamento no desenvolvimento das adaptações à mirmecofilia (Malicky 1970, Pierce & Mead 1981, Fiedler &

Maschwitz 1988, Fiedler 1991, DeVries 1991b). No entanto, as duas hipóteses não são excludentes e são difíceis de separar (Cottrell 1984, Pierce *et al.* 2002). Um avanço importante ao entendimento destas interações em Lepidoptera foi dado por DeVries (1991b), que levando em conta a biologia alimentar das formigas que atendem larvas, propôs um cenário ecológico para a evolução da mirmecofilia em Lepidoptera. Neste cenário, a mirmecofilia surgiu sobre a vegetação, inserida em um complexo sistema de interações que envolvem também plantas com nectários extraflorais, hemípteros produtores de exsudatos e, principalmente, formigas especializadas em alimentos líquidos. Apesar deste cenário não poder ser testado, experimentos simples envolvendo larvas e potenciais formigas atendentes podem ser úteis para entender a importância relativa do mutualismo e do apaziguamento para lepidópteros mirmecófilos (DeVries 1991b, ver também Kaminski 2008a).

As larvas mirmecófilas apresentam várias adaptações comportamentais e morfológicas para conviver com formigas, como por exemplo, uma cutícula cerca de 20 vezes mais espessa do que a apresentada por larvas não mirmecófilas (possivelmente uma proteção contra possíveis ataques das formigas) e a ausência de comportamento reflexo de se debater quando perturbada (tal comportamento geralmente provoca reações agressivas por parte das formigas) (Malicky 1970, Fiedler 1991, Freitas & Oliveira 1992, Pierce *et al.* 2002). Além disso, estas larvas apresentam alguns tipos de órgãos altamente especializados na interação ("*ant-organs*" ou órgãos mirmecofílicos), e que são de grande importância para a classificação e compreensão das relações entre diversos subgrupos de Lycaenidae e Riodinidae (Harvey 1987, DeVries 1991a, DeVries *et al*. 2004, Penz & DeVries 2006). No entanto, apesar de serem similares quanto à função, existem diferenças marcantes quanto à anatomia interna e posicionamento destes órgãos (DeVries 1991a, 1997, Kaminski 2006). Tais diferenças geraram dúvidas sobre uma origem única da mirmecofilia em Lepidoptera e, conseqüentemente, sobre o status taxonômico destas duas famílias. Neste sentido, o mapeamento da distribuição dos diferentes tipos de órgãos mirmecofílicos nas filogenias implica que a mirmecofilia teria surgido e desaparecido várias vezes em borboletas (Campbell & Pierce 2003). Uma hipótese alternativa e mais parcimoniosa para a falta de concordância no posicionamento dos órgãos seria a ocorrência de mutações em genes homeóticos (ver Campbell & Pierce 2003).

Segundo Pierce *et al.* (2002), as borboletas mirmecófilas podem manipular o comportamento das formigas de três formas: apaziguamento do comportamento agressivo, manutenção do interesse na simbiose, e através da indução de comportamento defensivo. Estas respostas comportamentais das formigas são mediadas pelos órgãos mirmecofílicos de três formas: (1) através de recompensas nutritivas produzidas por glândulas (também denominadas órgãos nectaríferos); (2) por comunicação química; (3) por comunicação sonora. As borboletas mirmecófilas podem interagir com formigas em todas as suas fases do desenvolvimento, mas as adaptações à mirmecofilia são mais efetivas e conspícuas no estágio larval, para o qual daremos mais ênfase.

Os órgãos nectaríferos estão presentes em Lycaenidae e Riodinidae, mas a morfologia interna e posicionamento variam grandemente entre as duas famílias (DeVries 1991a, 1997). Em Lycaenidae, a glândula é denominada órgão nectarífero dorsal (DNO) ou órgão de Newcomer, constituído por uma estrutura única posicionada dorsalmente no sétimo segmento abdominal (Newcomer 1912, Malicky 1970, Fiedler 1991). Em Riodinidae, a estrutura é denominada órgão nectário tentacular (TNOs), sendo composta por um par de glândulas eversíveis posicionadas dorsalmente no oitavo segmento abdominal (Ross 1964, DeVries 1988, Kaminski 2006). Em geral, tem sido sugerido que estes órgãos desempenham um papel fundamental na manutenção da simbiose com formigas (DeVries 1988, Fiedler 1991, Pierce *et al.* 2002, Daniels *et al.* 2005). Estudos sobre o conteúdo nutricional destas secreções têm evidenciado uma riqueza de aminoácidos superior à encontrada em outras fontes alimentares líquidas disponíveis para as formigas na vegetação (DeVries & Baker 1989).

Existe uma grande quantidade de estruturas larvais que têm sido apontadas como importantes na comunicação química com as formigas, mediando o apaziguamento, reconhecimento, e até mesmo manipulando o comportamento das formigas (ver Fiedler 1991, DeVries 1997, Pierce *et al.* 2002). No entanto, quase nada é conhecido sobre as substâncias que são produzidas por estes órgãos e seu real efeito nas interações larva-formiga. Dentre as estruturas mais importantes e menos compreendidas, estão os órgãos perfurados em forma de cúpula (PCOs). Os PCOs são glândulas epidermais unicelulares presentes em quase todos os Lycaenidae e Riodinidae (Malicky 1970, Kaminski 2006). Por sua constância, os PCOs têm sido considerados os primeiros órgãos a aparecer em larvas mirmecófilas e acredita-se que produzam voláteis (alomônios) importantes no apaziguamento das formigas (DeVries 1988, Fiedler 1991, Pierce *et al.* 2002).

Outra classe de órgão mirmecofílico importante na comunicação química larva-formiga são os órgãos tentaculares (TOs) eversíveis que aparecem pareados em algumas larvas de Lycaenidae, e os órgãos tentaculares anteriores (ATOs) em larvas de Riodinidae. Com base em observações comportamentais de interações entre larvas e formigas, tem sido sugerido que estes órgãos produzem substâncias voláteis que induzem comportamento de alerta e agressividade nas formigas atendentes (DeVries 1988, Fiedler 1991, Axén *et al.* 1996). Outros órgãos com função similar têm sido descritos para outras larvas mirmecófilas, como as cerdas baloniformes e a glândula cervical em alguns Riodinidae (DeVries 1997, DeVries *et al.* 2004). De forma geral, órgãos mirmecofílicos relacionados à sinalização química mais complexa estão presentes em larvas que tem interações obrigatórias e especificas com formigas (DeVries 1988, 1997, Fiedler 1991). Em algumas espécies, a sinalização química com as formigas pode não ser mediada especificamente por um órgão, como no caso de espécies parasitas de formigueiros que mimetizam hidrocarbonetos cuticulares de suas formigas hospedeiras (ver Akino *et al.* 1999, Nash *et al.* 2008, Hojo *et al.* 2008).

A habilidade de produzir som está presente em quase todas as larvas mirmecófilas e parece ser um aspecto chave na manutenção das interações com as formigas (DeVries 1990, 1991c). Os órgãos que produzem sons em larvas de Lycaenidae ainda são pouco conhecidos do ponto de vista funcional e morfológico, mas acredita-se que as larvas produzam sons através de estridulações nas áreas entre os segmentos (Travassos & Pierce 2000). Em Riodinidae, os sons podem ser produzidos de duas formas, mas basicamente envolve a estridulação de estruturas (papilas vibratórias ou uma placa membranosa) do protórax com a superfície corrugada da cápsula cefálica (DeVries 1988, 1990, 1991c, Travassos *et al.* 2008). DeVries (1988) demonstrou que larvas de *Thisbe irenea* (Riodinidae) que tinham suas papilas vibratórias removidas apresentavam uma menor capacidade de recrutar formigas.

Como é observado para outras larvas de lepidópteros, a vasta maioria dos Lycaenidae e Riodinidae é exclusivamente herbívora. No entanto, a interação simbiótica com formigas parece favorecer uma maior amplitude de plantas hospedeiras utilizadas, bem como uma mudança nos hábitos alimentares das larvas. Como resultado, nenhuma família de Lepidoptera apresenta tanta variação nos hábitos alimentares das larvas como é observado nestas duas famílias de borboletas mirmecófilas (ver Cottrell 1984, DeVries *et al.* 1994, Pierce 1995, Pierce *et al.* 2002). Esse padrão pode ser explicado pela vantagem obtida pelas larvas mirmecófilas ao ocuparem espaços livres de inimigos em suas plantas hospedeiras (Atsatt 1981a). Mudanças na dieta larval ocorrem primariamente pelo papel que a presença de formigas adquiriu na seleção da planta hospedeira, servindo muitas vezes como estímulo para a oviposição (Atsatt 1981b, Pierce & Elgar 1985, Fiedler 1991).

A primeira conseqüência da oviposição dependente de formigas é uma forte influência das formigas nos padrões de distribuição espacial dos imaturos e adultos (Smiley *et al.* 1988, Seufert & Fiedler 1996, Kaminski 2008a). Outra conseqüência possível é a expansão do espectro de plantas hospedeiras utilizadas pelas larvas (polifagia) (Pierce 1984, Pierce & Elgar 1985, DeVries *et al.* 1994, DeVries 1997, Kaminski 2008a). Tal padrão ocorreria porque ao utilizar formigas como pistas no processo de seleção de planta hospedeira, a fêmea poderia cometer "enganos" ao depositar seus ovos (Pierce 1984, Pierce & Elgar 1985). Desta forma, seria esperado que a polifagia aparecesse mais facilmente em espécies mirmecófilas do que em espécies não mirmecófilas (ver Fiedler 1994).

A evolução de outros tipos de hábitos alimentares está relacionada à exploração de interações simbióticas entre plantas e outros herbívoros (Maschwitz *et al.* 1984, DeVries & Baker 1989, Fiedler 1991). Interações facultativas entre plantas com nectários extraflorais e formigas têm sido exploradas por várias espécies de Riodinidae (DeVries & Baker 1989, DeVries 1997), e até mesmo interações obrigatórias de mirmecófitas podem ser parasitadas (Maschwitz *et al.* 1984).

Similarmente, interações entre hemípteros produtores de exsudatos e formigas têm sido exploradas consistentemente em várias linhagens de Lycaenidae e Riodinidae (Cottrell 1984, Pierce 1995, DeVries & Penz 2000, Oliveira & Del-Claro 2005). A extrema exploração destes sistemas pode levar ao surgimento de hábitos alimentares incomuns em Lepidoptera, como a alimentação especializada em exsudatos e/ou predação de hemípteros mirmecófilos (Pierce *et al.* 2002). O hábito predador nestas larvas pode estar associado a mudanças morfológicas e comportamentais, como o alongamento das pernas protorácicas, e/ou seleção de planta hospedeira mediada pela presença de hemípteros (DeVries & Penz 2000). Outra via para o surgimento do hábito predador em larvas mirmecófilas ocorre quando os sinais químicos utilizados pelas larvas se tornam tão específicos que as formigas reconhecem as larvas como se fossem outras formigas, propiciando o surgimento de parasitismo social. O sistema parasítico mais estudado é o que envolve o gênero *Phengaris* (= *Maculinea*, Lycaenidae), cujas lagartas habitam ninhos de formigas *Myrmica* durante uma parte da vida, e se alimentam de larvas de formigas ou até mesmo através de trofalaxis com as formigas hospedeiras (ver Thomas & Elmes 1998, Als *et al.* 2004).

CONCLUSÕES E PERSPECTIVAS

O estudo das interações entre plantas, formigas e lepidópteros pode ser importante para melhor compreendermos a ecologia evolutiva de sistemas multitróficos, especialmente em ecossistemas tropicais. As estratégias defensivas em Lepidoptera podem ser uma combinação de vários fatores, sendo difícil indicar contra qual inimigo ela foi desenvolvida (Dyer 1997). Além disso, os inimigos naturais mudam ao longo da ontogênese e conseqüentemente as táticas defensivas também devem mudar (Salazar & Whitman 2001). De fato, predadores invertebrados parecem ser mais importantes para larvas menores, enquanto larvas maiores são mais atacadas por vertebrados (Bernays 1997). Nesse sentido, é difícil identificar dentre uma gama de características defensivas qual responde especificamente a um determinado predador (Gross 1993, Gentry & Dyer 2002). Embora formigas possam usar sinais visuais, os principais sinais utilizados são químicos e táteis, e as táticas defensivas desenvolvidas contra formigas devem operar nestes dois universos sensoriais.

Segundo Hölldobler (1971), a análise comparativa de espécies mirmecófilas com diferentes níveis de associação com as formigas é a chave para compreender os detalhes da evolução das associações e dos sistemas de comunicação em organismos mirmecófilos. Além disso, o entendimento das variações nos padrões de utilização de plantas hospedeiras e mudanças nos hábitos alimentares das larvas requerem uma análise conjunta de todas as partes do sistema. Nos últimos anos, diferentes trabalhos têm analisado a evolução da mirmecofilia em Lepidoptera de maneira comparativa, com o apoio de análises cladísticas (Als *et al.* 2004, Megens *et al.* 2005). Este tipo de abordagem é útil para entender a evolução das modificações impostas aos organismos mirmecófilos. No entanto, tal enfoque nunca foi dado para se entender a evolução de características defensivas em larvas que utilizam plantas hospedeiras visitadas por formigas. Informações básicas de história natural que servem de ponto de partida para estes estudos comparativos ainda são escassas, principalmente na região Neotropical. Nesse sentido, esperamos que esta revisão estimule futuros estudos sobre as interações entre formigas, larvas de lepidópteros e suas plantas hospedeiras em ecossistemas brasileiros.

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CAPÍTULO II

IMMATURE STAGES OF *PARRHASIUS POLIBETES* (LEPIDOPTERA: LYCAENIDAE): HOST PLANTS, MYRMECOPHILY, AND CO-OCCURRENCE WITH HEMIPTERAN TROPHOBIONTS [*]

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Abstract

Natural history and immature stage morphology of the facultative myrmecophilous butterfly *Parrhasius polibetes* (Stoll) (Lepidoptera: Lycaenidae) are described and illustrated for the first time, through both light and scanning electron microscopy. Larvae developed through four instars. At the third instar, the dorsal nectar organ (DNO) becomes functional and larvae can be facultatively tended by several ant species, those also tending plants bearing extrafloral nectaries (EFNs), and honeydew-producing hemipterans. Larvae are florivorous and polyphagous at the individual level, using at least 28 species of plants in 16 families. Most host plants (78.57%) have some kind of ant attractive elements, as extrafloral nectaries and/or ant-tended treehoppers. Host range of this butterfly species allows it to use of floral resources throughout the year. Food sources that promote ant visitation, flower bud morphology and phenology appear to the rise and maintenance of polyphagy in this butterfly. We proposed *P. polibetes* as an exemplar model for studies of ant-butterfly evolutionary history in the Neotropics.

Keywords: Eumaeini; florivory; natural enemies; polyphagy; symbiosis

Introduction

The family Lycaenidae is composed of 4,000 species, accounting for approximately one quarter of the global butterfly species richness (Lamas 2008). An important feature present in several lycaenids is the symbiotic interactions with ants in the larval stage – termed myrmecophily (see reviews in Fiedler 1991; Pierce et al. 2002). Due to the recognized importance of myrmecophily in the immature biology and morphology of these butterflies, several authors have argued that much of the evolutionary history of Lycaenidae, including host plant use, would be explained by their interactions with ants (see Atsatt 1981a; Pierce 1984; Eastwood et al. 2006). However, most knowledge about the evolutionary ecology of lycaenids is based on studies of well known Palaearctic, Oriental, and Australian species (e.g. Eastwood and Fraser 1999; Fiedler 2001, 2006; Pierce et al. 2002), while little is known about the rich Neotropical fauna (see Brown 1993).

The majority (~90%) of the approximately 1,200 species of Neotropical Lycaenidae belongs to the tribe Eumaeini (Brown 1993; Robbins 2004). Eumaeini butterflies usually have small size, perform hilltopping and are skilled flyers; some species migrate in multi-specific groups (Robbins and Small 1981; Prieto and Dahners 2009). In general, adults have a common pattern consisting of wing dorsal surface shining blue and ventral side uniformly grayish brown, bearing tails and with dark spots at the anal angle resembling a false head (Robbins 1980). Although poorly studied, the immature stages of different species are apparently similar with respect to biology and morphology. Except for some oligophagous genera, most species are considered florivorous and polyphagous (Robbins and Aiello 1982; Fiedler 1991; Monteiro 1991; Brown 1993; Kaminski et al. 2010, Rodrigues et al. 2010). All known species are facultative myrmecophilous or non-myrmecophilous (e.g. Robbins 1991; Monteiro 1991; Ballmer and Pratt 1988; Duarte et al. 2005; Kaminski and Freitas 2010), and obligate myrmecophily was never reported for species in this tribe. Studies on the biology of Neotropical Eumaeini have brought novelty in the understanding of this group's evolution, as for example the recent description of detritivory in several species (Duarte et al. 2005; Duarte and Robbins 2009).

The genus *Parrhasius* Hübner, [1819] contains six species widely distributed in the Neotropical region, with only one species (*Parrhasius m-album* Boisduval & Le Conte, [1833]) occurring in the eastern USA (Nicolay 1979; Robbins 2004). Published information about the immature stages of *Parrhasius* is scattered and currently available only to Nearctic species (see Boisduval and Le Conte 1829-1937; Clench 1962; Downey and Allyn 1981; Sourakov 2008). There is no formal description of immature stages from any Neotropical species in this genus, and only a few host plant records are available in the literature (e.g. Zikán 1956; Diniz and Morais 2002; Torezan-Silingardi 2007; Beccaloni et al. 2008; Silva et al. 2009).

Parrhasius polibetes (Stoll, 1781) is the most widespread species of the genus and considered one of the most common Lycaenidae butterflies in the Neotropics (Nicolay 1979; Brown 1993), but surprisingly little is known about its biology and immature stages. Fortunately, this scenario has changed due to recent report on larval co-occurrence with honeydew-producing hemipterans in the Brazilian cerrado savanna (Oliveira and Del-Claro 2005). This finding, combined with the abundance of this butterfly species, has qualified it as a potential model for evolutionary studies on ant-butterfly interactions and host plant use (Rodrigues et al. 2010; Kaminski et al. submitted). In this sense, the aim of this study is to provide new basic information about natural history and morphology of immature stages of *P. polibetes*, including data from host plants, tending ants, natural enemies, and potential interactions with trophobiont insects.

Material and methods

Host plant records, collection and rearing of **Parrhasius polibetes**

Collections were carried out in two sites of cerrado savanna in São Paulo State, southeast Brazil: 1) Laboratório Nacional de Luz Síncrotron (22° 48'S, 47°03' W), Campinas; and 2) Reserva Biológica e Estação Experimental de Mogi-Guaçu (22°18'S, 47°10'W), Mogi-Guaçu. In both sites the vegetation consists of a dense scrubland of shrubs and trees, classified as cerrado *sensu stricto* (Oliveira-Filho and Ratter 2002). Samplings occurred monthly in 2007, and restricted to the months that correspond to the dry season (April–July) in the years of 2008 and 2009, when normally eumaeine adult butterflies were more abundant in southeast Brazil (see Brown 1992). Along marked trails in the sites and road edges, available host plants with inflorescences were checked for the presence of *P. polibetes* immatures. Plants with immatures were identified *in situ* or collected for identification. We also recorded the presence of food sources that may promote visitation of ants on the leaves, such as extrafloral nectaries (EFNs), and/or honeydew-producing hemipterans (HPHs). Additional host plant records, flowering phenology data, and presence of ant attractives were complemented with previously published data available in the literature (see Table 1). At the time of immature collection, it was also recorded whether tending ants, well as the natural enemies where present, and those still unknown were collected for identification.

Immatures of *P. polibetes* for the morphological description were collected in the field and reared as follows: eggs were placed in Petri dishes and observed daily until the eclosion; newly-hatched larvae were reared individually in transparent 250 ml plastic pots under controlled conditions (25 ± 2 °C; 12h L: 12h D). Only the newly-laid eggs (i.e. the eggs that were seen being laid by females at the moment of collection) were taken into account for determining egg development time. Branches with *Schefflera vinosa* flower buds were offered *ad libitum*, and larvae were checked daily for food replacing and cleaning when necessary. As rearing on different host plant species as well presence of tending ants affected the larval development time of each instar were taken on the larvae reared on *S. vinosa*. Immatures for morphological analysis were separated by stage, fixed in Dietrich's fluid, and then preserved in 70% ethanol. Shed head capsules were collected and preserved for measurements. Voucher specimens of the immature stages were deposited at the Museu de Zoologia "Adão José Cardoso" (ZUEC), Universidade Estadual de Campinas, Campinas, São Paulo, Brazil.

Morphology

Measurements and general aspects of morphology were assessed using a Leica® MZ7.5 stereomicroscope equipped with a micrometric scale. Egg size is given as height and diameter.

Head capsule width of larvae is the distance between the most external stemmata; maximum total length for both larvae and pupae corresponds to distance from head to posterior margin of the tenth abdominal segment in dorsal view (as in Freitas 2007). Color patterns *in vivo* of immature stages were recorded using a Nikon® Coolpix 4500 digital camera. Images of the eggs and early instar larvae were taken through a digital camera attached to the stereomicroscope. Scanning electron microscopy (SEM) was conducted using a JEOL® JSM-5800 microscope, and samples were prepared in accordance with the following protocol: Critical point dried in a Bal-tec® - CPD030 equipment and attached with double stick tape to aluminum stubs; gold/palladium coated with a Bal-tec® - SCD050 sputter coater. Terminology for early stage descriptions followed Downey and Allyn (1981, 1984a) for eggs; Stehr (1987) for general morphology of larvae; Downey and Allyn (1984b), Duarte et al. (2005), and Ballmer and Wright (2008) for chaetotaxy; Mosher (1916) and Duarte et al. (2005) for pupae; and Fiedler (1991) for ant-organs.

Results

Natural history of Parrhasius polibetes

Both eggs and larvae of *P. polibetes* were found on 28 host plants species in 16 families (Table 1). The eggs were deposited only on reproductive tissues of host plants, especially inflorescences not blooming (Figs. 1B-C). Floral bud size and coloration strong varied among host plans, from small and brownish in *Schefflera vinosa* to large green in *Pyrostegia venusta* (see Rodrigues et al. 2010). Most of the observed host plants of *P. polibetes* present some kind of liquid food potentially used by ants (78.57%), either honeydew-producing hemipterans and/or extrafloral nectaries (see Table 1). Oviposition occur in the warmest period of the day, from 11:00 to 14:00 (n = 11). Females normally fluttered around the host plant before oviposition (pre-alighting phase). In the post-alighting phase, females repeatedly touch the flower bud surface with the tip of the abdomen before laying 1 to 10 eggs during an oviposition event. While eggs were found either isolated or in clusters on a given flower bud, larvae presented a solitary habit.

The development time from egg to adult last approximately 36 days. Larvae developed through four instars and pupation probably occurs off the host plant, given that no pupae were found in the field. In general, larvae fed externally to the flower bud; sometimes the protractile head extended into the internal parts of the flower buds (Figs. 1F-H). Usually, larvae were found off the flower buds (i.e., underneath the leaves) during the molting process. From the second instar on, larvae

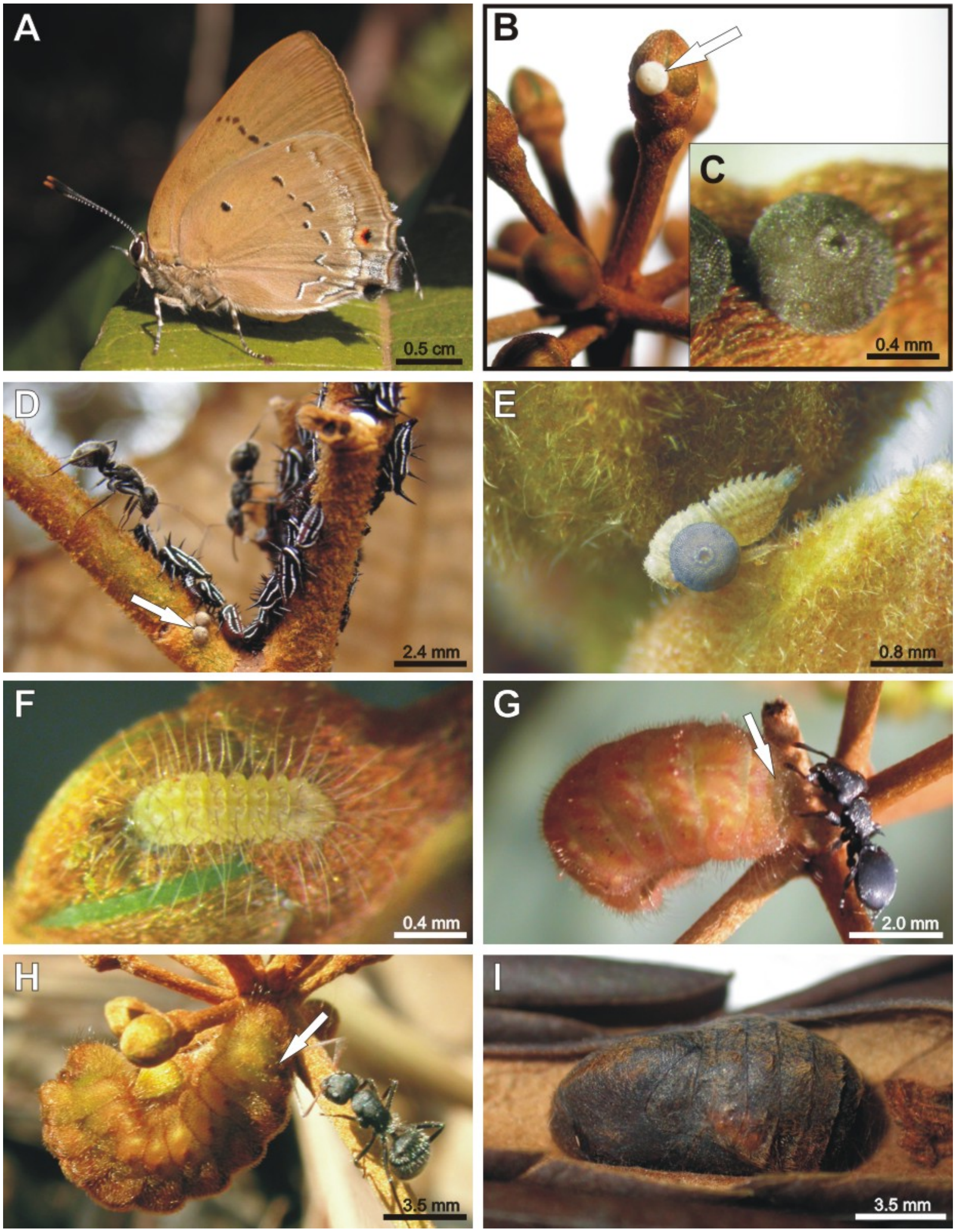

Figure 1. Life stages of *Parrhasius polibetes* on *Schefflera vinosa* (A-C; F-G) and on *Luehea grandiflora* (D-E). (A) Adult female; (B) newly-laid egg (arrow); (C) egg after 24 hours; (D) eggs (arrow) laid near an aggregation of *Guayaquila xiphias* treehoppers tended by *Camponotus crassus* workers; (E) egg accidentally laid on an ant-tended treehopper nymph of *Enchenopa gracilis*; (F) first instar; (G) third instar being tended by a worker of *Cephalotes pusillus*; (H) fourth (last) instar being tended by a worker of *C. crassus*; (I) pupa.

Table 1. Summary of host plant records for *Parrhasius polibetes*, including the flowering periods (for cerrado plant species only) and type of sources of liquid food from ants available (HPHs, honeydew-producing hemipterans; EFNs, extrafloral nectaries). The nomenclature for plant families follows APG II (Angiosperm Phylogeny Group) (2003).

Host plant	**Flowering period**	**Sources of liquid food**	**References**
Araliaceae			
Schefflera macrocarpa	Feb-May[1]	HPHs[1,7]	Present study Silva et al. (2009)
Schefflera vinosa	Mar-Jul[1,2]	HPHs[1,2,7]	Present study, Rodrigues et al. (2010) Oliveira and Del-Claro (2005)
Bignoniaceae			
Pyrostegia venusta	May-Nov[1,3,4]	-	Present study Rodrigues et al. (2010)
Caryocaraceae			
Caryocar brasiliensis	Sep-Nov[1,4]	EFNs[8]	Diniz and Morais (2002)
Chrysobalanaceae			
Licania humilis	Jun-Oct[1,4]	EFNs[8]	Present study
Combretaceae			
*Terminalia catappa**	-	EFNs, HPHs[1]	Present study
Euphorbiaceae			
Croton floribundus	-	-	Zikán (1956)
Fabaceae			
Bauhinia variegata	Apr-Jun[1]	HPHs, EFNs[1]	Present study
Erythrina speciosa	Apr-Aug[1]	HPHs, EFNs[1]	Present study
Inga uruguensis	Aug-Nov[1]	EFNs[1]	Present study
Inga sp.	-	-	Beccaloni et al. (2008)
Malpighiaceae			
Banisteriopsis argyrophylla	Mar-May[1, 2, 3]	EFNs[1]	Present study
Banisteriopsis campestris	Jan-Apr[1,5]	EFNs[1,5,8]	Present study
Banisteriopsis malifolia	Mar-Jun[5]	EFNs[5]	Torezan-Silingardi (2007)
Banisteriopsis muricata	-	-	Beccaloni et al. (2008)
Peixotoa tomentosa	Jan-Aug[1,5]	EFNs[1,5]	Present study

			Torezan-Silingardi (2007)
Heteropterys cf. *byrsonimifolia*	Apr-Sep[1]	EFNs, HPHs[1,7,8]	Present study
Malvaceae			
Luehea grandiflora	May-Aug[1]	HPHs[1]	Present study Rodrigues et al. (2010)
Melastomataceae			
Miconia ferruginea	Jun-Sep[1]	HPHs[1]	Present study
Miconia ferruginata	-	-	Silva et al. (2009)
Myrtaceae			
Myrcia cf. *albo-tomentosa*	Apr-Jul[1]	HPHs[1,7]	Present study
Proteaceae			
Roupala montana	Sep[4]	HPH[1,9]	Silva et al. (2009)
Sapotaceae			
Pouteria torta	Jun-Sep[1,4]	HPHs[1]	Present study
Sapindaceae			
Serjania caracasana	Mar-Jun[1,3]	EFNs[1]	Present study
Serjania cf. *erecta*	Aug-Sep[1]	EFNs[1]	Present study
Styracaceae			
Styrax camporum	Apr-Jul[1]	HPHs[1]	Present study
Styrax ferrugineus	Fev-Sep[4]	HPHs[1,7]	Silva et al. (2009)
Vochysiaceae			
Vochysia elliptica	Apr-Sep[6]	-	Diniz and Morais (2002)

*Native of southern India and coastal south-east Asia.

Phenology and sources of liquid food references: [1]present study; [2]Del-Claro and Oliveira (1999); [3]Morellato and Leitão-Filho (1996); [4]Batalha and Mantovani (2000); [5]Torezan-Silingardi (2007); [6]Oliveira and Gibbs (1994); [7]Lopes (1995); [8]Machado et al. (2008); [9]Maravalhas and Morais (2009).

Table 2. Summary of tending ant specie records for *Parrhasius polibetes* larvae, including ant activity period and the type of sources of liquid food used by tending ants (EFNs, extrafloral nectaries; HPH, honeydew-producing hemipterans).

Ant species	**Activity period***	**Sources of liquid used**
Formicinae		
Camponotus atriceps[1]	night[3]	EFNs, HPHs
Camponotus aff. *blandus*[1]	day[1,3]	EFNs, HPHs
Camponotus crassus[1]	day[1,3]	EFNs, HPHs
Camponotus lespesi[1]	crepuscular[1,3]	EFNs, HPHs
Camponotus leydigi[1]	day[1]	EFNs, HPHs
Camponotus renggeri[1]	day/night[1,3]	EFNs, HPHs
Camponotus rufipes[1]	day/night[1,3]	EFNs, HPHs
Camponotus sericeiventris[1]	day[1,3]	EFNs, HPHs
Camponotus melanoticus[1]	day/night[1]	EFNs, HPHs
Camponotus sp. 1[1]	day[1]	EFNs, HPHs
Camponotus sp. 2[1]	day[1]	EFNs, HPHs
Myrmicinae		
Cephalotes clypeatus[2]	day/night[3]	EFNs, HPHs
Cephalotes pusillus[1]	day[1,3]	EFNs, HPHs
Crematogaster sp. [1]	day[1]	EFNs, HPHs
Ectatomminae		
Ectatomma edentatum[2]	day/night[3]	EFNs, HPHs

*Diurnal activity (ca 07:00 – 17:00 h); nocturnal activity (ca 18:00 – 06:00 h).

Tending ant records, and ant activity period references: [1]present study; [2]Oliveira and Del-Claro 2005; [3]Del-Claro and Oliveira 1999.

showed cryptic polychromatism that is related to the host plant coloration. This polychromatism was observed in larvae using all host plants, resulting in tones of green, yellow, brown among others (Figs. 1H, 2). In a few cases (~10%), with no apparent causes larvae reared in the laboratory had a conspicuous reddish color and an apparent inability to reproduce the color substrate (Fig. 2B). These red larvae were more conspicuous on the green surface of *P. venusta* buds.

From the third instar on, the dorsal nectar organ (DNO) became functional. A total of 15 ant species distributed in three subfamilies Formicinae, Myrmicinae, and Ectatomminae (see Table 2) were recorded tending larvae of *P. polibetes*. The *Camponotus* genus was the most common with eleven recorded species. On average, 1.2 *Camponotus* tends a single *P. polibetes* late instar larva in the field (n = 25 larvae). Larvae were found with ants with a low frequency (~5%) and encounters between larvae and ants in the field are relatively short (less than 1 min.), i.e. larvae are not intermittently tending by ants as described for obligate myrmecophily interactions. However, the frequency and probability of an encounter between larva and prospective tending ants increased significantly (~20%) when the former developed in the vicinity of ant-tended treehoppers (Kaminski et al. submitted).

Immature stages of *P. polibetes* are attacked by a variety of natural enemies. In the egg stage the only recorded natural enemy was the parasitoid wasp *Telenomus* sp. (Hymenoptera: Scelionidae; Fig. 3A). Infestation rate by this microhymenopteran ranged from 4.15% (n = 193 eggs; Mogi-Guaçu) to 46.15% (n = 78 eggs; Campinas), depending on host plant species and study site. During the larval stage, *P. polibetes* is parasitized by at least five species of wasps. Early instars (1st and 2nd instars; Fig. 3B) are attacked by Braconidae wasps, while late larvae (3rd and 4th instars; Figs. 3C-D) are parasitized mainly by Chalcididae and Ichneumonidae wasps. Late instar larvae were rarely parasitized by a Tachinidae fly that emerge when *P. polibetes* reached the pupal stage. In the field, late instar larvae were frequently observed being sucked by the ectoparasitic biting midge *Forcipomyia* sp. (Diptera: Ceratopogonidae; Fig. 3E), but the fly attacks are apparently harmless. Several spider species in the families Araneidae, Thomisidae, and Salticidae, as well as the predatory bugs *Podisus nigrispinus* (Asopinae) were also recorded predating the larval stage (Figs. 3F-G).

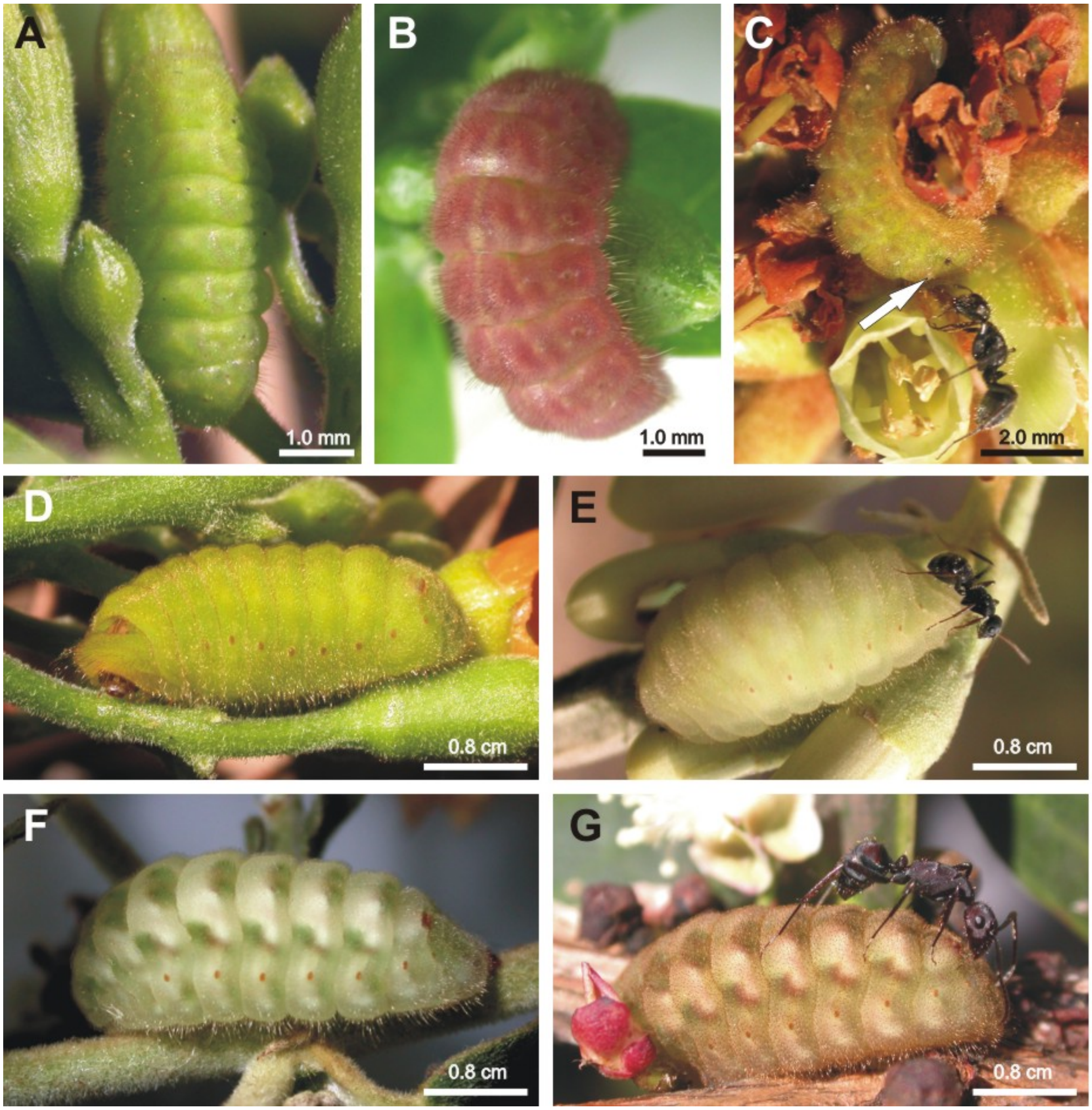

Figure 2. Larval color patters of *Parrhasius polibetes* on different host plants. (A) Third instar on *Pyrostegia venusta*; (B) third instar "red morph" on *P. venusta*; (C) third instar on *Pouteria torta* being tended by a worker of *Camponotus crassus*; (D) fourth (last) instar on *P. venusta*; (E) fourth instar on *Styrax camporum* being tended by a worker of *Camponotus* sp.; (F) fourth instar on *Banisteriopsis campestris*; (G) fourth instar on *Myrcia* cf. *albo-tomentosa* being tended by a worker of *Camponotus leydigi*.

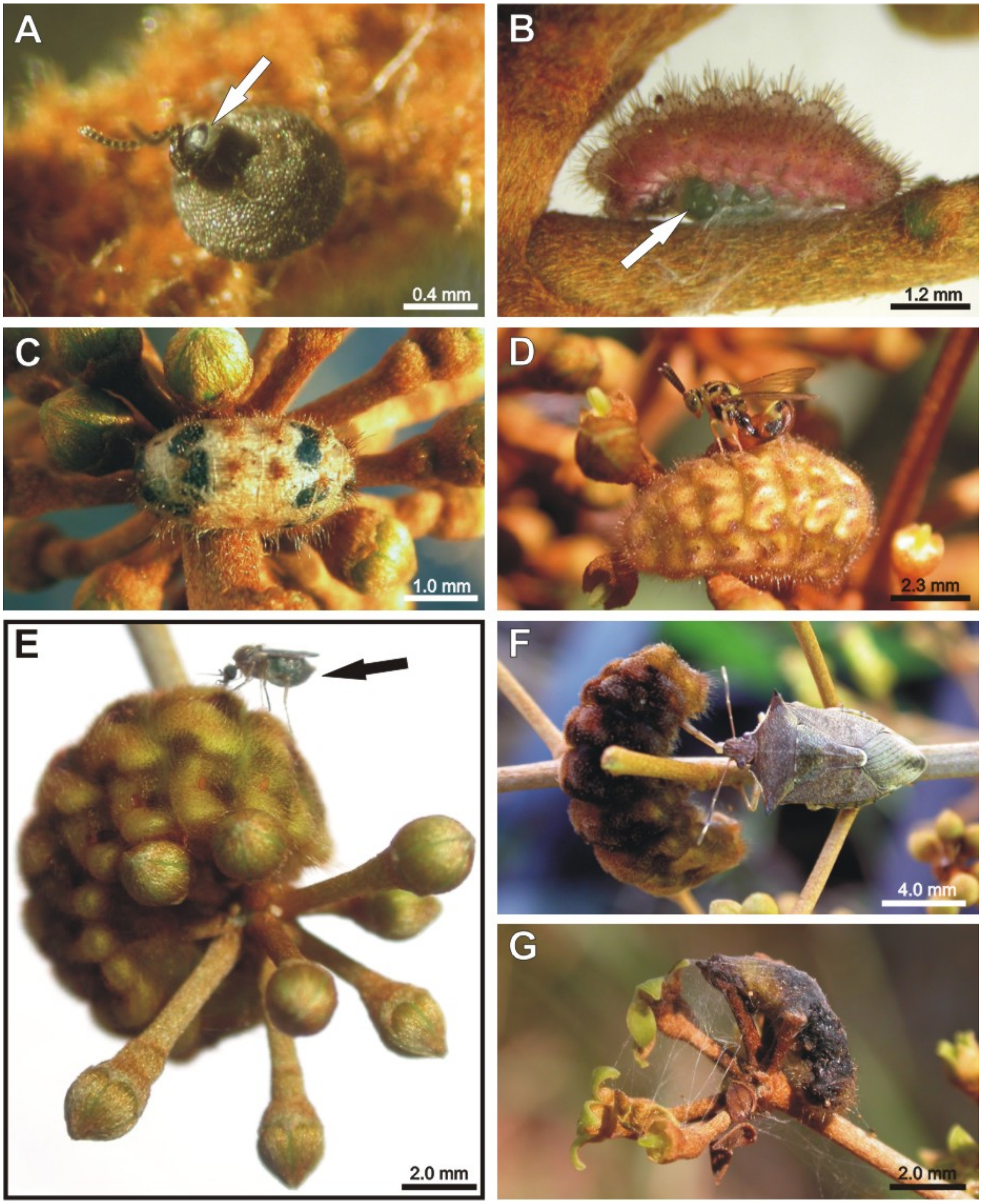

Figure 3. Natural enemies of *Parrhasius polibetes*. (A) *Telenomus* sp. wasp (arrow) emerging from the an egg; (B) second instar parasitized by a braconid wasp (arrow); (C) ichneumonid cocoon under third instar host remains; (D) wasp (*Conura* sp.; Chalcididae) parasitizing a fourth (last) instar; (E) fourth instar being attacked by a ceratopogonid biting midge (arrow); (F) predatory bug (*Podisus nigrispinus*; Asopinae) sucking a fourth instar larva; (G) remains of a fourth instar larva preyed by an araneid spider.

Description of the immature stages

Egg

Mean development time: 5.4 ± 0.24 days (n = 5). Height 0.60 – 0.62 mm, diameter 0.80 – 0.82 mm (n = 10). White coloration when newly laid, turning to a leaden gray after 24 hours (Figs. 1B-E). General shape sub-conical “bun shaped”, circular in anterior view (Fig. 4A). Exochorion with elevated ribs outlining hexa- and heptagonal cells; which can be separated in two parts: a basal with irregular surface formed by well delimited ribs and cells centrally depressed; and an apical constituted by slight ribs and cells. Micropilar area on the top of anterior region and composed by soft cells compared to the other egg cells (Fig. 4B). Aeropyles opening located on the rib intersections (Fig. 4C).

First instar

Mean development time: 3.7 ± 0.13 days (n = 20). Head capsule width 0.32 – 0.34 mm (n = 10), maximum length 1.50 mm. Head capsule brown, and prothoracic shield black. Body whitish yellow at the eclosion (Fig. 1F); turning to white with longitudinal red bands on the following day. Larvae onisciform with hypognathous protruded head (Figs. 6A-B). Cuticle with microtrichia, setae, and perforated cupola organs (Figs. 5, 6C-D). Spiracles elevated with circular peritrema (Fig. 6E). Proleg with uniordinal crochets in uniserial mesoseries, interrupted near center by conspicuous fleshy pad (Fig. 6F).

Head chaetotaxy (Figs. 5A-B) with 17 pairs of setae (A1, A2, AF1, C1, C2, CD1, CD2, CD3, F1, MG1, P1, S1, S2, S3, SS1, SS2, SS3), and 14 pairs of pores (Aa, AFa, Ca, CDa, Fa, La, MGa, Pa, Pb, Sa, Sb, SSa, more two unnamed pores located ventrally near antenna, probably related to substemmatal (SS) group.

Body chaetotaxy (Fig. 5C) consisted of 140 pairs of primary setae and 32 pairs of perforated cupola organs distributed as follows:

Prothorax with 11 pairs of setae directed forwards: five on the prothoracic shield (D1, D2, SD1, XD1, XD2), and one pair of PCO (DL); three pairs of “fringed setae” (=MSD1, MSD2, and L1; but see discussion in Ballmer & Wright 2008), more L2, SV1, SV2. Mesothorax with 10 pairs of setae (MD1, D1, D2, SD1, SD2, L1, L2, L3, SV1, SV2), and one pair of PCO (=DL). Metathorax with 11 pairs of setae similar to mesothorax, but with SD3, and one pair of subdorsal PCO (= SDL).

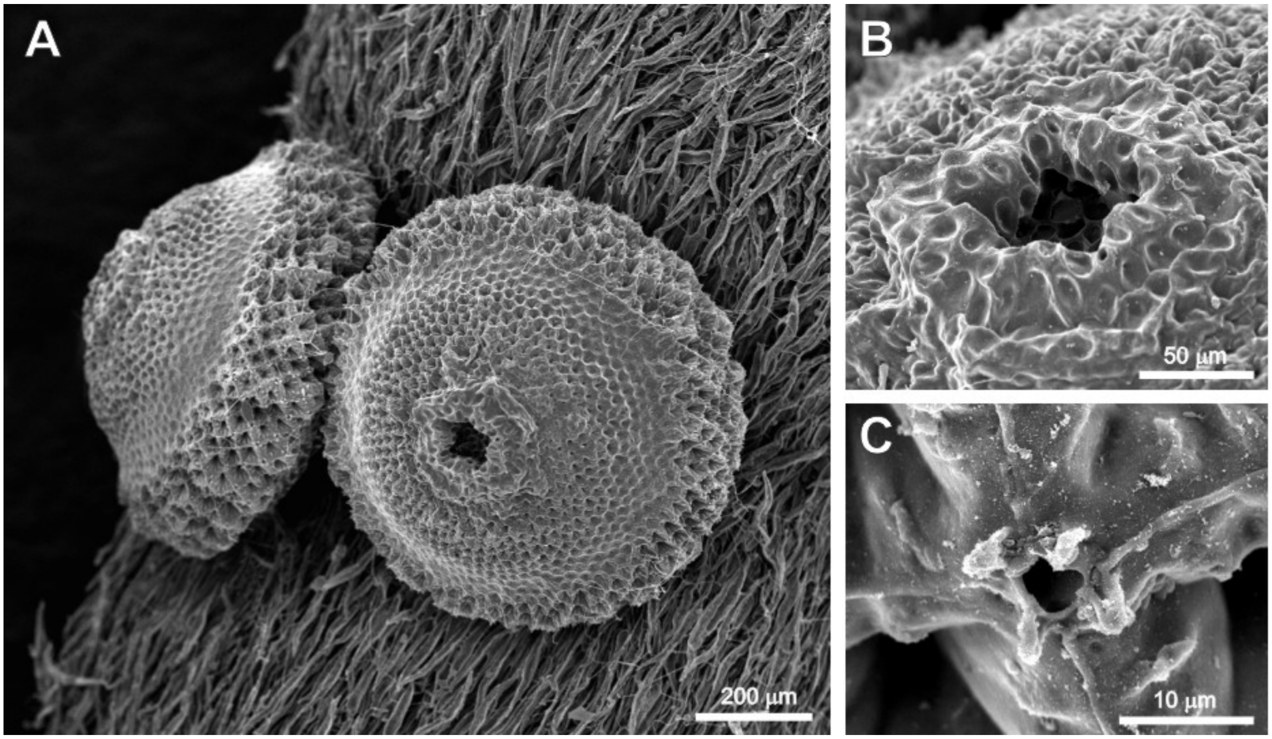

Figure 4. Scanning electron microscopy of *Parrhasius polibetes* egg. (A) Anterior and lateral view of two eggs; (B) micropylar area; (C) detail of an aeropyle on a rib intersection.

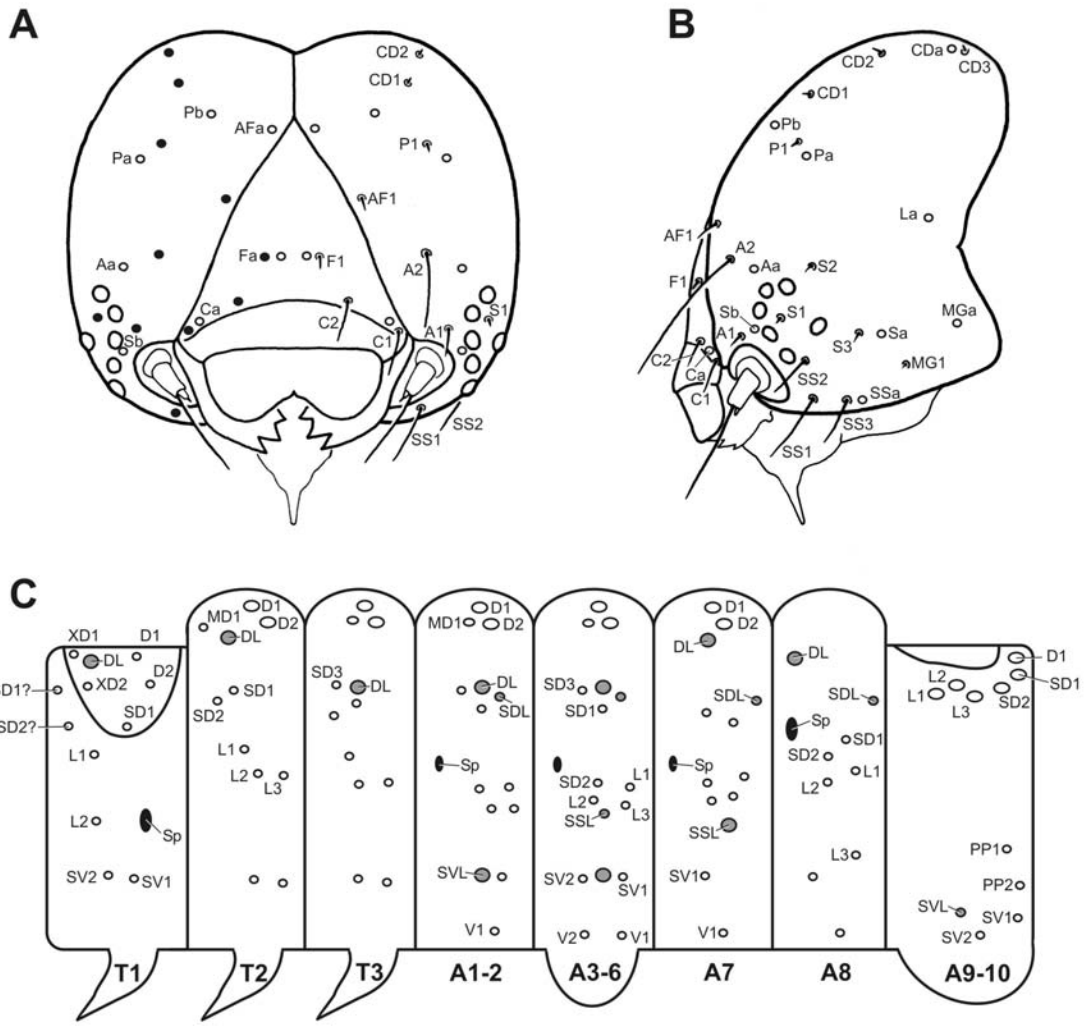

Figure 5. Chaetotaxy of first instar larva of *Parrhasius polibetes*. (A) Head in frontal view; (B) head in lateral view; (C) body diagram in lateral view. See text for abbreviations.

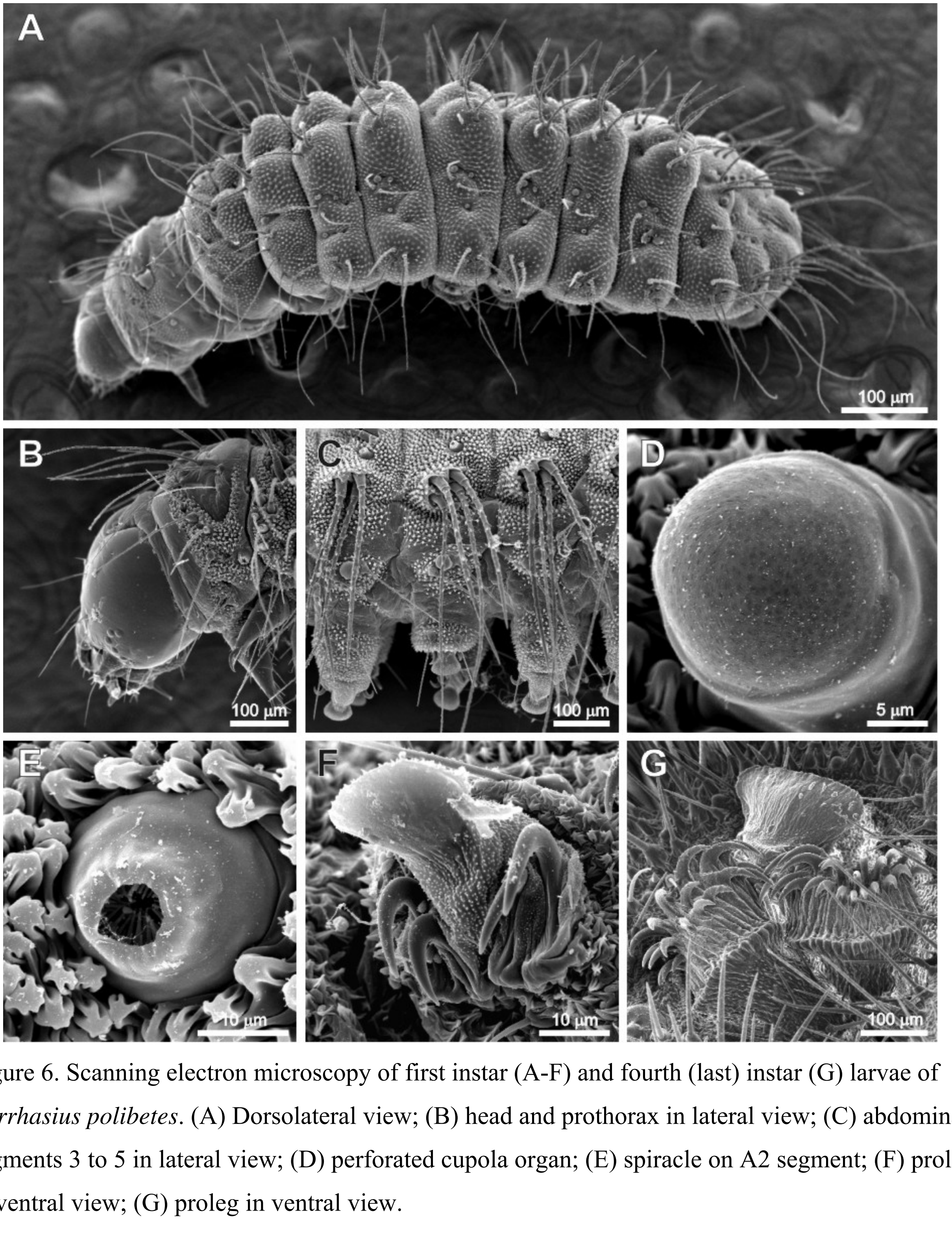

Figure 6. Scanning electron microscopy of first instar (A-F) and fourth (last) instar (G) larvae of *Parrhasius polibetes*. (A) Dorsolateral view; (B) head and prothorax in lateral view; (C) abdominal segments 3 to 5 in lateral view; (D) perforated cupola organ; (E) spiracle on A2 segment; (F) proleg in ventral view; (G) proleg in ventral view.

Abdominal segments A1 and A2 with 11 pairs of setae (MD1, D1, D2, SD1, SD2, SD3, L1, L2, L3, SV1, and V1), and three pairs of PCOs (DL, SDL, and SVL). Abdominal segments A3 to A6 with 14 pairs of setae (MD1, D1, D2, SD1, SD2, SD3, L1, L2, L3, SV1, SV2, V1, and V2, more V3 ventrally) and four pairs of PCOs (DL, SDL, SSL, and SVL). Abdominal segment A7 with ten pairs of setae (D1, D2, SD1, SD2, SD3, L1, L2, L3, SV1, and V1) and three pairs of PCOs (DL, SDL, and SSL). Abdominal segment A8 with seven pairs of setae (SD1, SD2, L1, L2, L3, SV1, and V1) and two pairs of PCOs (DL and SDL). Abdominal segment A9 and A10 with 13 pairs of setae (D1, SD1, SD2, L1, L2, L3, PP1, PP2, SV1, and SV2, more SV3, SV4, and SV5 ventrally), and one pair of PCO associated to subventral group.

Second instar

Mean development time: 3.5 ± 0.15 days (n = 20). Head capsule width 0.54 – 0.60 mm (n = 10), maximum length 4.81 mm. Head capsule and prothoracic shield brown. Cryptic polychromatism related to feeding started to take place, although some color patterns of the typical first instar still remained. Tegument covered by several light brown short setae, all of similar size.

Third instar

Mean development time: 3.3 ± 0.16 days (n = 20). Head capsule width 1.02 – 0.96 mm (n = 10), maximum length 6.26 mm. Head capsule, prothoracic shield, and spiracles brown. Body color uniform and similar to the substrate (Figs. 1E, 2A-C). Body setae similar to those described for the second instar, but enlarged. Dorsal nectar organ (DNO) opening medially located on the A7, with clusters of perforated cupola organs associated.

Fourth (last) instar

Mean development time: 7.05 ± 0.18 days, from which 2.55 ± 0.51 days corresponded to the pre-pupal period (n = 20). Head capsule width 1.56 – 1.86 mm (n = 10), maximum length 1.72 cm. Color pattern similar to third instar but less uniform (Figs. 1H, 2D-G). Head capsule, prothoracic shield, and spiracles brown. Body softly sliced and covered by light brown translucent short setae, giving a velvety aspect to larva. In the pre-pupa, larvae acquire a translucid brownish pink aspect. Protruded head smooth with few setae associated to frontoclypeus, and mouthparts; legs relatively short with some tarsal setae and a claw (Fig. 7A). Prothoracic shield subrectangular with some

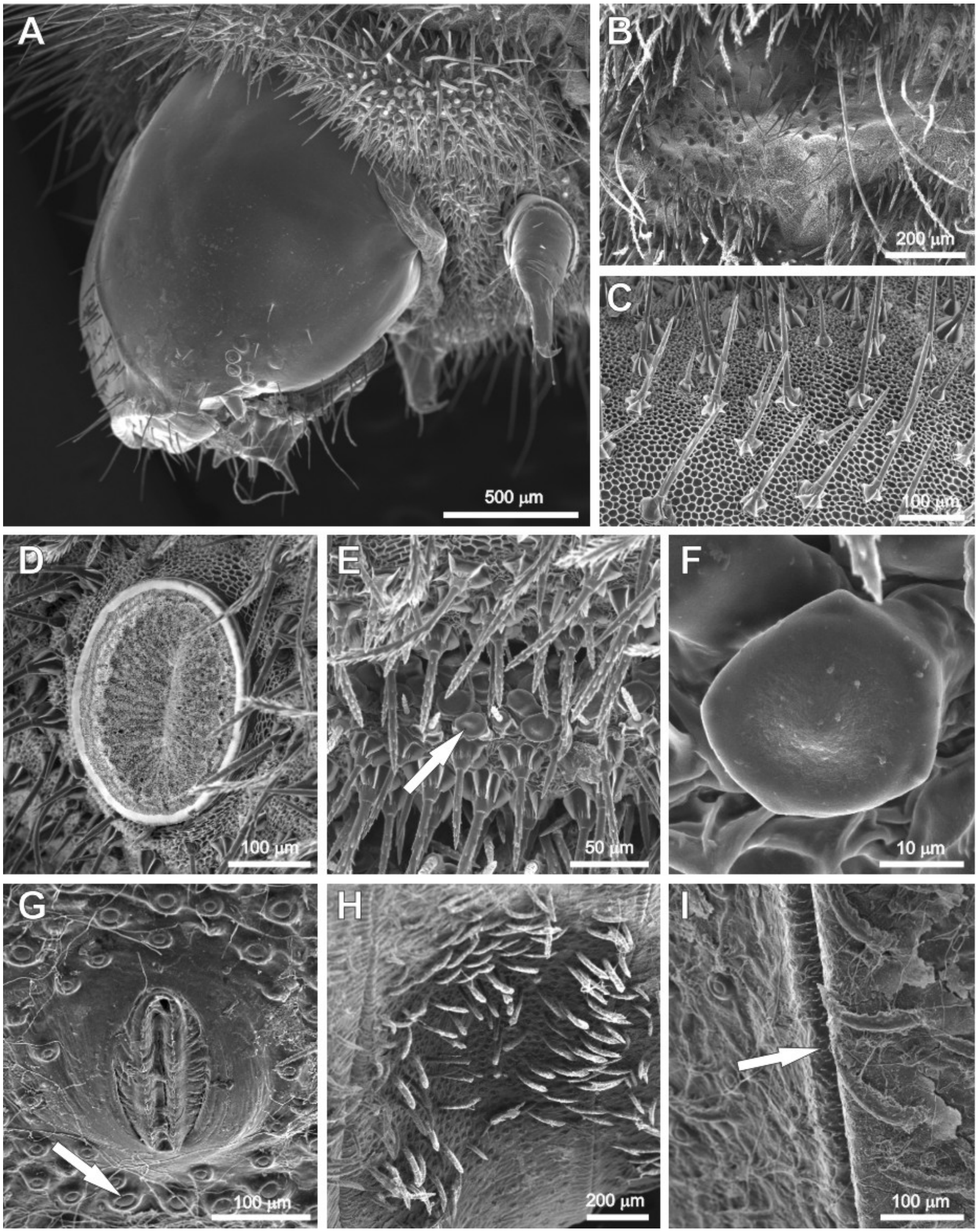

Figure 7. Scanning electron microscopy of fourth (last) instar (A-F) and pupae (G-I) of *Parrhasius polibetes*. (A) head in lateral view; (B) prothoracic shield; (C) detail of the abdominal tegument in dorsal view; (D) spiracle on A2 segment; (E) opening of the dorsal nectar organ (DNO) with perforated cupola organs (arrows); (F) detail of the perforated cupola organ; (G) spiracle on A5 segment, note the perforated cupola organs (arrows); (H) detail of the abdominal tegument in lateral view; (I) detail of the stridulating area between A5-A6 segments (arrow).

PCOs and setae; the SD1 tactile retain the primary chaetotaxy (Fig. 7B). Tegument covered with microtrichia; general body setae erect and similar in size (Fig. 7C), and stellate chalaza (sensu Ballmer and Pratt 1988). Spiracles openings aligned on the prothorax and A1-A8 segments; the format is semi elliptical with an elevated margin (Fig. 7D). Prolegs with several apically pointed setae; biordinal crochets arranged in uniserial mesoseries, interrupted near center by a fleshy pad (Fig. 6G). Dorsal nectar organ in the same position as the third instar, but with a reddish color and surrounded by more numerous and larger PCOs (Figs. 7E-F).

Pupa

Mean development time: 12.95 ± 0.11 days (n = 20). Maximum length 14.25 mm, and width at A3 5.48 mm. Color initially brownish pink, turning dark brown after some hours (Fig. 1I). Tegument covered by several short brown setae, with addition of some additional dorsal and lateral clusters of golden brown setae. Mesothoracic spiracle white; others dark brown, with a semi elliptical margin and surrounded by PCOs (Fig. 7G). Silk-girdle crossing the pupa on the 2A segment. Cuticle sculptured with several setae and PCOs. Abdominal lateral area above spiracles on A5 presents a smooth concavity with several setae and PCOs (Fig. 7H). Intersegmental area between A5-6 and A6-7 functioning as a stridulatory organ (Fig. 7I). The abdominal segment A10 with a ventrally flat cremaster.

Discussion

Morphology of the immature stages

In general, the eggs of *Parrhasius polibetes* resemble those of other species of Eumaeini, with upright shaped, round when viewed from above, and micropylar area centered on the top surface (see Downey and Allyn 1981, 1984a). Compared with other related genera (*Panthiades* section *sensu* Robbins 2004), as *Michaelus* Nicolay, 1979, *Oenomaus* Hübner, [1819], and *Panthiades* Hübner, [1819], the eggs of *Parrhasius* differ by the lack of spine-like protuberances on the rib intersections (Downey and Allyn 1984a; S.C. Thiele and L.A. Kaminski unpbl.). The *P. polibetes* exochorion is superficially similar to those of *Parrhasius m-album* and *Parrhasius orgia* (Hewitson, 1867), but differs from the later because it is more corrugated and less rounded in the lateral view in the former (Downey and Allyn 1984a; L.A. Kaminski unpbl.). Moreover, the leaden gray color seems to occur only in *P. polibetes*. The existence of morphological variation at both, generic and inter-generic levels, suggests that egg characters may be useful in phylogenetic studies in these Eumaeini lineages.

As already recorded for most Neotropical Eumaeini (e.g. Robbins and Aiello 1982; Ballmer and Pratt 1988; Calvo 1998; Kaminski and Freitas 2010), the larvae of *P. polibetes* have four instars. The first instar chaetotaxy of *P. polibetes* appear to be consistent with information available about other genera of Eumaeini (see a proposal of generalized chaetotaxy of eumaeine larvae in Ballmer and Pratt 1992). The main exception is the presence of a pair of frontal setae (F1) on the head capsule, although these setae are common in Lepidoptera (see Stehr 1987), had not yet been described in Lycaenidae. The dorsal perforated cupola organ (DL) on the larval A7 abdominal segment recorded in *Calycopis* Clench, 1961, by Duarte and Robbins (2009) is present in *P. polibetes*, but is less conspicuous. The specialized chaetotaxy present in Eumaeini yet requires further comparative studies in order to accurately determine the homologies.

Late instar larvae of *P. polibetes* have the tegument dorsally covered by microtrichia and several setae with similar size and stellate chalaza. This pattern appears to be consistent within the genus *Parrhasius* (Clench 1962; L.A. Kaminski unpbl.), and is shared with other genera of the *Panthiades* section (L.A. Kaminski unpbl.). Conversely, such pattern contrasts with those observed for other Eumaeini genera such as *Allosmaitia* Clench [1964], *Laothus* Johnson, Kruse & Kroenlein, 1997, and *Rekoa* Kaye, 1904; all which have a smooth cuticula and a few groups of long setae on the dorsal and lateral areas (see Monteiro 1991; Janzen and Hallwachs 2010; Kaminski and Freitas 2010). Although the presence of long setae or scoli seem to be related to defense against natural enemies in nonmyrmecophilous species (Kaminski 2008a; Kaminski et al. 2009), the current knowledge on the morphological variation in Eumaeini larvae not allow generalizations on this matter. For example, in *Cyanophrys* Clench, 1961, there are both, species with developed dorsal tubercles and setae, and species with smoother bodies (Kaminski et al. 2010). Furthermore, species in recognized nonmyrmecophilous genera may have tegument without projections, e.g. *Atlides* Hübner, [1819], *Calycopis*, *Eumaeus* Hübner, [1819], *Oenomaus*, *Pseudolycaena* Wallengren, 1858, and *Theritas* Hübner, 1818 (see Ballmer and Pratt 1988; Calvo 1998; Contreras-Medina et al. 2003; Duarte et al. 2005; Duarte and Robbins 2009; Janzen and Hallwachs 2010; L.A. Kaminski unpbl.).

The pupa of *P. polibetes* is indistinguishable from others species of *Parrhasius* at the gross morphology level, as well as pupae of other Eumaeini genera as *Michaelus* and *Oenomaus* (Clench 1962; Janzen and Hallwachs 2010). In Neotropical Eumaeini, the dark coloration and inconspicuous pupae appears to be associated with the habit of pupate outside the host plant, usually in the litter; while species that pupate on the host plant have more variation in the color pattern (L.A. Kaminski unpbl.). Although the *P. polibetes* pupa has a larger number of perforated cupola organs, and the ability to produce audible sounds, they are ignored by tending ants. Myrmecophily in the pupal stage is still not recorded for the Neotropical Eumaeini.

Host plant use and myrmecophily

As observed for several species of Neotropical Eumaeini, larvae of *P. polibetes* are highly polyphagous and feed only on reproductive parts of their host plants (Robbins and Aiello 1982; Fiedler 1991; Monteiro 1991; Brown 1993; Kaminski et al. 2010; Rodrigues et al. 2010). The evolution of polyphagy, and whether it is related with the florivory in eumaeins, all remain open questions. Some hypothesis has been proposed to explain this pattern: 1) relative lower toxicity of reproductive tissues (Robbins and Aiello 1982; Chew and Robbins 1984), however this hypothesis is refuted by the optimal-defense theory of allocation in plants (see Zangerl & Bazzaz 1992); 2) predilection for nitrogen-rich plant parts related to costs of producing reward secretions for ants (Pierce 1985); 3) flower bud morphology as a strong visual cue for egg-laying in flower-feeding butterflies (Chew and Robbins 1984; see also Rodrigues et al. 2010); 4) selective advantage of polyphagy related to escape predation in species with cryptic coloration through apostatic selection (Monteiro 1991, 2000); 5) strategy to decrease competition with other florivorous at the community level (Rodrigues et al. 2010); and 6) increase host plant range due to temporal restriction of floral resources supply (Monteiro 1991). However, such hypotheses are not mutually exclusive, and more information on the biology of eumaeine species is necessary to understand the host plant use patterns of these butterflies.

In obligate myrmecophilous butterflies, interactions with ants have been considered an important selective pressure affecting the host plant selection (e.g. Atsatt 1981a, b; Pierce 1984; Pierce and Elgar 1985; Fiedler 1994; DeVries et al. 1994; Kaminski 2008b). However, the role of myrmecophily in the rise of polyphagy has been ruled out in Neotropical Eumaeini, due to the inexistence of obligate myrmecophilous species (see Chew and Robbins 1984). For *P. polibetes*, recent studies have shown that females are able to use ant-treehopper associations as cues for host plant selection (Kaminski et al. submitted). In addition, larvae growing near ant-tended treehoppers have more chances to be found by prospective tending ants, and survive better compared to those growing on plant locations free from these trophobionts. In other words, the presence of honeydew-producing hemipterans may increases ant abundance and predictability on the host plant, resulting in an increase of myrmecophily degree in *P. polibetes*. Interestingly, this evolutionary scenario was predicted by Atsatt (1981a), who suggested that species using early successional herbaceous plants and/or ephemeral plant parts (buds, flowers, and fruits) are less dependent upon ants, but exceptions would be expected in plants which have traits that increase the ant predictability (extrafloral nectaries or ant-treehopper associations).

Our findings suggest that ant-treehopper may be visual cues for egg-laying butterflies, resulting in oviposition “mistakes” and use of plants that either confers “enemy-free space” or are of high quality. As a consequence, the above factor may have played a role in the rise and maintenance of polyphagy in *P. polibetes* (Rodrigues et al. 2010). On the other hand, feeding restricted to flower buds and use of host plants little visited by ants (e.g. *Pyrostegia venusta*) suggests that plant traits, such as the flower bud morphology may be also important for the oviposition selection decision making processes (Rodrigues et al. 2010). Moreover, *P. polibetes* uses a large set of host plants, assuring availability of resources throughout the year (see Table 1). However, it is hard to say, without an elucidated phylogeny whether the use of host plants with different flowering phenology is a cause or a consequence of florivory. Curiously, the oligophagy may be possible in Eumaeini florivorous that use plant families whose species present sequential flowering periods (Kaminski and Freitas 2010; Schmid et al. 2010).

Our results were obtained from populations of the Brazilian cerrado savanna – a biome known by the richness of interactions among plants, ants, and herbivores (see Oliveira and Freitas 2004). It would be interesting to know how the host plant use and myrmecophily degree vary in other biomes with different flora and ant mutualism systems. In addition, laboratory and field experiments on *P. polibetes* host preferences may be useful for understanding the evolution of specialized feeding behaviors seen in other myrmecophilous butterflies, as aphytophagy, carnivory, and feeding on hemipterans exudates (see Lohman and Samarita 2009). In this sense, hopefully that this work will stimulate further studies on ant-butterfly interactions in the Neotropics.

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CAPÍTULO III

SPECIES-SPECIFIC LEVELS OF ANT ATTENDANCE MEDIATE DEVELOPMENTAL COSTS IN A FACULTATIVE MYRMECOPHILOUS BUTTERFLY [*]

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Abstract. 1. Trophobiont butterfly larvae offer caloric rewards to ants through specialised glands, and in return gain ant-derived protection from natural enemies. Thus, from the larva's perspective, the major cost of myrmecophily is the reward production.

2. Larvae of the butterfly *Parrhasius polibetes* (Lycaenidae) are facultatively tended by several ant species, which differ in the intensity of tending behaviour. We examined the performance costs of *P. polibetes* when tended by two ant species differing in size and foraging strategies (*Camponotus melanoticus* and *Camponotus crassus*), and recorded the corresponding intensity of tending behaviour towards late instar larvae. Untended larvae were used as controls.

3. Larvae tended by *C. melanoticus* took longer to pupate compared to both *C. crassus* and control larvae. In contrast, pupae whose larvae were tended by *C. crassus* were lighter than control larvae but did not differ from those tended by *C. melanoticus*. No differences were found in the adult stage, indicating compensation in all cases.

4. Both at short and long term scales, *C. melanoticus* tended larvae of *P. polibetes* more intensely than *C. crassus*. The increased tending activity of *C. melanoticus* presumably delays the development time of larvae tended by this ant species.

5. Our results show that tending intensity varies depending on the ant species, and that *P. polibetes* has compensatory mechanisms to minimize myrmecophily costs regardless of tending intensity. To our knowledge this is the first experimental evidence that the intensity of ant tending behaviour is species-specific and affects performance in a trophobiont insect.

Key words. *Camponotus*, conditional mutualism, trade-offs, Eumaeini, tending behaviour, larval reward secretions, performance traits.

Introduction

Mutualism can be defined as an interspecific interaction that results in positive (beneficial) effects on per capita reproduction and/or survival of the interacting populations (Bronstein, 1994). Among lepidopterans, two butterfly families (Lycaenidae and Riodinidae) have developed symbiotic interactions with exudate-feeding ants (see review in Pierce *et al.*, 2002). These interactions are usually considered to be mutualistic in nature: the ants receive nutritional rewards produced by the larvae and in return protect the trophobionts from natural enemies (Pierce & Mead, 1981; DeVries & Baker, 1989; DeVries, 1991; Daniels *et al.*, 2005).

The degree of larval dependency on tending ants lies within a continuum (Pierce *et al.*, 2002), which ranges from larvae being totally dependent on specific tending ants (obligate myrmecophily) to occasional association with ants (facultative myrmecophily). Although there are many cases of obligate interactions between butterflies and ants, most associations are facultative and the corresponding balance between costs and benefits may vary with factors such as the nutritional quality of host plants, co-occurrence with other symbionts, and/or the ant species involved (Pierce *et al.*, 1991; Robbins, 1991; Fiedler & Hölldobler, 1992; Fraser *et al.*, 2001; Kaminski *et al.*, submitted).

From the larva's standpoint, the benefits of myrmecophily have been revealed through ant-exclusion experiments (e.g. Pierce & Mead, 1981; Pierce & Easteal, 1986; DeVries, 1991; Wagner & Kurina, 1997; Weeks, 2003). In general, these studies have shown that the presence of tending ants increases larval survival through protection against natural enemies, mainly parasitoids and predatory wasps (Pierce *et al.*, 2002 and included references). On the other hand, a key factor related to the cost of myrmecophily is the production of larval reward secretions that are rich in sugars and amino acids (DeVries & Baker, 1989; Pierce *et al.*, 1991; Daniels *et al.*, 2005). The production of secretions may affect both pupal mass and development time, which may ultimately reduce butterfly fitness (Pierce *et al.*, 1987; Elgar & Pierce, 1988; Robbins, 1991; Baylis & Pierce, 1992). In addition, some studies have shown that developmental costs related to myrmecophily are sex-dependent (Pierce *et al.*, 1987; Fiedler & Hölldobler, 1992; Fraser *et al.*, 2001).

Since myrmecophily is mediated by the production of liquid rewards by the trophobiont (Fig. 1), one might expect that ant species with distinct sizes and foraging strategies (i.e. different energy requirements) may incur different costs to ant-tended butterfly larvae. In fact, this has been demonstrated for some facultative butterfly-ant systems (Fiedler & Hölldobler, 1992; Wagner, 1993; Fraser *et al.*, 2001). However, no link has so far been made between the costs of myrmecophily for a trophobiont species and the intensity of tending behaviour imposed by different ant species.

The present study examines the developmental costs of myrmecophily for larvae of the facultative ant-tended butterfly *Parrhasius polibetes* (Stoll) (Lycaenidae) in relation to two species of *Camponotus* that differ in the intensity of tending behaviour. *Camponotus* ants are among the most important partners engaged in facultative associations with lycaenids (Fiedler, 2001), including *P. polibetes* (mentioned as *Panthiades polibetes* in Oliveira & Del-Claro, 2005) (Fig. 1).

These ants are extremely diverse with respect to several morphological and ecological attributes (Wilson, 1987; Hölldobler & Wilson, 1990).

Material and methods

Study system

Parrhasius polibetes is a Neotropical polyphagous lycaenid whose larvae feed on reproductive plant parts (flower buds and flowers) (Rodrigues *et al.*, 2010). The larval stage consists of four instars; from the third instar on the dorsal nectar organ (DNO) becomes functional and myrmecophily takes place. Late instar larvae can be tended by over ten ant species in the cerrado savannah of southeast Brazil. This butterfly species has been shown to use ant-treehopper associations as cues for oviposition, which significantly improves larval survival due to enhanced ant attendance (Kaminski *et al.*, submitted).

Schefflera vinosa (Cham. and Schltdl.) (Araliaceae) is the main food plant of *P. polibetes* larvae in cerrado (Oliveira & Del-Claro, 2005). Shrubs often also host aggregations of the honeydew-producing treehopper *Guayaquila xiphias* Fabr. (Hemiptera: Membracidae), which are tended day and night by over twenty ant species (Del-Claro & Oliveira, 1999). Prospective tending ants are attracted to the treehoppers after finding scattered droplets of flicked honeydew on lower leaves and ground (Del-Claro & Oliveira, 1996). As observed in most facultative ant-based mutualisms (e.g. DeVries, 1991; Fiedler, 2001; Rico-Gray & Oliveira 2007), *Camponotus* is the most representative ant genus tending *G. xiphias* aggregations and *P. polibetes* larvae (Oliveira & Del-Claro, 2005). Field observations have shown that on average 1.2 *Camponotus* tend a single *P. polibetes* late instar larva on *S. vinosa* shrubs (Kaminski *et al.*, submitted).

The two ant species used in this study, *Camponotus crassus* Mayr and *Camponotus melanoticus* Emery (Fig. 1), are commonly associated with plants bearing extrafloral nectaries and honeydew-producing hemipterans in cerrado areas (Oliveira & Brandão, 1991; Del-Claro & Oliveira, 1999). The two species, however, differ both in size and behaviour: *Camponotus crassus* is relatively small (length ~ 5.0 mm) and has a diurnal habit, whereas *C. melanoticus* is larger (length ~ 7.0 mm) and mostly nocturnal. Both species are commonly seen tending late instar larvae of *P. polibetes* at daylight (L.A. Kaminski, pers. obs.).

Parrhasius polibetes eggs, *Schefflera vinosa* flower buds, and *Camponotus* colonies were collected in the cerrado area of the Laboratório Síncrotron in Campinas (22°48’S 47°03’W; São

Fig. 1. Fourth (last) instar of *Parrhasius polibetes* being tended by a *Camponotus melanoticus* worker, note arrow pointed a secretion drop on the dorsal nectar organ (DNO).

Paulo, southeast Brazil) during May-July 2008, which corresponds to the dry season (fall-winter).Butterfly eggs were placed on Petri dishes lined with moistened filter paper and observed daily until eclosion. Captive ant colonies (~ 50-70 workers) were reared in artificial nests consisting of 3 test tubes (2.2 cm diameter x 15 cm length) with water trapped behind a cotton plug. Colonies were fed daily *ad libitum* with a honey/water solution and weekly with mature larvae of the palatable noctuid moth *Spodoptera frugiperda* L.

Performance in the presence or absence of ants

Newly-hatched larvae of *P. polibetes* were individually reared in transparent 250 ml plastic pots under controlled conditions (25 ± 2 °C; 12h L: 12h D). Unopened *S. vinosa* flower buds were offered *ad libitum*, and larvae were checked daily to replace food and clean containers. After the third instar each larva was randomly assigned as: (1) control (no ants), (2) tended by a single *C. crassus* worker, or (3) by a single *C. melanoticus* worker (n = 20 per treatment). Because worker size varies widely within both *Camponotus* species, we used mid-sized ant workers in the experiments of larval performance (*C. crassus* ~ 5.0 mm; *C. melanoticus* ~ 7.0 mm). Individual ants were fed only with *P. polibetes* secretion during trials, and were replaced every 48 hours. After pupation, all ants were removed and placed back in their corresponding colonies. Newly-emerged butterflies were frozen and placed in an incubator (70 °C) for 48 hours to assess adult dry mass. Mass of both pupae and adults were taken using an analytical Toledo ABS 104 meter® balance (precision = 0.1 mg). We recorded also development time of third and fourth instars, survivorship until the adult stage, pupal mass, and adult dry mass.

Ant tending behaviour

Ant tending behaviour was examined through both instantaneous and continuous observations (Altmann, 1974) of *P. polibetes* larvae and the two *Camponotus* species. Tending behaviour was defined as the active antennation by the ants on any part of the larva's body, that is, palpation (*sensu* Hinton, 1951). Instantaneous observations of third and fourth instar larvae were made three times a day: morning (9 - 10 a.m.), afternoon (2 - 3 p.m.) and evening (7 - 8 p.m.) (n = 20 larvae per ant species). Evening observations were made with the aid of a flash light covered with red cellophane paper to avoid light incidence on both ants and larvae (see Del-Claro & Oliveira, 1999). Continuous observations were performed in the afternoon for 1 hour, during which we recorded frequency and duration of both *C. melanoticus* and *C. crassus* tending behaviour on fourth instar larvae (*n* = 10 larvae per ant species). Tending occasions lasting less than one second were not included in the analysis.

Statistical analysis

Survivorship of *P. polibetes* among treatments was compared using Chi-Square tests. Effects of treatment and sex on performance traits were analyzed with a two-way ANOVA followed by Bonferroni post tests. Frequency and duration of tending behaviour by the two *Camponotus* species were compared with Mann-Whitney tests. Instantaneous observations of tending behaviour by *C. crassus* and *C. melanoticus* at different times of the day were analyzed with a three-way repeated measures ANOVA.

Results

Performance in the presence or absence of ants

Larval survivorship did not differ among treatments (Table 1; Chi-Square test, $\chi^2_2 = 0.07$, $P = 0.96$). There was no effect of sex on development time (two-way ANOVA) (Tables 1, 2). Larvae tended by *C. melanoticus* took longer to pupate compared to both untended controls and larvae tended by *C. crassus* (Bonferroni post tests, $P < 0.01$). No difference was found between larvae tended by *C. crassus* and control larvae (Bonferroni post tests, $P > 0.05$). In contrast, pupal weight was affected by both sex and treatment (two-way ANOVA) (Tables 1, 2). Male pupae were significantly heavier than female pupae in all treatments. Pupae whose larvae were tended by *C. crassus* were lighter than pupae from untended control larvae (Bonferroni post tests, $P < 0.05$). Pupal weight, however, did not differ between larvae tended by *C. melanoticus* vs. untended controls, or between larvae tended by *C. melanoticus* vs. *C. crassus* (Bonferroni post tests, $P > 0.05$). Pupal development time did not vary with sex or treatment (data not shown). Adult females were heavier than males (two-way ANOVA) (Tables 1, 2). Treatment had no significant effect on adult weight, but adults emerging from *C. crassus*-tended larvae were generally lighter compared to the other treatments (two-way ANOVA) (Tables 1, 2).

Ant tending behaviour

Both continuous and instantaneous observations revealed that *C. melanoticus* tended larvae more persistently than *C. crassus* (Fig. 2) (Mann-Whitney tests; continuous observations, time spent

Table 1. Performance traits of third and fourth instar larvae of *Parrhasius polibetes* assigned to the following treatments: no ants (control), tended by *Camponotus melanoticus*, tended by *Camponotus crassus*. For statistical analysis, see Table 2.

	Control (n = 20)	*C. melanoticus* (n = 20)	*C. crassus* (n = 20)
Survivorship (%)	100 (20)	90 (18)	100 (20)
Development time (days)			
Females	10.20 ± 0.92 (10)	11.33 ± 0.71 (9)	10.11± 0.60 (11)
Males	10.50 ± 0.20 (10)	11.11 ± 0.79 (9)	9.73 ± 0.47 (9)
Pupal mass (mg)			
Females	26.79 ± 0.03 (10)	26.51 ± 0.04 (9)	24.27 ± 0.03 (11)
Males	31.29 ± 0.04 (10)	29.21 ± 0.04 (9)	28.09 ± 0.03 (9)
Adult mass (mg)			
Females	5.12 ± 0.01 (10)	5.33 ± 0.01 (9)	4.71 ± 0.01 (11)
Males	4.74 ± 0.01 (10)	4.48 ± 0.01 (9)	3.99 ± 0.05 (9)

Table 2. Two-way ANOVA of the effects of treatment and sex on *Parrhasius polibetes* performance. Treatments: no ants (control), tended by *Camponotus melanoticus*, tended by *Camponotus crassus*. Asterisks denote significant factors. See also Table 1.

Performance trait	Factor	Degrees of Freedom	Sum of Squares	F	*P*
Development time	Treatment	2	16.86	18.17	<0.01*
	Sex	1	0.14	0.31	0.58
	Interaction	2	1.21	1.31	0.28
	Error	52	24.13		
Pupal weight	Treatment	2	0.01	3.44	0.04*
	Sex	1	0.02	16.24	<0.01*
	Interaction	2	0.00	0.44	0.71
	Error	52	0.06		
Adult weight	Treatment	2	0.01	3.06	0.06
	Sex	1	0.02	8.95	<0.01*
	Interaction	2	0.00	0.40	0.67
	Error	52			

Table 3. Three-way repeated measures ANOVA of the effects of treatment (tended by *Camponotus melanoticus* or *C. crassus* ants), sex, and time of the day (morning, afternoon, evening) on *Parrhasius polibetes* larvae. Asterisk denotes the significant factor.

Factor	Degrees of Freedom	Sum of Squares	F	*P*
Treatment	1	4584.24	24.03	< 0.01*
Sex	1	3.34	0.018	0.90
Treatment vs. Sex	1	106.02	0.56	0.46
Error	34	6486.95		
Time	2	190.02	0.59	0.55
Time vs. Treatment	2	53.09	0.17	0.85
Time vs. Sex	2	69.76	0.22	0.80
Treatment vs. Time vs. Sex	2	449.21	1.40	0.25
Error	68	10872.71		

tending: U = 0.00; *P* < 0.01; number of tending occasions: U = 5.5; *P* < 0.01; instantaneous observations: U = 41.0; *P* < 0.01). Intensity of tending behaviour by the two ant species, however, did not vary at different times of the day, or with sex of *P. polibetes* (three-way ANOVA, Table 3).

Discussion

Tending behaviour by the two *Camponotus* species incurs different costs and benefits for different life stages of *P. polibetes*. *Camponotus melanoticus* antennated larvae more frequently than *C. crassus* and, as a consequence, larvae took more time to pupate compared to control larvae and to those tended by *C. crassus*. Such a delay presumably increases susceptibility to natural enemies and postpones the first reproduction (see Robbins, 1991; Fiedler & Hummel, 1995). Presumably to compensate, larvae attained a greater mass at pupation, thus reflecting a trade-off between development time and size.

The same trade-off occurs with larvae tended by *C. crassus*, but in the opposite direction. In this case they develop as fast as control larvae but at the same time gain protection against natural enemies through ant tending (Kaminski *et al.*, submitted). The corresponding cost took place in the next stage: the energy spent to produce secretion rewards for *C. crassus* resulted in decreased pupal weight. Thus the trade-off between development time and size in *P. polibetes* larvae is resolved in contrasting ways depending on the species of tending ant. In the adult stage, control and treatment individuals no longer differed in weight (forewing length also did not differ among control and treatment groups; data not shown). This indicates that development time-size trade-offs operate for the immature stages only, with no detectable effects on adults.

Previous studies have found different effects of ant attendance on butterfly life history traits, and frequently showed a trade-off between larval development time and pupal size. The corresponding outcomes ranged from compensation (Robbins, 1991; Wagner, 1993; current study) to overcompensation (DeVries & Baker, 1989; Fiedler & Hölldobler, 1992; Wagner, 1993; Fiedler & Hummel, 1995) in facultative systems (but see Trager & Daniels, 2009, for no detectable effect). On the other hand, in obligate systems the outcomes apparently does not vary as in the facultative systems, since the interacting species are more tightly coevolved (Pierce *et al.*, 1987; Baylis & Pierce, 1992; Cushman *et al.,* 1994).

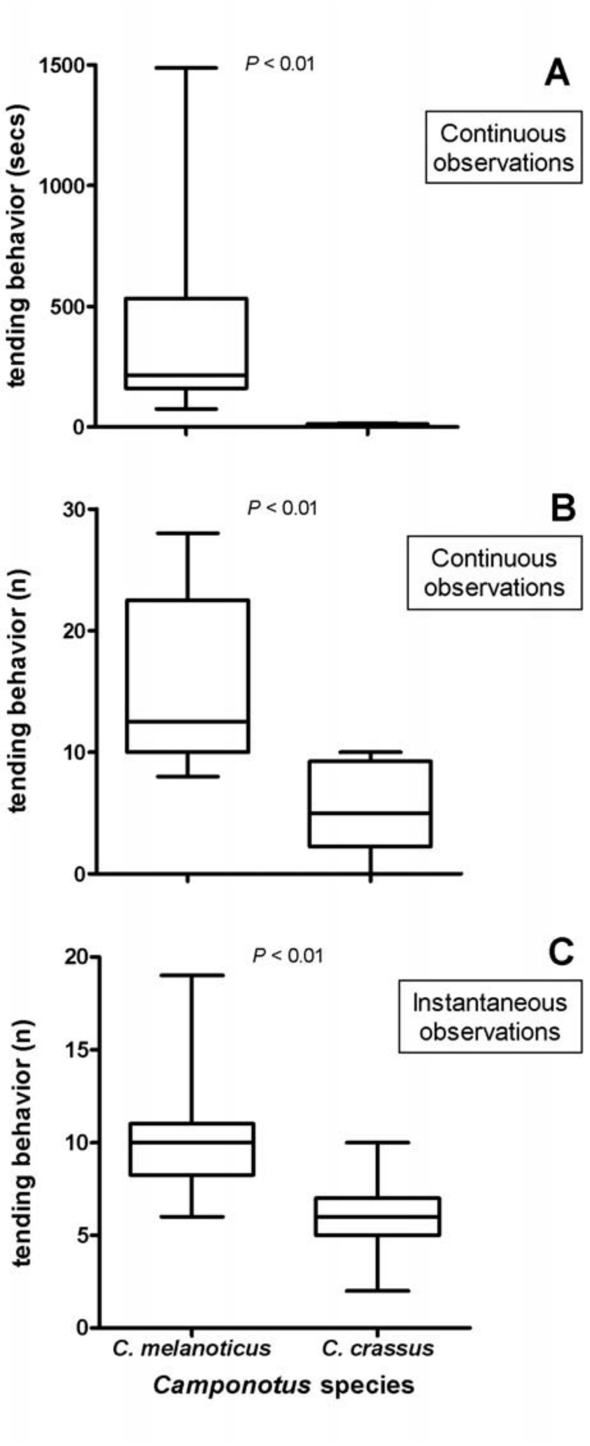

Fig. 2. Tending behaviour of *C. melanoticus* and *C. crassus* in relation to *P. polibetes* late instar larvae. **A**, time spending tending and **B**, number of tending events recorded through continuous observations. **C**, number of tending events recorded through snapshots observations. Lines represent medians, boxes show the lower and upper quartiles, whiskers show total range.

Apart from ant species, sex also needs to be taken into account for addressing the effects of ant tending behaviour on *P. polibetes* performance. Male pupae were heavier than females, regardless of the treatment. Since pupal weight is usually related to water content, or meconium, male pupae may produce more liquid (which in turn is lost at adult emergence). Adult females, however, were larger than males in all treatments. For animals in general and particularly in invertebrates, females become larger than males (see Fairbairn, 1997). Studies on the costs of myrmecophily in lycaenids have mostly focused on pupal rather than adult mass, making comparisons difficult in this regard. The few studies that have analyzed both pupal and adult stages found similar results to our study (Wagner & Del-Rio, 1997).

The delay in development time of *P. polibetes* larvae tended by *C. melanoticus* may be caused by two factors that are not mutually exclusive. First, intense tending may stimulate a substantial production of larval secretion, creating the need to consume more food than usual, which in turn leads to a delay in larval development time. Second, intense tending may disrupt normal feeding, impairing growth rate and thus development. Tending intensity in terms of ant number and persistency of contact has been suggested as a key factor determining the extent of the costs imposed on myrmecophilous larvae (see Fiedler & Hummel, 1995). Studies on food consumption and assimilation efficiency in lycaenid larvae facultatively tended by different ant species were not conclusive in this regard (Wagner & Del-Rio, 1997). Therefore the sources of variation in larval response to patterns of ant attendance remain unclear.

Secretion production has been considered a major cost for myrmecophilous larvae, thus mechanisms to avoid ant overexploitation are expected to occur (Agrawal & Fordyce, 2000). In fact myrmecophilous larvae have been shown to produce the secretion only when properly tended by ants (Daniels *et al*., 2005). Moreover, some lycaenid species are able to control secretion levels under variable circumstances: they offer increased amounts to ants in dangerous situations (Leimar & Axën, 1993; Agrawal & Fordyce, 2000), at the beginning of ant recruitment, and at different larval instars (DeVries, 1988; Fiedler & Hummel, 1995).

Camponotus melanoticus tended late instar larvae of *P. polibetes* more persistently than *C. crassus* at both short and long term time scales, which may be a result of differing nutritional requirements between species. As workers of *C. melanoticus* are on average larger than *C. crassus*, they are likely more nutritionally demanding than *C. melanoticus*. Indeed, *C. melanoticus* workers have a greater capacity to expand their abdomen than *C. crassus*, which results in increased storage of secretions (see Hölldobler & Wilson, 1990). Alternatively, such variation may be related to gustatory responses of each ant species (see Hojo *et al.*, 2008). Although the time of the day did not influence ant tending behaviour in captivity, *C. melanoticus* is seen mostly at night in the field, whereas *C. crassus* is diurnal (Oliveira & Brandão, 1991; Del-Claro & Oliveira 1999; Schoereder *et al.,* 2010). Invariable activity rhythm in the laboratory by either ant species was probably caused by their confinement with *P. polibetes* larvae under a limited foraging range compared to field conditions.

The moderate levels of attendance displayed by *C. crassus* on *P. polibetes* larvae under laboratory conditions probably mirrors levels in nature. Indeed, the probability of finding a late instar larva of *P. polibetes* being attended by ants in the field is low. There is about a 20% chance of a given *P. polibetes* larva be attended by ants when it is near an ant-tended treehopper aggregation, a value that drops significantly when treehoppers are absent (L.A. Kaminski, submitted). Therefore, it is expected that the costs of myrmecophily related to reward production would be even lower in the field than in the laboratory. In addition, even under overexploitation by tending ants (i.e., when larvae are confined with workers of *C. melanoticus*), compensatory mechanisms take place to minimize the costs of myrmecophily. It is usually thought that facultative myrmecophilous associations incur low costs for larvae, thus reflecting an evolutionary stable strategy (see Fiedler & Hölldobler, 1992; Fiedler & Hummel, 1995). Our findings strongly support this suggestion.

To our knowledge this is the first demonstration that species-specific variation in ant tending behaviour incurs different costs for a trophobiont insect. Pairwise comparisons provide only a limited view of the variation in the costs of ant-butterfly interactions that may occur in nature (see Wagner, 1993). Our results bring to light the need for assessing the costs of facultative myrmecophily, taking into account a multispecific scenario and its inherent variation. Assessing variable patterns of ant tending behaviour in the field, as well as characterizing patterns of reward secretion by larvae of *P. polibetes* constitute promising research venues in ant-butterfly interaction systems.

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CAPÍTULO IV

INTERACTION BETWEEN MUTUALISMS: ANT-TENDED BUTTERFLIES EXPLOIT ENEMY-FREE SPACE PROVIDED BY ANT-TREEHOPPER ASSOCIATIONS [*]

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ABSTRACT: Although mutualisms have been intensively investigated, demonstration of indirect effects between co-occurring mutualistic systems is rare. For instance, the ecological consequences of co-occurrence of ant-tended insects on a plant have never been examined for survival effects on either trophobiont species. Here, we assess the selective pressures mediating co-occurrence of a facultative ant-tended butterfly (*Parrhasius polibetes*) with ant-tended treehoppers (*Guayaquila xiphias*) on *Schefflera vinosa* shrubs. We evaluated host plant selection and caterpillar survival in *P. polibetes* in the presence and absence of ant-treehopper associations. Paired trials revealed that butterflies preferably oviposit on branches hosting ant-tended treehoppers than on naturally unoccupied ones, or from which the interaction was removed. Presence of ant-tended treehoppers on a branch reduced the abundance of *P. polibetes*' natural enemies, and improved caterpillar survival both in pre-myrmecophylic and ant-tended phases. Thus ant-tended treehoppers create an enemy-free space on foliage that butterflies exploit to protect larval offspring. These findings connect two widely documented ant-trophobiont mutualisms and highlight the importance of considering multiple interactions for a proper understanding of ant-plant-herbivore systems. Detection of other ant-based mutualisms upon oviposition to improve offspring survival may have represented an important evolutionary step in the process of host plant selection in facultative myrmecophilous butterflies.

Keywords: Ant-plant-herbivore interactions; cerrado savanna; insect trophobionts; natural enemies; oviposition behavior; trophic and non-trophic indirect effects.

Introduction

A species niche dimensions are determined by many variables, including abiotic factors, the nature and rate of available food resources, interspecific competition for limiting resources such as food or space, and natural enemies (Jeffries and Lawton 1984). For insect herbivores, natural enemies (predators and parasitoids) are recognized as one of the most important factors determining niche dimensions (see Price et al. 1980; Singer and Stireman 2005). Ants are extremely abundant on foliage and are considered major predators of insect herbivores in tropical habitats (Jeanne 1979; Floren et al. 2002). A main factor accounting for the remarkable dominance ants on plant surface is the high occurrence of predictable liquid food sources such as extrafloral nectaries and honeydew-producing insects (Rico-Gray and Oliveira 2007). The frequent presence of liquid-feeding ants on foliage represents a constant threat to herbivore insects because exudatefueled ant foragers of particularly dominant species complement their diets by actively preying on herbivores (Davidson et al. 2003). Thus insect herbivores face a major problem in order to feed on plant tissue: they need to find a safe spot on foliage, that is, an “enemy-free space” (Price et al. 1980). In this scenario, the capacity to make appropriate colonization decisions in the process of host plant selection is an important behavioral trait in insect herbivores (Thompson and Pellmyr 1991). Hence information about predation risks can be critical and natural selection may favor the ability of herbivores to detect and avoid predators before oviposition, especially if offspring mortality risk is high (Schmitz et al. 2004). This was recently demonstrated for a tropical butterfly that feeds on a risky ant-visited plant (Sendoya et al. 2009).

Some types of insect herbivores, however, not only circumvent ant predation but even attract them for their own benefit. Myrmecophily (i.e., life associated with ants) is widespread among numerous insect taxa, especially in the Hemiptera and Lepidoptera (Hölldobler and Wilson 1990). By producing liquid nutritional rewards, such insects attract aggressive ants that collect the exudate and in return act as bodyguards by warding off their natural enemies (a relationship known as trophobiosis; see Stadler and Dixon 2008). As a result of intense patrolling activity in the vicinity of their exudate-producing partners, aggressive ants create an enemy-free space around the trophobionts. Due to this important benefit, natural selection on trophobiont herbivores may favor behavioral abilities to detect mutualistic ants before oviposition and select more protected (i.e., ant-occupied) foliage that improve offspring survival. This is the opposite behavioral pattern recorded for non-myrmecophilous herbivores (e.g., Sendoya et al. 2009).

In Lepidoptera, myrmecophily is widespread in two butterfly families (Lycaenidae and Riodinidae) whose larvae produce nutritional liquid rewards to tending ants (Fiedler 1991; Pierce et al. 2002). Butterfly-ant symbiosis probably arose on plants that commonly have liquid food sources for ants such as extrafloral nectaries or honeydew-producing hemipterans (DeVries 1991), and it is expected that these ant attractants should affect oviposition decisions and host plant use in myrmecophilous butterflies (Atsatt 1981a; Thompson and Pellmyr 1991). Indeed, species from different lineages of myrmecophilous butterflies exploit plants that are constantly visited by ants, either because they have ant attractants and/or because they regularly house ant colonies (e.g., Cottrell 1984; Maschwitz et al. 1984; DeVries and Baker 1989).

Although ant-based mutualistic systems frequently include multiple participants (see Bronstein and Barbosa 2002), the range of indirect effects among interacting species remains poorly documented. For instance the ecological consequences of co-occurrence with hemipteran trophobionts have been only marginally treated with respect to host plant selection by myrmecophilous butterflies (see Atsatt 1981b; Pierce and Elgar 1985), and have never been examined for effects on larval survival. Recently, Oliveira and Del-Claro (2005) found evidence of spatial-temporal co-occurrence between larvae of the facultative myrmecophilous butterfly *Parrhasius polibetes* (mentioned as *Panthiades polibetes*; Lycaenidae) and ant-tended treehopper aggregations (*Guayaquila xiphias*; Membracidae) (figs. 1, 2*A*-*B*). This system offers an ideal opportunity to investigate how the presence of an ant-tended herbivore on a plant can affect colonization decisions by a myrmecophilous butterfly. Our hypothesis is that *P. polibetes* butterflies would prefer to lay eggs near honeydew-producing treehoppers because the enemy-free space generated by tending ants in the vicinity of such trophobionts significantly improves larval survival.

We conducted a series of field experiments to assess the selective pressures mediating the co-occurrence of *P. polibetes* larvae with ant-treehopper associations. Specifically, we addressed the following questions: (1) Do butterflies use ant-treehopper associations as a cue for host plant selection? (2) Does larval survival improve in the vicinity of ant-treehopper associations? (3) Does the presence of ant-treehopper associations decrease the abundance of potential natural enemies on a plant, thus creating an “enemy-free space” for butterfly larvae? (4) Does co-occurrence with ant-tended treehoppers improve discovery of butterfly larvae by prospective tending ants? A full assessment of the reciprocal indirect interactions between the two coexisting trophobiont species is beyond the scope of this study, although the whole scenario is addressed in the discussion.

Methods

Study site and system

The study was carried out in a site of cerrado savanna of the Laboratório Nacional de Luz Síncrotron (22° 48’S, 47°03’ W) in Campinas, southeast Brazil. The vegetation consisted of a dense scrubland of shrubs and trees, classified as cerrado *sensu stricto* (Oliveira-Filho and Ratter 2002). Experiments were performed in 2008 and 2009 during the dry season (May–July), when adult butterflies are abundant and larval host plants have plenty of inflorescences (Del-Claro and Oliveira 1999; Rodrigues et al. 2010).

The study system includes the gregarious honeydew-producing treehopper *Guayaquila xiphias* which commonly occurs on shrubs of *Schefflera vinosa* (=*Didymopanax vinosum*; Araliaceae) in

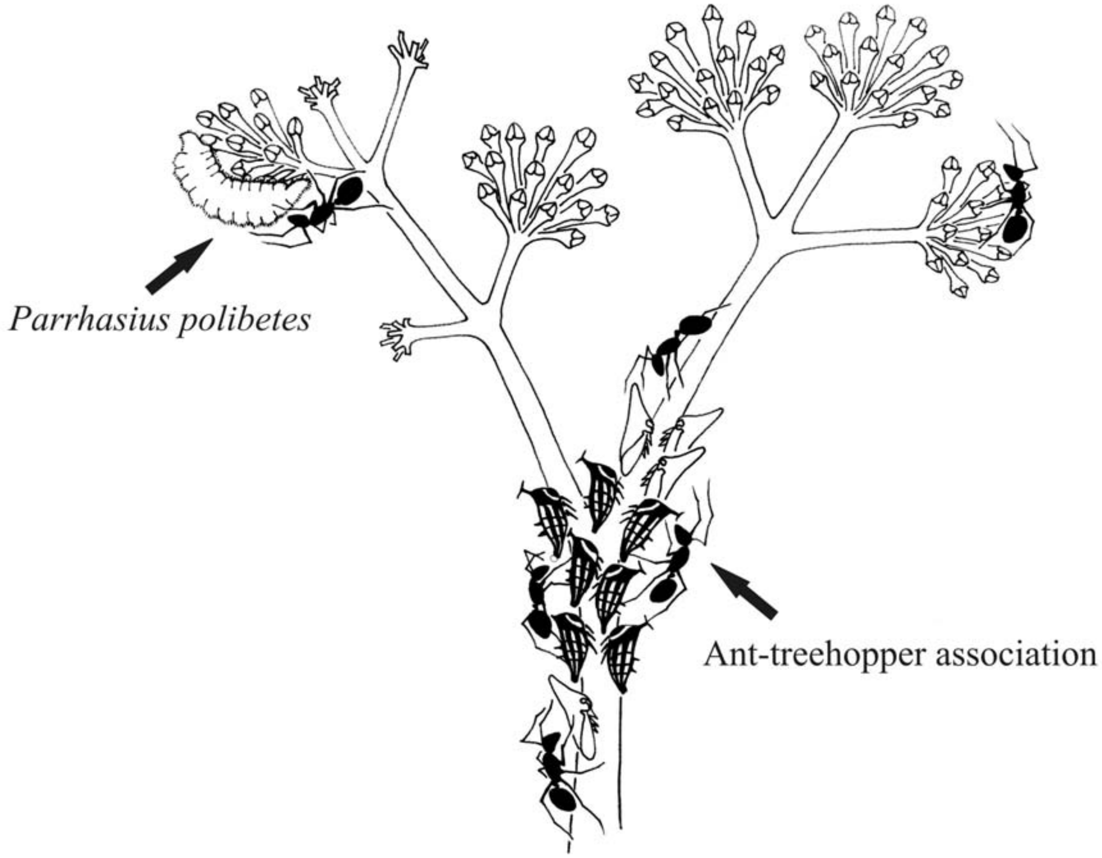

Figure 1: Schematic representation of the study system involving ant-tended *Guayaquila xiphias* treehoppers (adults and nymphs), myrmecophilous larvae of the butterfly *Parrhasius polibetes*, and the host plant *Schefflera vinosa*. Ants (*Camponotus rufipes*) from the same colony attend both trophobiont species on the inflorescence branch.

cerrado areas of southeast Brazil (fig. 2*B*). The treehopper can be tended day and night by more than 20 species of honeydew-gathering ants, which climb onto host plants after finding scattered droplets of flicked honeydew on the ground (Del-Claro and Oliveira 1996, 1999). The aggressive behavior of ants near *G. xiphias* aggregations decreases the incidence of natural enemies (salticid spiders, syrphid flies, and mymarid parasitoid wasps) on the host plant, and increases treehopper survival (Del-Claro and Oliveira 2000). Moreover, patrolling behavior by honeydew-gathering ants can reduce plant damage by other herbivores (Oliveira and Del-Claro 2005). Plants with *G. xiphias* aggregations, however, are more infested by *Parrhasius polibetes* butterflies, whose ant-tended larvae feed on reproductive plant tissue (buds and flowers) (Oliveira and Del-Claro 2005; Rodrigues et al. 2010). Female butterflies lay about 3 eggs on the inflorescences per oviposition event; the larvae are solitary and develop in four instars (Kaminski 2010). Early non-myrmecophylic instars (1st and 2nd) present numerous morphological and behavioral defensive traits to appease and/or hide from ants (Malicky 1970). The dorsal nectar organ (DNO) becomes functional in the 3rd instar and caterpillars can be facultatively tended by the same ants that attend *G. xiphias* aggregations on a plant (fig. 1). Immature stages of *P. polibetes* are attacked by a variety of natural enemies (figs. 4*A*-*B*), but larvae are mostly attacked by spiders (Araneidae, Thomisidae, and Salticidae) and parasitoid wasps (Braconidae, Chalcididae, and Ichneumonidae) (Kaminski 2010).

The impact of ant-treehopper associations on host plant selection by Parrhasius polibetes

To evaluate the role of ant-tended treehoppers as a cue used for host plant selection by *P. polibetes*, we carried out a series of paired oviposition trials in the field (see also Freitas and Oliveira 1996; Sendoya et al. 2009). For each tagged shrub of *S. vinosa* we selected a pair of branches at approximately the same height (1 - 2m), and with similar inflorescence size, and number of leaves. The distance between branches of a pair ranged from 0.4 to 1 m. Each branch of a selected pair was designated as "occupied" by an ant-*Guayaquila* association, or "unoccupied" by such association. Two groups of experimental host plants were set simultaneously for the oviposition trials. In one group of plants, we did not manipulate insect presence within paired branches: one branch was naturally occupied by ant-tended treehoppers and the other was unoccupied (n = 20 plants "without manipulation"). In a second group of plants, however, both paired branches were already occupied by ant-treehopper associations upon our arrival. We then manipulated the presence of ant-treehopper associations by manually removing them from one of the branches (assigned by the flip of a coin). Trials consisted of experimental pairs formed by a branch occupied by ant-tended treehoppers and a branch from which the latter had been recently manually removed (n = 16 plants "with manipulation"). We used only *G. xiphias* aggregations tended by *Camponotus rufipes* or *Camponotus renggeri* (Formicinae). Both species are similar in size (~ 0.8 cm) and tending behavior, behave aggressively toward intruders, and monopolize day and night the *G. xiphias* aggregations (Del-Claro and Oliveira 1999). Unoccupied branches were applied at their base a sticky barrier of Tanglefoot® (Tanglefoot Co., Grand Rapids, MI) to prevent ant access. Occupied branches had resin applied on only one side so that ants could still reach the foliage. To control for unknown effects of common insect visitors other than ants and treehoppers on butterfly oviposition, we pinned one dried honeybee specimen (*Apis mellifera*, common flower visitor) next to the inflorescence of each experimental branch (for a similar method see Sendoya et al. 2009). Vegetation bridges providing aerial ant access to experimental plants were removed. Nearby branches with inflorescences were clipped off so as to induce prospective ovipositing butterflies to choose between selected branches during oviposition experiments. Except for treehoppers and tending ants, all eggs and larvae of *P. polibetes* as well as all other arthropods were removed from the branches before trials (but see above trials "with manipulation"). Experimental branches were set up at 14:30 h, and checked after 48 hours. Only plants receiving at least one egg on either branch were considered for the analyses (n = 36). Whenever an oviposition event was seen, all behavioral aspects of host plant selection by female *P. polibetes* were reported (fig. 2*A*). Because experiments were performed during the period of highest butterfly abundance, oviposition decisions were assumed to be independent (i.e., made by different females).

Indirect effects of ant-treehopper associations on larval survival

The indirect effects of the presence of ant-treehopper associations on *P. polibetes* larvae were evaluated through two field experiments, in which caterpillars were placed on *S. vinosa* host plants and regularly checked for survival in subsequent days. For both experiments we selected one pair of similar-sized branches, in which one branch was naturally "occupied" by ant-tended treehoppers and one branch was naturally "unoccupied" by the latter. As with the oviposition experiment, we used only *G. xiphias* aggregations tended day and night by *Camponotus rufipes* or *C. renggeri*.

Experiment I: Larval survivorship. This 25-day experiment evaluated the indirect effect of ant-treehopper associations on larval survival in *P. polibetes* and on the abundance of its potential natural enemies on host plants (n = 25). The experiment included both the pre-myrmecophylic early larval phase (~ 12 days), as well as the 3rd and 4th myrmecophylic instars. Branches occupied by ant-tended treehoppers received Tanglefoot resin on only one side so that ants could still reach the foliage, whereas unoccupied branches had resin applied at the base to prevent ant access. Neighboring plant bridges were clipped to impede aerial ant access to plants. On each branch of a pair we placed one newly-hatched *P. polibetes* larva (~ 0.2 cm) obtained from field-collected eggs. Larval survival on paired branches was checked daily for up to 5 min per plant (9:00-14:00 h) over 25 days. Because the larvae pupate off the host plant, caterpillars were removed from experimental plants on the fifth day of the last instar. Missing larvae were considered dead, although we continued to check the experimental branches until the end of the experiment, when live caterpillars were collected for adult emergence in the laboratory. Potential natural enemies of *P. polibetes* larvae (spiders and parasitoid wasps) were checked every other day for up to 10 min per plant (9:00-14:00 h).

Experiment II: Levels of ant tending. In this 10-day experiment we assessed the indirect effects of ant-treehopper associations on larval survival in *P. polibetes* during the myrmecophylic period (3rd and 4th instars) in which caterpillars can potentially be tended by ants on host plants. In this experiment, however, ants had free access to both branches in a pair and thus were able to find *P. polibetes* larvae on either type of foliage: “occupied” or “unoccupied” by ant-tended treehoppers (n = 25 plants). One newly-hatched 3rd instar larva (~ 0.8 cm; obtained from field-collected eggs) was placed on each branch of a pair. Larval survival on either branch was checked daily for up to 5 min per plant (9:00-14:00 h) over 10 days; missing larvae were considered dead. We also recorded the number of scout ants walking on foliage or tending *P. polibetes* larvae on either type of branch, as well as the abundance of potential natural enemies (inspections of up to 10 min; 9:00-14:00 h).

Statistical analyses

The proportion of experimental branches receiving eggs, and the number of eggs oviposited on each branch category were evaluated with *G* and Mann-Whitney *U* tests, respectively. Survival curves of *P. polibetes* larvae were analyzed with log-rank (Mantel-Cox) tests, both for the pre-myrmecophylic larval phase in Experiment I and for the entire extent of Experiments I and II. Abundance data of natural enemies (spiders and parasitoid wasps) on branch pairs were analyzed with repeated-measures ANOVAs, fitting paired experimental branches as a blocking factor and treatment (presence or absence of ant-tended treehoppers) as a fixed effect. Separate ANOVAs were performed for the pre-myrmecophylic larval phase. We performed square-root transformations on the data to stabilize treatment variances for the statistical analyses. Mean numbers of ants on branches or tending experimental *P. polibetes* larvae (Experiment II) were analyzed using Mann-Whitney *U* tests.

Results

Presence of ant-tended treehoppers and host plant selection by butterflies

Parrhasius polibetes females normally flutter around the host plant for 5 to 15 s (n = 11) before oviposition (pre-alighting phase). In the post-alighting phase, however, the butterflies take 5 to 60 s (n = 11) and in this process they repeatedly touch the flower bud surface with the tip of the abdomen before ovipositing (fig. 2*A*). Direct contact of egg-laying females with foliage-dwelling ants was never observed. Paired oviposition experiments revealed that *P. polibetes* females prefer to lay eggs on branches of *S. vinosa* hosting ant-tended treehoppers compared to locations without such associations. This behavioral trend occurred in the experiments either without (*G*-test, $G = 7.43$, df = 1, $P < .01$; fig. 2*C*) or with the manipulation of ant-treehopper associations (*G*-test, $G = 9.46$, df = 1, $P < .01$; fig. 2*E*). Additionally, on average butterflies oviposited more eggs on branches with ant-tended treehoppers, both without (Mann-Whitney test, $U = 50.50$, df = 1, $P < .05$; fig. 2*D*) or with the manipulation procedure (Mann-Whitney test, $U = 38.50$, df = 1, $P < .05$; fig. 2*F*).

Indirect effects of ant-treehopper associations on larval survival

Parrhasius polibetes larvae survive better when developing on branches of *S. vinosa* hosting ant-tended treehoppers than on branches without these associations (Log-rank (Mantel-Cox) test, $\chi^2 = 4.54$, $P < .001$; fig. 3*A*). After 25 days, survivorship of butterfly larvae in the vicinity of ant-tended treehoppers was approximately 6-fold higher than away from trophobionts. In addition, survival differences between paired branches were already significant in pre-myrmecophylic phase, when the dorsal nectar organs are non-functional (Log-rank (Mantel-Cox) test, $\chi^2 = 4.02$, $P < .05$; fig. 3*A*). This early difference in larval survival may be related to the indirect effects of the presence of ant-treehopper associations on occupied branches, which reduced the abundance of

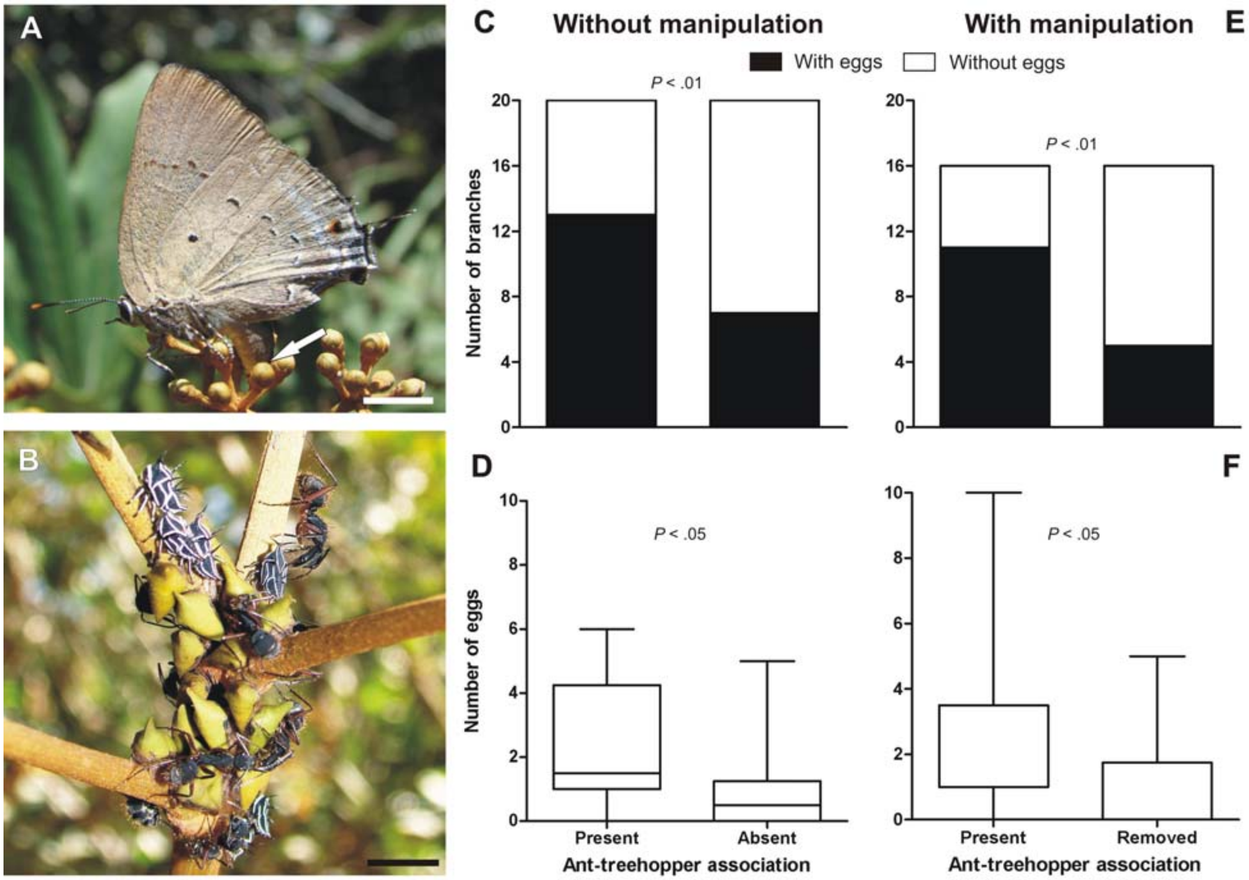

Figure 2: *A*, *Parrhasius polibetes* butterfly laying eggs on a *Schefflera vinosa* inflorescence; note abdomen tip curved (arrow). *B*, *Guayaquila xiphias* aggregation (adults and nymphs) tended by *Camponotus rufipes* ants on *S. vinosa*. Scale bars = 0.6 cm. *C-F*, Oviposition pattern by *P. polibetes* butterflies during choice experiments (48-h trials) using paired branches of *S. vinosa. C-D*, Branches were naturally occupied by ant-treehopper associations, or unoccupied by the latter (Without manipulation). *E-F*, Both branches were occupied by ant-treehopper associations prior to trials; after manipulation the experimental pairs consisted of one branch occupied by ant-tended treehoppers and one branch from which the latter were manually removed (With manipulation). *C-E*, Selection of plant location by egg-laying butterflies. *D-F*, Number of eggs laid per branch. Boxes show the lower and upper quartiles; whiskers show total range. Low quartile and the median are 1 in the control; minimum value, lower quartile and the median are 0 in the treatment.

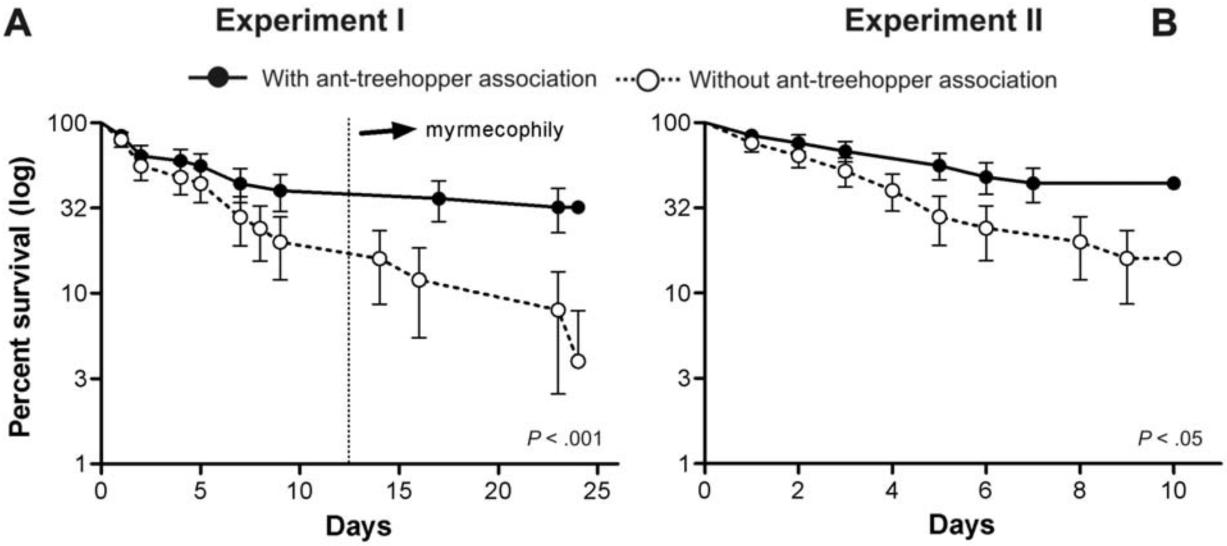

Figure 3: *A, B,* Survival curves of *Parrhasius polibetes* larvae on paired branches of *Schefflera vinosa* through time, as a function of the presence or absence of ant-treehopper associations. Experiment I (*A*) included both the pre-myrmecophylic early larval phase (~12 days), as well as the 3rd and 4th myrmecophylic instars (dashed line indicates when myrmecophily begins); occasional scout ants were excluded from unoccupied branches. Experiment II (*B*) included only the myrmecophylic larval instars (3rd and 4th), and ants had free access to either branch category. Values are means ± EP.

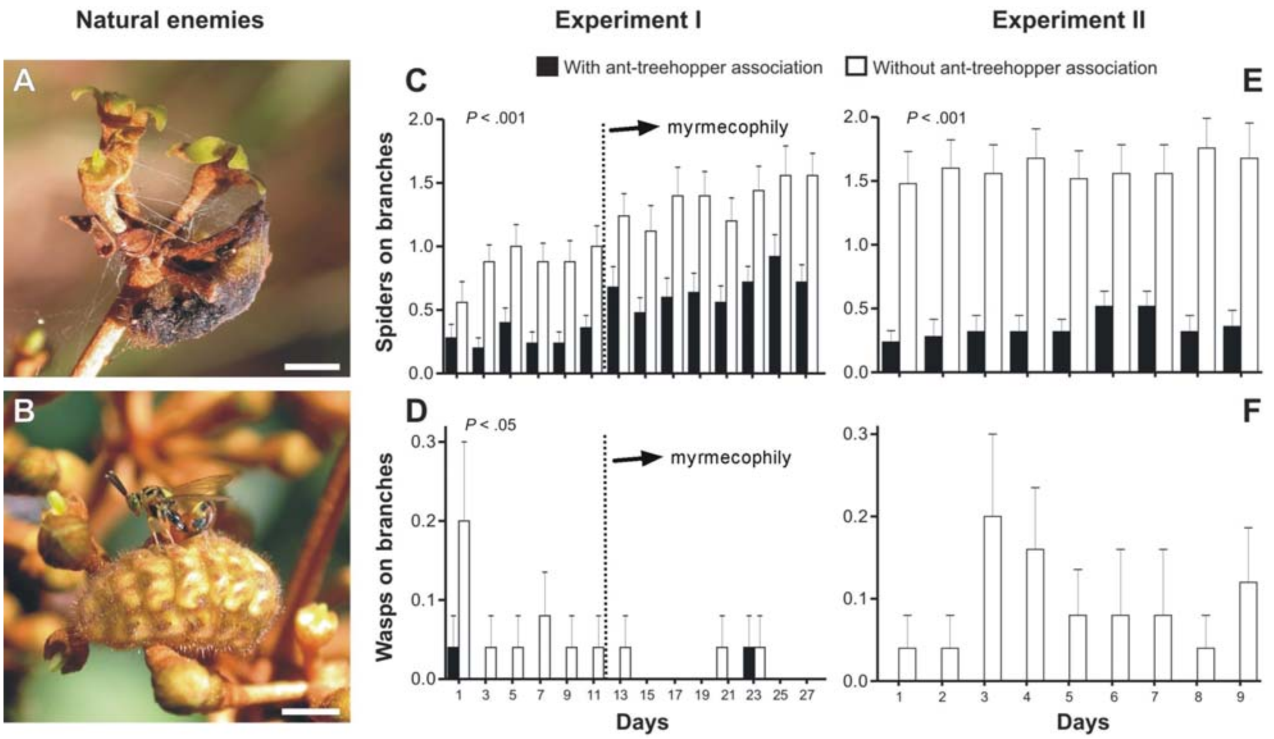

Figure 4: *A*-*B*, Natural enemies of *Parrhasius polibetes* butterflies. *A*, Remains of a caterpillar preyed by an araneid spider. *B*, Wasp (*Conura* sp.; Chalcididae) parasitizing a caterpillar. Scale bars = 0.3 cm. *C*-*F*, Number of natural enemies (spiders and parasitoid wasps) of *P. polibetes* larvae on experimental branches of *Schefflera vinosa* through time, as a function of the presence or absence of ant-treehopper associations. Experiment I (*C*-*D*) included both the pre-myrmecophylic early larval phase (~12 days), as well as the 3rd and 4th myrmecophylic instars (dashed lines indicate when myrmecophily begins); occasional scout ants were excluded from unoccupied branches. Experiment II (*E*-*F*) included only the myrmecophylic larval instars (3rd and 4th), and ants had free access to either branch category. Values are means ± EP. See also Table 1.

Table 1: Repeated-measures ANOVAs performed on the number of natural enemies (spiders and parasitoid wasps) of *Parrhasius polibetes* larvae through time, per experimental plant hosting (occupied) or not (unoccupied) an ant-treehopper association. For Experiment I, a separate analysis was performed for the pre-myrmecophylic larval phase. Calculations were performed on square-root-transformed data. Significant *P* values are in bold. See also fig. 4.

Source	SS	*df*	MS	*F*	*P*
Experiment I (Pre-myrmecophylic phase)					
Spiders					
Ant-treehopper treatment	15.58	1	15.58	13.34	**<.005**
Plant	18.13	24	.75	0.65	.854
Error 1	28.03	24	1.17		
Time	1.88	5	.38	2.89	.050
Interaction time x treatment	1.51	5	.22	1.72	.131
Error 2	92.09	240	.13		
Parasitoid wasps					
Ant-treehopper treatment	.29	1	.29	7.54	**<.005**
Plant	1.13	24	.05	1.21	.854
Error 1	.94	24	.04		
Time	.31	5	.06	1.70	.134
Interaction time x treatment	.10	5	.02	0.53	.750
Error 2	8.79	240	.04		
Experiment I (Whole experiment)					
Spiders					
Ant-treehopper treatment	9.43	1	9.43	15.58	**<.001**
Plant	14.29	24	.60	0.98	.516
Error 1	14.53	24	.60		
Time	5.42	13	.48	11.45	**<.001**
Interaction time x treatment	.34	13	.03	.71	.756
Error 2	22.74	624	.04		
Parasitoid wasps*					
Ant-treehopper treatment	.20	1	.20	6.41	**<.050**
Plant	.86	24	.04	1.15	.36
Error 1	.74	24	.60		
Time	.34	8	.48	1.24	.276
Interaction time x treatment	.24	8	.03	.86	.546
Error 2	13.11	384	.04		
Experiment II					
Spiders					
Ant-treehopper treatment	69.83	1	69.83	56.58	**<.001**
Plant	52.76	24	2.20	1.78	.822
Error 1	29.62	24	1.23		
Time	1.02	8	.13	0.92	.500

Interaction time x treatment	1.59	8	.20	1.43	.183
Error 2	53.31	384	.14		
Parasitoid wasps**					
Ant-treehopper treatment	.82	1	.82	4.10	.054
Plant	4.81	24	.02	1.00	.500
Error 1	4.81	24	.02		
Time	.29	8	.04	1.50	.154
Interaction time x treatment	.29	8	.04	1.50	.154
Error 2	9.16	384	.02		

* Days in which parasitoid wasps were not recorded on either experimental branch were excluded from the analysis (see fig. 4*D*).

**Due to the complete absence of parasitoid wasps on branches occupied by ant-treehopper associations (see fig. 4*F*), an appropriate statistical treatment with ANOVA was not possible. However, when considering the number of branches in each category with at least one wasp record during the whole experiment, a significant negative effect of ant-tended treehoppers on wasp occurrence is detected (G = 12.62, df = 1, $P < .001$).

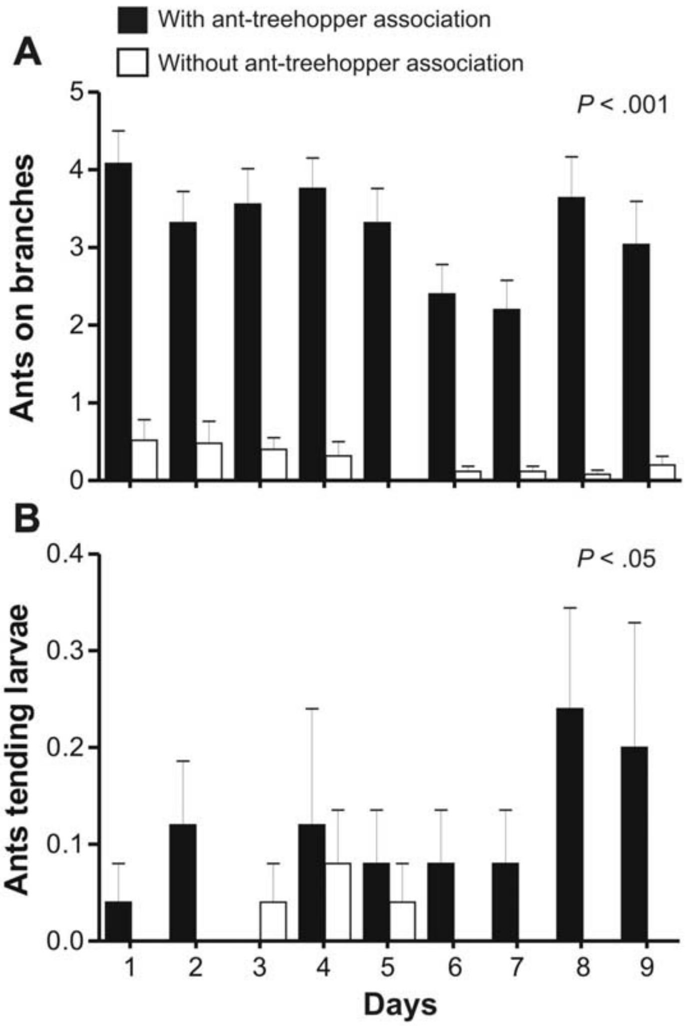

Figure 5: *A*-*B*, Ant foraging pattern on the host plant *Schefflera vinosa*. *A*, Number of ant foragers walking on experimental branches. *B*, Number of ants tending *Parrhasius polibetes* larvae (3rd and 4th instars) through time, as a function of the presence or absence of ant-treehopper associations. Values are means + EP.

natural enemies during the first 9 days when caterpillar are unattended by ants (Table 1; figs. 4*C*-*D*). In the myrmecophylic phase, due to the combined ability to attract ants by treehoppers and larvae, survival differences between paired branches persisted consistently until the end of the experiment (fig. 3*A*). Indeed predation by spiders and parasitism by wasps accounted, respectively, for 20.8% and 12.5% of the identifiable death causes of *P. polibetes* larvae developing on branches without ant-treehopper associations (see figs. 2*A*-*B*, 3*A*; total mortality of 96%). We were unable to detect mortality sources on branches occupied by ant-tended treehoppers (fig. 3*A*; total mortality of 68%).

Experiment II revealed that larval survival in the vicinity of ant-tended treehoppers is nearly 3-fold higher than away from such associations (Log-rank (Mantel-Cox) test, $\chi^2 = 4.62$, $P = .05$; fig. 3*B*). As in Experiment I, branches hosting ant-tended treehoppers had lower numbers of potential natural enemies of *P. polibetes* larvae than branches free from these associations (Table 1; fig. 4*E*-*F*). Although ant access was allowed to either branch category, branches hosting ant-treehopper associations had increased numbers of ant foragers on leaves and inflorescences (Mann Whitney test, $U = 0.00$, df = 8, $P < .001$; fig. 5*A*). Consequently, *P. polibetes* larvae growing on branches with ant-tended treehoppers had a higher probability of being discovered by prospective tending ants than those developing on plant locations visited only by occasional scout ants (Mann Whitney test, $U = 14.00$, df = 8, $P < .05$; fig. 5*B*).

Discussion

Although mutualisms have been intensively investigated in the past decades, very few studies have focused on the interaction between co-occurring mutualistic systems despite their common occurrence in nature (Stanton 2003). Indeed some mutualisms can only be understood within a broad context since pairwise interactions are relatively rare (Bronstein and Barbosa 2003; Holland et al. 2005). The current study is important by unveiling some of the selective pressures acting at the interface of two widely documented ant-based mutualisms that hitherto have been treated mostly as separate systems. We provide the first experimental evidence that an ant-treehopper mutualism can mediate behavioral decisions by a facultative myrmecophilous butterfly, with relevant fitness-related consequences for the latter.

Experimental results show that *Parrhasius polibetes* uses the presence of another ant-trophobiont interaction as an oviposition cue. We also demonstrate that butterfly larvae developing in the vicinity of ant-tended *Guayaquila xiphias* treehoppers survive better compared to those growing on plant locations free from these trophobionts (fig. 3*A*), and that mortality is greater where the butterfly larva relies solely on its own ability to attract ants rather than on the additional pulling power of the treehoppers (fig. 3*B*). Our data show that honeydew-gathering ants around treehopper aggregations create an "enemy-fee space" (Price et al. 1980) for butterfly larvae in the more vulnerable pre-myrmecophylic phase. In addition to growing in a safer place due to the ants' negative impact on natural enemies, caterpillars have an increased chance of being discovered by prospective tending ants if treehoppers are nearby. Thus the spatio-temporal co-occurrence between *P. polibetes* and ant-tended *G. xiphias* treehoppers previously reported by Oliveira and Del-Claro (2005) can be explained by both host plant selection by ovipositing females and increased larval survival near hemipteran trophobionts.

Ant-mediated host plant selection in myrmecophilous butterflies has been suggested for many species, but so far it has only been demonstrated experimentally for a few obligate ant-tended species (e.g., Atsatt 1981b; Pierce and Elgar 1985). For facultative myrmecophilous species, there is only one study providing evidence of ant-mediated oviposition (Wagner and Kurina 1997), although the authors were unable to separate the effects of host plant quality and of nearby ant-tended trophobionts in the choice experiments (see also Oliveira and Del-Claro 2005; Collier 2007). Both these factors were controlled in our experiment by using paired branches of the same plant individual. Moreover, by manipulating the presence of ant-tended treehoppers within paired branches, we discarded the possibility that butterflies and treehoppers merely preferred branches with the same traits (see fig. 2). We have not identified, however, what kind of signal (visual and/or chemical) and which component of the association (ants and/or treehoppers) is most critical in the selection process by egg-laying butterflies. Additional experiments using dried insect specimens should help clarify these issues (see Sendoya et al. 2009).

Host plant selection by phytophagous insects is carried out by the adult female and is often linked to components of immature performance (Price et al. 1980; Thompson and Pellmyr 1991). From this point of view, our results for host plant selection can be explained by improved larval survival on plants offering enemy-free space. The positive effect by tending ants on larval survival through the provision on an enemy-free space on foliage has already been demonstrated for obligate myrmecophilous species (e.g., Pierce et al. 1987). For facultative ant-tended species such as *P. polibetes*, however, there is no consensus on the existence of such benefits (see Pierce and Easteal 1986; DeVries 1991; Peterson 1993; Wagner and Kurina 1997; Weeks 2003). The difficulty in detecting benefits in facultative ant-tended butterfly larvae is probably related with the usual conditionality of facultative mutualisms, since cost/benefit relationships vary over time and space by a number of factors (Bronstein and Barbosa 2002). However, since the association between *G. xiphias* treehoppers and tending ants is relatively stable in cerrado savanna (Del-Claro and Oliveira 1993, 1999), it should provide a favorable environment to maintain the benefits to a nearby ant-tended trophobiont. Indeed, Atsatt (1981a) has suggested that host plant traits such as the presence of honeydew-producing treehoppers may increase ant abundance and predictability, and thus improve the co-occurrence of ants with other insect trophobionts (such as lepidopteran larvae), which may promote myrmecophily.

The main benefit afforded by tending ants to myrmecophilous butterfly larvae is protection against natural enemies, including insect parasitoids, predatory wasps, and spiders (Pierce and Mead 1981; Pierce et al 1987; DeVries 1991). Our results are meaningful by showing that protection to *P. polibetes* larvae from parasitoid wasps and spiders can also be indirectly provided by nearby ant-treehopper associations in the pre-myrmecophylic phase, and persist in the late ant-tended instars. Because Tanglefoot resin also decreased the abundance of walking predators (see Dempster 1967), it is likely that protective effects from ants and differential larval survival were underestimated by our design (fig. 3*A*).

As suggested for other ant-hemipteran associations (see Styrsky and Eubanks 2007), the multitrophic system involving honeydew-producing *G. xiphias* on *S. vinosa* shrubs should be seen as a “keystone interaction”, and can be depicted under the perspective of a non-trophic, indirect interaction web (fig. 6; see also Ohgushi 2005, 2007). Ants not only benefit honeydew-producing treehoppers by reducing the abundance of their natural enemies on *S. vinosa* host plant, but also deter non-trophobiont herbivores. Thus the direct negative effect of sap-feeding treehoppers on the plant is counterbalanced by the indirect positive effect of herbivore deterrence by tending ants (fig. 6; Oliveira and Del-Claro 2005). The bud-destroying lycaenid *P. polibetes*, on the other hand, uses ant-tended treehoppers as a cue for host plant selection and improves larval survival by exploiting the ant-generated enemy-free space in their vicinity. Thus ant-tended *P. polibetes* can ultimately be considered as opportunistic exploiters of other ant-based mutualisms occurring on foliage. Previous data show that presence of a nearby liquid food source has no effect on ant-attendance levels to *G. xiphias* treehoppers (Del-Claro and Oliveira 1993), suggesting that competition for ant mutualists may not be critical in the study system. Whether the arrival of butterfly larvae on the plant has any consequence (positive or negative) for resident treehoppers awaits further investigation.

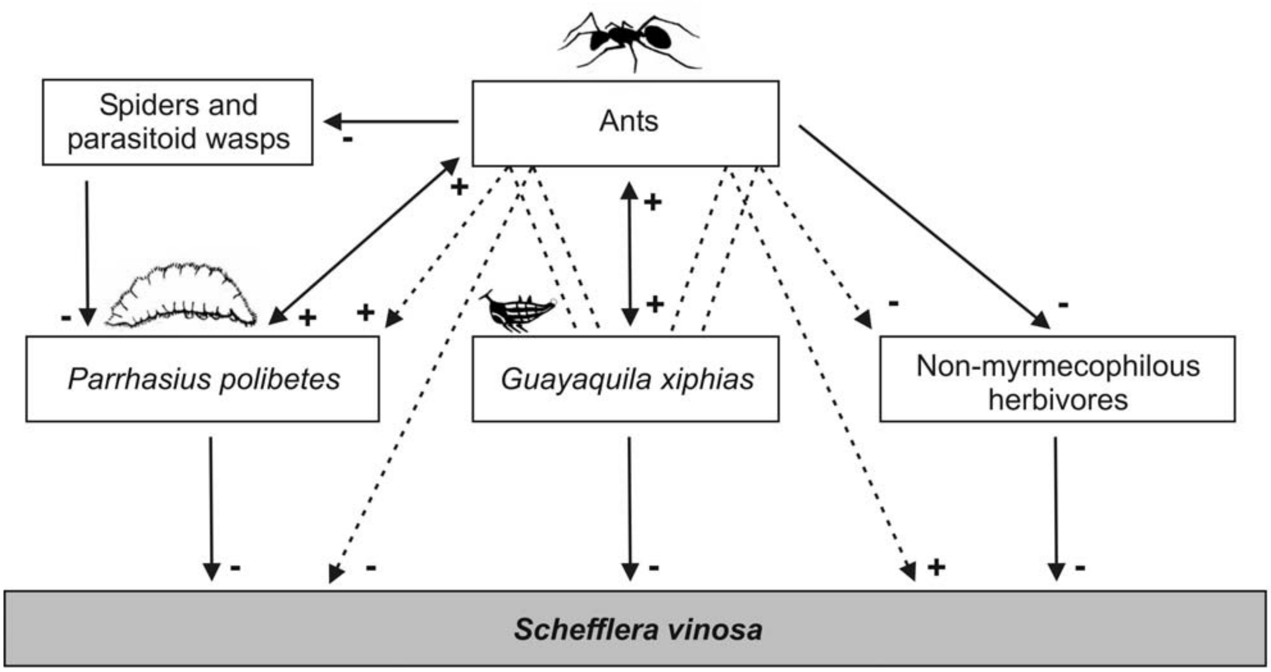

Figure 6: Indirect interaction web of the study system involving foliage-dwelling ants, herbivorous insects, and natural enemies on the host plant *Schefflera vinosa*. Solid and broken lines show direct and indirect effects, respectively. Plus and minus signs indicate positive and negative effects from an initiator to a receiver species, respectively. Depicted relationships are based field experiments by Del-Claro and Oliveira (2000), Oliveira and Del-Claro (2005), and the current study.

It seems clear that the traditional pairwise approach commonly used in studies of ant-based mutualisms would not have allowed us to properly assess some of the selective pressures operating within our study system. Indeed, research on ant-plant-herbivore interactions in cerrado savanna shows that the frequent occurrence of plant and insect exudates on vegetation effectively promotes ant activity on foliage, which in turn produces a range of direct and indirect effects (positive and negative) among participant species from multiple trophic levels (Oliveira and Freitas 2004; Kaminski 2008; Sendoya et al. 2009; Silveira et al. 2010).

In conclusion, this study points out the importance of considering the multitude of interactions occurring on foliage for a proper understanding of the origin and maintenance of symbiotic associations between butterflies and ants. Although previously ignored, detection of other ant-based mutualisms on foliage to the benefit of larval offspring may have represented an important evolutionary step in the process of host plant selection in facultative myrmecophilous butterflies.

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CONSIDERAÇÕES FINAIS

De maneira geral, os objetivos traçados no início da elaboração do projeto de Tese foram na sua maioria alcançados. Vários aspectos da biologia de *Parrhasius polibetes* foram desvendados e, possivelmente, após a publicação dos resultados aqui apresentados poderemos dizer que *P. polibetes* é o Lycaenidae Neotropical mais bem conhecido. Embora reconhecendo que isso possa ser apenas uma consequência indireta da carência de estudos.

Para as populações estudadas de *P. polibetes* nós sabemos agora que:

1) As larvas utilizam apenas tecidos reprodutivos das plantas hospedeiras (florívoras), comendo principalmente botões ainda fechados.

2) Imaturos ocorrem em várias famílias de plantas hospedeiras (polífagas).

3) Larvas de terceiro e quarto instar são atendidas facultativamente por várias espécies de formigas que também visitam nectários extraflorais e hemípteros trofobiontes.

4) O atendimento por formigas incorre em custos, mas esse custo é dependente da espécie de formiga atendente, sendo compensado ao longo da ontogenia.

5) Fêmeas são capazes de detectar e selecionar plantas que apresentam formigas e membracídeos, tal capacidade pode explicar a preponderância de plantas hospedeiras que apresentam atributos que promovem a atividade de formigas.

6) Larvas que se desenvolvem em plantas com associação membracídeos-formigas sobrevivem melhor do que em plantas sem associação.

7) Presença de formigas atendendo membracídeos cria um "espaço livre de inimigos" em algumas plantas hospedeiras que podem ser detectados e explorados por *P. polibetes*.

Tendo como base essas informações podemos levantar novas questões. Por exemplo, uma vez que *P. polibetes* é amplamente distribuída na região Neotropical, como será que varia geograficamente o padrão de utilização de plantas hospedeiras? Podemos pensar tanto no nível de tipo de tecido vegetal utilizado pelas larvas (vegetativo *versus* reprodutivo) quanto na amplitude taxonômica de hospedeiras (polifagia *versus* oligofagia). Possíveis variações nessas preferências podem ser úteis para entender a evolução da florivoria e sua ligação com a polifagia. Interessantemente, há informações de que a espécie Neártica do gênero, *Parrhasius m-album* come folhas de Fagaceae (Sourakov 2008) – seria essa uma característica apomórfica ou plesiomórfica para o gênero? Será que esta diferença esta relacionada com mirmecofilia? Num sentido mais amplo, *P. polibetes* e outras espécies do gênero poderiam ser também utilizadas como modelos para testar hipóteses vigentes sobre a evolução da dieta em insetos herbívoros, como por exemplo, a "hipótese da oscilação" (Janz & Nylin 2008).

A utilização de características relacionadas à presença de formigas na seleção da planta hospedeira pode promover *trade-offs* entre qualidade da planta hospedeira e benefícios de um "espaço livre de inimigos". Estudos nessa linha já estão sendo desenvolvidos, Rodrigues *et al.* (2010) encontraram esses *trade-offs* ao analisar comparativamente desempenho em laboratório, características físicas das plantas, taxas de parasitismo em campo e frequência de espaço livre de inimigos em três espécies de hospedeiras. Nesse trabalho foi mostrado que *Luehea grandiflora* (Malvaceae) proporciona um espaço livre de inimigos para as larvas (~90% plantas possuem associação membracídeos-formigas), mas ao mesmo tempo os botões apresentam características físicas (dureza e espessura) que provocam desgaste mandibular e mortalidade larval (100% mortalidade). Por outro lado, *Pyrostegia venusta* (Bignoniaceae), uma planta sem associação membracídeos-formigas e com maior taxa de mortalidade por parasitóides pode ser utilizada com relativo sucesso pelas larvas (~50% mortalidade). Com resultados menos contrastantes, *Schefflera vinosa* (Araliaceae) proporciona um considerável "espaço livre de inimigos" (~50% plantas com membracídeos; ver também Capítulo IV), e comparativamente ótimos parâmetros para o desempenho (100% sobrevivência; desenvolvimento mais rápido e adultos maiores).

Outra questão em aberto é porque as fêmeas de *P. polibetes* não ovipositam em determinadas famílias de plantas hospedeiras? Por exemplo, Asteraceae é uma família utilizada com relativa frequência por outros Eumaeini florívoros (e.g. *Chalybs*, *Cyanophrys*, *Laothus*, *Rekoa*) (Monteiro 1991, L. A. Kaminski dados inéditos), mas ainda não foi registrada para *P. polibetes*. Os 50% de mortalidade encontrada para larvas criadas em *P. venusta* (Rodrigues *et al.* no prelo), sugerem que características químicas das plantas hospedeiras são importantes para no desempenho das larvas. Nesse sentido, a interface química da relação entre polifagia e florivoria constitui uma área inexplorada. Ainda ao que se refere à química, foi demonstrado recentemente que os membracídeos mirmecófilos presentes em *S. venusta* possuem padrão de hidrocarbonetos cuticulares similar à planta e se protegem das formigas por camuflagem química (Silveira *et al.* 2010). Para borboletas com mirmecofilia facultativa, como *P. polibetes*, ainda é desconhecido o papel dos hidrocarbonetos cuticulares nas interações entre larvas e formigas atendentes.

Quanto aos custos da mirmecofilia, nossos resultados corroboram outros estudos com espécies mirmecófilas facultativas (*e.g.* Robbins 1991; Fiedler & Hölldobler 1992). Tais estudos têm mostrado que a interação com formigas é relativamente pouco custosa para as larvas. Em campo a frequência e intensidade de atendimento por formigas é baixa (~5%), aumentando quando a larva se desenvolve próximo à associação membracídeos-formigas (~20%), mas comparado com situações de mirmecofilia obrigatória, a frequência ainda é baixa (ver Kaminski 2008). Assim, mesmo sob a situação "forçada" do nosso desenho experimental, em que foi confinado todo o tempo uma formiga com a larva, *P. polibetes* conseguiu compensar os custos para os parâmetros de peso pupal e tamanho dos adultos. Os baixos custos da mirmecofilia sugerem que a interação com formigas é uma estratégia evolutivamente estável. Estudos que avaliem os custos do mutualismo podem auxiliar a responder questões importantes sobre a evolução e manutenção da mirmecofilia e dos seus diferentes graus. Nesse sentido, uma questão em aberto é porque não existem registros de mirmecofilia obrigatória nos Eumaeini neotropicais? Haveria alguma restrição filonegética nestas linhagens? Muitas espécies de Riodinidae se associam obrigatoriamente com gênero de formigas neotropicais dominantes, como *Azteca*, *Dolichoderus*, *Crematogaster* e *Pheidole* (ver DeVries *et al.* 1994, Kaminski 2008), mas não se sabe por que tais associações ainda não foram registradas em Eumaeini.

Um dos aspectos mais importante desta Tese é a demonstração de que uma borboleta mirmecófila facultativa pode e utiliza características relacionadas à presença de formigas como pistas na seleção de plantas hospedeiras. Recentemente, foi demonstrado que uma borboleta não mirmecófila (*Eunica bechina*, Nymphalidae) é capaz de identificar e evitar ovipositar na presença de espécies de formigas agressivas que podem representar um perigo maior para as larvas (Sendoya *et al.* 2009). Nesse sentido, é plausível esperar que uma borboleta pertencente a uma família com uma longa história evolutiva de interações com formigas, como são os Lycaenidae, tenha desenvolvido mecanismos para reconhecimento e seleção de parceiros mutualistas. A seleção de planta hospedeira mediada por formigas é aceita para as espécies mirmecófilas obrigatórias, mas até o presente trabalho, tal possibilidade era descartada para espécies facultativas (ver Pierce *et al.* 2002). Nesse sentido, as descobertas para *P. polibetes* trouxe evidências para novas hipóteses, proporcionando novas perspectivas para o estudo de interações borboletas-formigas e suas consequências no uso de plantas hospedeiras.

Provavelmente, as diferenças obtidas nos nossos experimentos para seleção de planta hospedeira e sobrevivência larval só tenham sido detectadas porque foi utilizado um enfoque multitrófico – diferente do enfoque pareado comum ao estudo de mutualismos (veja uma crítica em Stanton 2003). Os custos e benefícios em sistemas mutualísticos facultativos podem ser sutis e difíceis de serem detectados, ao estudar a mirmecofilia em *P. polibetes* na presença de um segundo organismo mutualista, os benefícios da mirmecofilia se tornaram mais óbvios e puderam ser detectados. Dessa forma, fica claro que um melhor entendimento sobre a evolução e manutenção da mirmecofilia em Lepidoptera passa por um melhor conhecimento sobre as plantas com nectários extraflorais e formigas atendentes, bem como a presença de outros organismos mirmecófilos sobre a vegetação. Apesar da interconexão entre estes sistemas já ter sido proposta anteriormente (Atsatt 1981, DeVries 1991), ela permanece ainda pouco explorada nos estudos sobre mirmecofilia.

Finalmente, a presente Tese de Doutorado está inserida dentro de uma linha de pesquisa consolidada na Unicamp, que aborda a interface da interação entre plantas, formigas e herbívoros no cerrado (revistos em Oliveira & Freitas 2004). Dentre os sistemas estudados, o que envolve o membracídeo *Guayaquila xiphias* e suas formigas atendentes sobre *S. vinosa* parece infinito quanto à riqueza de interações existentes (Oliveira & Del-Claro 2005). Dessa forma, como sugerido para outros sistemas membracídeos-formigas (ver Styrsky & Eubanks 2007), *G. xiphias* pode ser considerada uma “keystone interaction” ao nível de comunidade porque a sua presença afeta direta e indiretamente várias espécies de diferentes níveis tróficos. Em relação aos Lycaenidae, em especial, *P. polibetes* não é a única espécie que utiliza *S. vinosa*, existe uma comunidade de pelo menos 10 espécies de Eumaeini utilizando as inflorescências em simpatria (Fig. 1). Dentre estas, existem tanto espécies não mirmecófilas quanto espécies com grau de mirmecofilia semelhante a *P. polibetes*. Porque tantos licenídeos utilizam essa planta, e como elas respondem à presença de associação membracídeos-formigas são questões que podem ser exploradas futuramente.

No trabalho de Brown (1993) – única revisão disponível com informações ecológicas sobre os Lycaenidae Neotropicais – o autor escreve que “(...) *love for the lycaenids, is still an affair destined to frustration. Thus, the following attempts at generalisations and a particularisation are very fragile, begging for more field work, laboratory study and experimentation.*”. Nesse sentido, espero que os resultados apresentados nesta Tese contribuam para um melhor entendimento sobre os Lycaenidae Neotropicais, e que sirva de estímulo para que mais estudos sejam realizados.

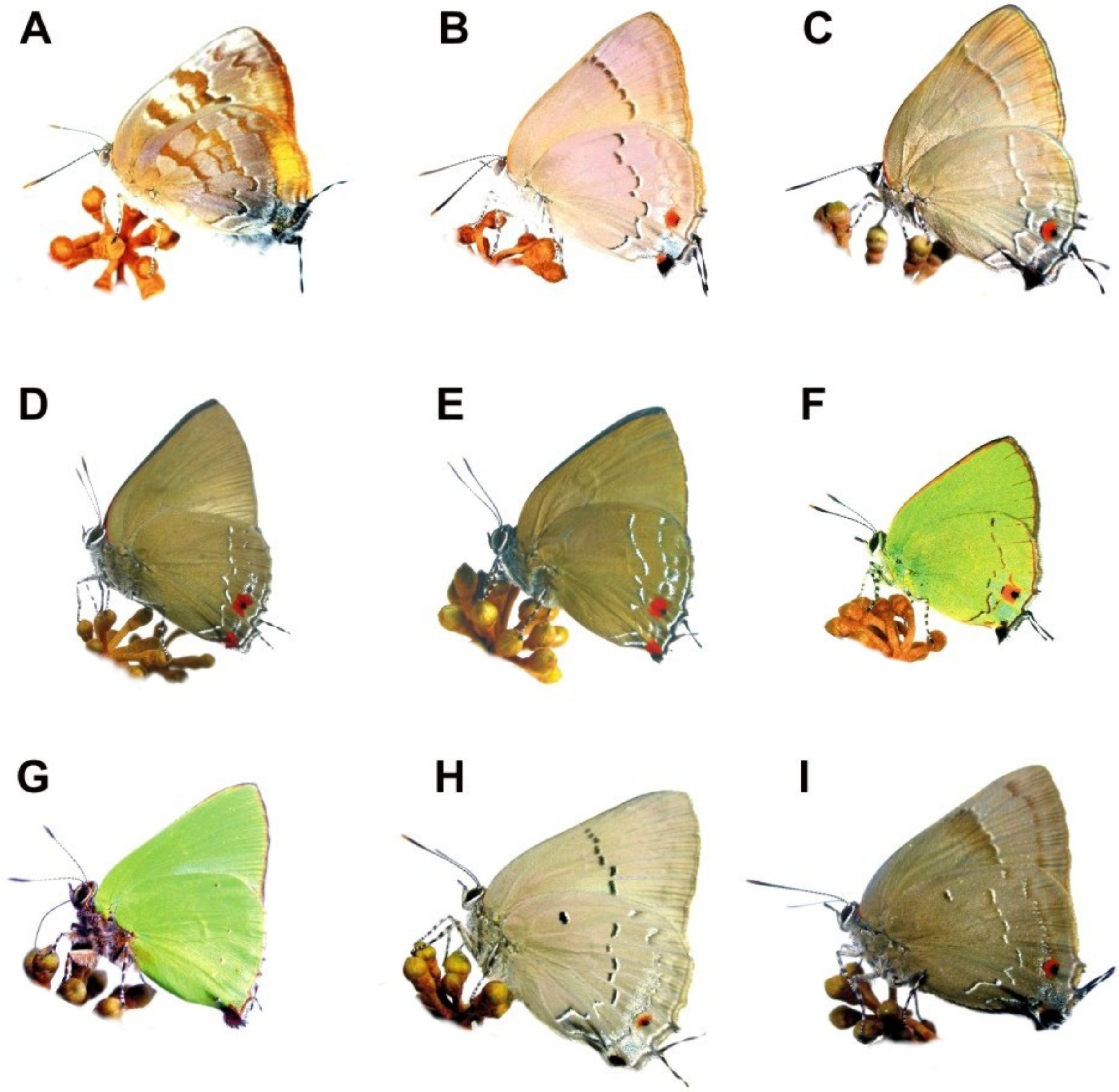

Figura 1. Exemplos de Eumaeini (Lepidoptera: Lycaenidae) criados em inflorescências de *Schefflera vinosa* em áreas de cerrado do Estado de São Paulo. A, *Rekoa palegon*; B, *Rekoa marius*; C, *Rekoa stagira*; D, *Kolana ergina*; E, *Kolana ligurina*; F, *Chalybs janias*; G, *Cyanophrys acaste*; H, *Parrhasius polibetes*; I, *Parrhasius orgia*.

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ANEXO I

NATURAL HISTORY AND MORPHOLOGY OF IMMATURE STAGES OF THE BUTTERFLY *ALLOSMAITIA STROPHIUS* (GODART) (LEPIDOPTERA: LYCAENIDAE) ON FLOWER BUDS OF MALPIGHIACEAE[*]

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Abstract

The natural history and morphology of immature stages of *Allosmaitia strophius* (Godart) are described and illustrated for the first time, using both light and scanning electron microscopy. The available host plant records for the genus were reviewed suggesting a feeding specialization on reproductive structures of Malpighiaceae. Both concentration of resources in the reproductive tissue of Malpighiaceae and the existence of sequential flowering periods may be important factors involved in the evolution of oligophagy in *Allosmaitia*. Field and laboratory observations showed that larvae of *A. strophius* are ignored by tending ants besides the presence of the dorsal nectar organ (DNO). Additionally, larvae present some behavioral and morphological adaptations that were proposed as preventing ant attacks, such as dendritic setae, thick cuticle, perforated cupola organs and absence of a "beat reflex".

Keywords: Ant-organs; *Camponotus*; Cerrado; Eumaeini; florivory; myrmecophily; Theclinae.

Introduction

The family Lycaenidae contains about of 1,200 species in the Neotropics. Lycaenidae is one of the richest families of true butterflies in the region (Lamas 2004; Robbins 2004a). In South America the family is represented by two subfamilies: Polyommatinae and Theclinae. Despite great diversity, little is known about the natural history and early stages of the Neotropical Lycaenidae when compared with the fauna of other biogeographical regions (*e.g.* Fiedler 1991; 2001; Eastwood & Fraser 1999; Pierce et al. 2002; Heath & Claassens 2003).

The genus *Allosmaitia* Clench 1964 contains only five species (Robbins 2004b), three restricted to the West Indies, and two widespread across the American continent. Butterflies belonging to the genus present the Eumaeini basic pattern, with dorsal wing surface blue and ventral side uniformly grayish brown, bearing tails and with a typical pattern of spots at the anal angle resembling a false head (see review in Robbins 1980). There is limited published information about the immature stages of *Allosmaitia*, including some anecdotal notes on host plants records (Dewitz 1879; Gundlach 1881; Monteiro 1990; Fernandez 2001; Armas 2004), and no information on morphology and behavior of immature stages.

Allosmaitia strophius (Godart, 1824) (Figs. 1A-B) is widely distributed from the southern USA to southern Brazil (Brown 1992; D'Abrera 1995; Brown & Freitas 2000; Emery et al. 2006; Prieto

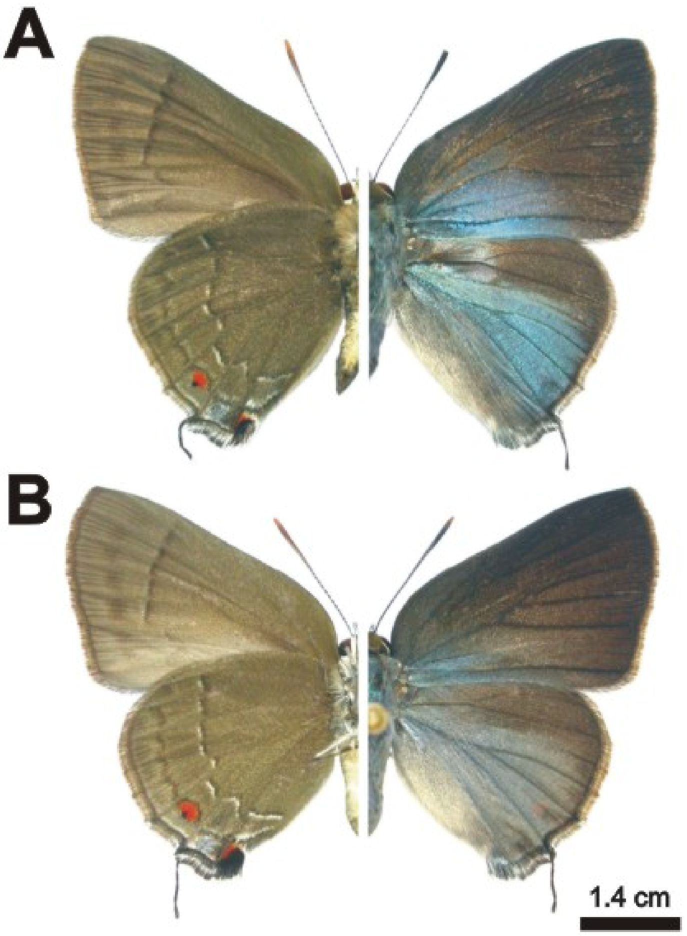

Fig. 1. Adults of *Allosmaitia strophius*. A, male; B, female (left: ventral view, right: dorsal view).

& Dahners 2006). The purpose of this study is to describe for the first time the morphology and behavior of the immature stages of *A. strophius*.

Materials and methods

Collection and rearing of Allosmaitia strophius

Host plant records for *A. strophius* were obtained in the field from several localities in southeast Brazil, complementing the available literature records (see Table 1). Collecting specimens and behavioral observations were performed during May and June 2007 in an area of Cerrado *sensu stricto* in the Reserva Biológica e Estação Experimental de Mogi-Guaçu (22°18'S, 47°10'W), municipality Mogi-Guaçu, São Paulo State, Brazil. For floristic details of the study area, see Mantovani & Martins (1993).

Larvae were observed in the field (for all instars) to check the presence of tending ants (n = 23). In addition, to assess the functionality of the larval ant-organs and the ability to form a symbiosis with ants (myrmecophily), last instars were tested with ants under laboratory conditions (n = 10 larvae), following procedures described in Robbins (1991). We used two ant species: *Camponotus crassus* Mayr and *Camponotus melanoticus* Emery (Formicinae). These species were chosen because they were frequently observed engaged with trophobiont insects in Cerrado vegetation (Oliveira & Brandão 1991), including facultative myrmecophilous butterflies (L.A. Kaminski unpublished).

Immatures of *A. strophius* used in the morphological analysis were collected in the field. Eggs were incubated in Petri dishes. Larvae were reared until the adult stage in 400 ml plastic containers with fresh branches of the host plant bearing floral buds. Branches were changed daily and offered *ad libitum*. Immatures for morphological analysis were separated by stage, fixed in Dietrich's fluid, and then preserved in 70% ethanol. Shed head capsules were collected and preserved for measurements. Voucher specimens of the immature stages were deposited at the Museu de Zoologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil.

Morphology

Measurements and general aspects of morphology were studied using a Leica® MZ7.5 stereomicroscope equipped with a micrometric scale. Egg size is given as height and diameter. The head capsule width of larvae is the distance between the most external stemmata (as in Freitas 2007). The total length for both larvae and pupae was measured in dorsal view. Color patterns *in* *vivo* of immature stages were recorded using a Nikon® Coolpix 4500 digital camera. Images of the eggs and initial larval instars were taken with the camera attached to the stereomicroscope. Scanning electron microscopy (SEM) was conducted using a JEOL® JSM-5800, with samples prepared according to standard techniques (for details, see Kaminski et al. 2008). Terminology for early stage descriptions follows: Downey & Allyn (1981, 1984a) for eggs; Stehr (1987) for general morphology of larvae; Downey & Allyn (1984b), Duarte et al. (2005), and Ballmer & Wright (2008) for chaetotaxy; Mosher (1916) and Duarte et al. (2005) for pupae; and Fiedler (1991) for ant-organs.

Results

Natural history of Allosmaitia strophius

Adults of *A. strophius* are commonly observed feeding upon the nectar of small flowers, especially *Schefflera vinosa* (Araliaceae). All known host plant records for *A. strophius* are species of Malpighiaceae with larvae always found using flowers or flower buds (Table 1). Females lay several eggs per inflorescence, but no more than two eggs per bud (Fig. 2A-B). The development from egg to adult was approximately 40 days. Larvae developed through four instars (Figs. 2C-F). Pupation probably occurs off the host plant, no pupae were observed in the field.

First instar larvae eat part of the exochorion after hatching, subsequently feeding upon reproductive tissue (androecia and gynoecia) and on the epithelial oil glands (elaiophores) that occur in pairs in the sepals of most Malpighiaceae species. Larvae generally feed with the retractile head extending into the plant tissue. Starting from the second instar, larvae can use almost all parts of buds and flowers. From the last half of first instar, larvae show cryptic larval polychromatism that is “food dependent”. The larval polychromatism was observed on all host plants, resulting in larvae with many different ground colors, including tones of green, yellow, orange and pink. Despite the abundance of secretion-harvesting ants on the host plants, no symbiotic interactions between *A. strophius* larvae and ants were observed in the field. Under laboratory conditions the two *Camponotus* species tested ignored the larvae most of the time. However, on some occasions the ants tending the larvae performed antennal palpation on the dorsal nectary organs. Production of liquid secretions was never observed.

Table 1. Summary of host plant records for *Allosmaitia* larvae, including flowering periods (for *Allosmaitia strophius*), localities and references. Abbreviations: MG: Minas Gerais, RJ: Rio de Janeiro, SP: São Paulo.

Host plant (Malpighiaceae)	**Flowering period**	**Localities**	**References**
A. coelebs			
Byrsonima crassifolia		Cuba	Fernandez (2001)
Malpighia punicifolia		Cuba	Armas (2004)
Stigmaphyllon diversifolium		Cuba	Fernandez (2001)
Stigmaphyllon sagraeanum		Cuba	Gundlach (1881) Fernandez (2001) Beccaloni et al. (2008)
A. fidena			
Tetrapterys citrifolia		Puerto Rico	Beccaloni et al. (2008)
A. strophius			
Banisteriopsis argyrophylla	Mar – May [1, 2, 3]	Mogi Guaçu (SP), Brazil	Present study
Banisteriopsis campestris	Jan – Apr [4]	Uberlândia (MG), Brazil	Torezan-Silingardi (2007)
Banisteriopsis laevifolia	Apr – Sep [1,4]	Uberlândia (MG), Brazil	Present study Torezan-Silingardi (2007)
Banisteriopsis malifolia	Mar – Jun[3,4]	Uberlândia (MG), Brazil	Torezan-Silingardi (2007)
Banisteriopsis stellaris	Jan – Jul[1,3]	Itirapina (SP), Brazil	Present study
Byrsonima sp.	Jun – Jul[1]	Bauru (SP), Brazil	Present study
Byrsonima intermedia	Sep – May[1, 4]	Campinas (SP), Brazil	Present study
Byrsonima sericea	Oct – Jul[5,6]	Maricá (RJ), Brazil	Monteiro (1990)
Heteropterys sp.	Apr – Sep[1]	Mogi Guaçu (SP), Brazil	Present study
Heteropterys chrysophylla	Mar – Aug[7]	Maricá (RJ), Brazil	Monteiro (1990)
Jubelina wilburii	Dec – Mar[8]	Panama	Beccaloni et al. (2008)
Lophanthera lactescens	Jun – Aug[1]	Campinas (SP), Brazil	Present study
Malpighiaceae sp.	May[1]	Conceição do Mato Dentro (MG), Brazil	Present study
Peixotoa hispidula	Oct – Jun[7]	Maricá (RJ), Brazil	Monteiro (1990)
Peixotoa sp.	Jul - Aug[1]	São João Batista do Gloria (MG), Brazil	Present study
Peixotoa tomentosa	Jan - Aug[1,4]	Uberlândia (MG), Brazil	Present study

			Torezan-Silingardi (2007)
Stigmaphyllon paralias	Nov – Mar[6]	Maricá (RJ), Brazil	Monteiro (1990)
Niedenzuella glabra	May – Aug[1]	Campinas (SP), Brazil	Present study

Phenology references: [1]present study; [2]Morellato & Leitão-Filho (1996); [3]Gaglianone (2000); [4]Torezan-Silingardi (2007); [5]Teixeira & Machado (2000); [6]Costa et al. (2006); [7]Monteiro (1990); [8]Anderson (1990).

Description of immature stages of **Allosmaitia strophius**

Because of the cryptic larval polychromatism related to feeding upon different host plants species, all descriptions and measurements are based on material reared at Mogi Guaçu, on the yellow flower buds of *Heteropterys* sp. (Fig. 2A).

Egg

Duration 5 – 6 days (n = 5). Height 0.40 – 0.42 mm, diameter 0.64 – 0.72 mm (n = 7). Color light green when laid, changing to yellowish before hatching (Fig. 2B). General shape spherical, with upper surface convex and bottom surface flattened. Exochorion with elevated ribs outlining hexa- and heptagonal cells with smooth surface (Fig. 3A). Ribs with punctuated surface. Aeropyles open on the rib intersections without protuberances (Fig. 3A-C). On top of the egg the micropylar area is located inside an octagonal cell well demarcated by elevated ribs (Fig. 3A-B). Micropylar area depressed and composed by soft cells; micropyles surrounded by petal-shaped cells.

First instar

Duration 5 – 6 days (n = 5). Head capsule width 0.26 – 0.30 mm (n = 6), maximum length 1.40 mm. Initially with head, body and setae whitish yellow (Fig. 2C), changing to yellow after two days. Larvae onisciform with hypognathous head, having the ability to retract into the thorax (Figs. 3D, E). Cuticle covered with microtrichia (Figs. 3D-F).

Head chaetotaxy with 15 pairs of setae (A1, A2, AF1, C1, C2, CD1, CD2, MG1, P1, S1, S2, S3, SS1, SS2, SS3), and 14 pairs of pores (Aa, AFa, Ca, CDa, Fa, La, MGa, Pa, Pb, Sa, Sb, SSa, two more unnamed pores located ventrally near antenna, probably related to substemmatal (SS) group).

Body chaetotaxy with 123 pairs of primary setae and 27 pairs of perforated cupola organs distributed as follows:

Prothorax with 12 pairs of setae directed forwards: five on the prothoracic shield (D1, D2, SD1, XD1, XD2, and one pair of PCO; four pairs of “fringed setae” (*sensu* Ballmer & Wright 2008), with more three pairs of setae (SV1, SV2, and V1). Mesothorax with nine pairs of setae (D1, D2, SD1, L1, L2, L3, SV1, SV2, V1), and one pair of PCO (=DL). Metathorax similar to mesothorax with addition of D3, and one pair of PCO (= SDL).

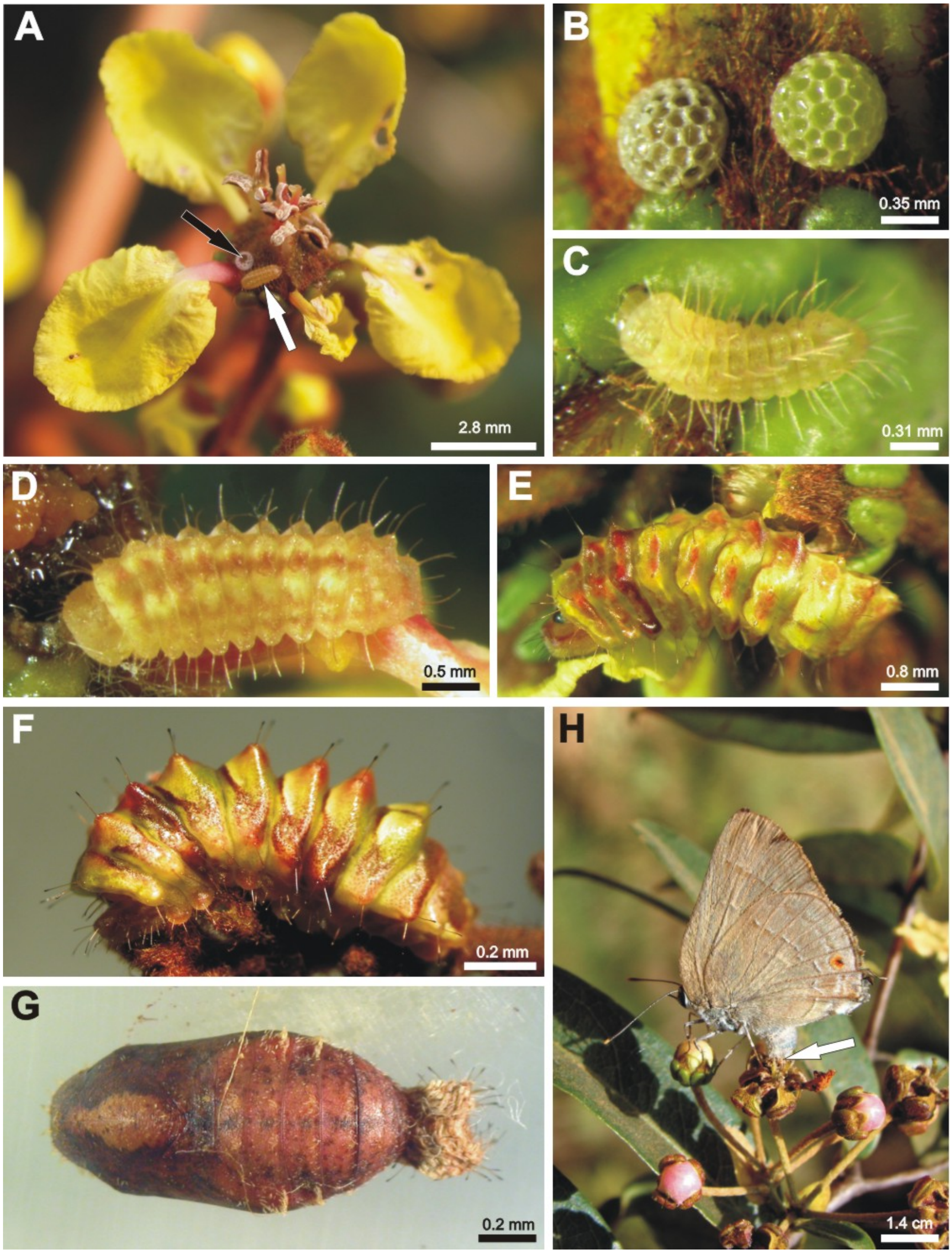

Fig. 2. Life stages of *Allosmaitia strophius* on *Heteropterys* sp. (A-G). A, flower with egg (black arrow) and first instar larva (white arrow); B, eggs on flower bud, note that the left egg its parasitized; C, first instar feeding on elaiophore; D, second instar; E, third instar; F, fourth (last) instar; G, pupa; H, butterfly laying eggs on *Banisteriopsis stellaris*; note abdomen tip curved (arrow).

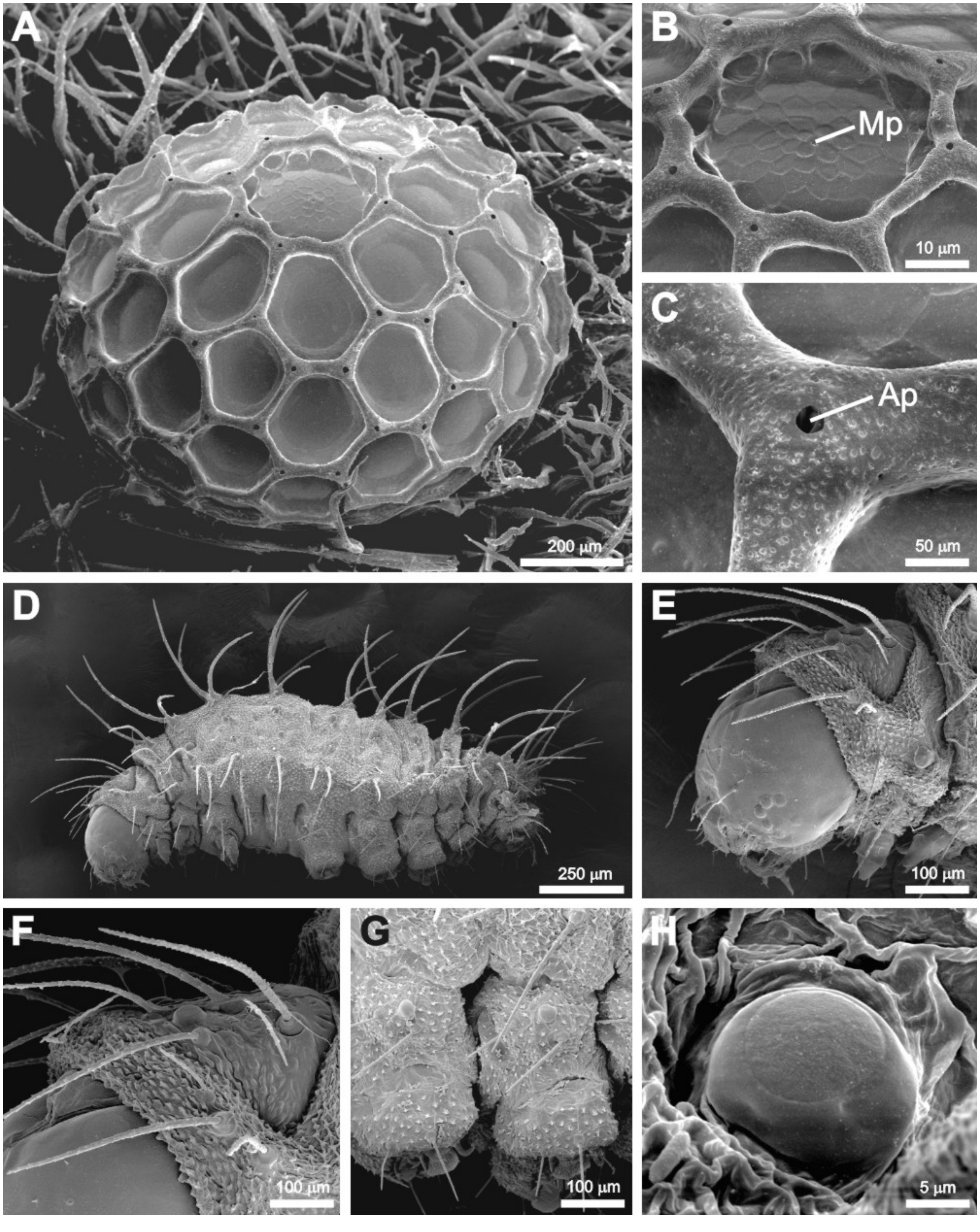

Fig. 3. SEM micrographs of eggs (A-C) and first instar (D-H) larvae of *Allosmaitia strophius*. A, dorso-lateral view; B, micropylar area; C, detail of aeropyle on the rib intersection; D, lateral view; E, head and prothorax in dorso-lateral view; F, prothoracic shield; G, abdominal segments 4 and 5 in lateral view; H, perforated cupola organ. Ap, aeropyle; Mp, micropyles.

Abdominal segments 1 and 2 with ten pairs of setae (D1, D2, D3, SD1, SD2, L1, L2, L3, SV1, and V1), and three pairs of PCOs (SDL, SDL, and SVL). Abdominal segments 3 to 6 with 12 pairs of setae (D1, D2, D3, SD1, SD2, L1, L2, L3, SV1, SV2, V1, and V2) and three pairs of PCOs (SDL, SDL, and SVL). Abdominal segment 7 with seven pairs of setae (D1, SD1, L1, L2, L3, SV1, and V1) and three pairs of PCOs (DL, SDL, and SVL). Abdominal segment 8 with five pairs of setae (L1, L2, L3, SV1, and V1) and two pairs of PCOs (SDL and SVL). Abdominal segment 9 with apparently only one seta (SV2). Abdominal segment 10 with 14 pairs of setae (D1, SD1, SD1, SD2, L1, L2, L3, PP1, PP2, SV1, SV2, SV3, SV4, and SV5), and one pair of PCO anterior to the suranal plate.

Second instar

Duration 5 – 6 days (n = 5). Head capsule width 0.46 – 0.48 mm (n = 5), maximum length 3.02 mm. Head capsule, prothoracic shield and body with different tones of yellow and orange (Fig. 2D). Body with prominent setae on the thoracic and abdominal segments, constituted by groups of two setae in the lateral area, and one isolated seta in the dorsal area.

Third instar

Duration 5 – 6 days (n = 5). Head capsule width 0.74 – 0.82 mm (n = 7), maximum length 5.08 mm. Head capsule light brown and prothoracic shield black. Body yellow with orange spots, first abdominal segment with a prominent red band (Fig. 2E). This dark band is present in all polychromatic patterns. Principal groups of body setae similar to described for second instar, but enlarged.

Fourth (last) instar

Duration 8 – 10 days (n = 5). Head capsule width 1.28 – 1.44 mm (n = 6), maximum length 1.34 cm. Color pattern similar to third instar with a characteristic dark red band in the first abdominal segment (Fig. 2F). Body with a general “sliced” appearance resulting from profound clefts between the segments (present in third instar, but less conspicuous). Prothoracic shield with some PCOs and setae, but only SD1 tactile retain the primary chaetotaxy (Fig. 4A). Cuticle smooth with several small clavate-capitate setae (*sensu* Ballmer & Pratt 1988) and PCOs (Figs. 4C-D). The PCO surface is convex with some punctuations and elevated margins (Fig. 4E). Principal body setae similar to those of second and third instar, with pairs of setae in the lateral area and isolated in the dorsal area. These setae have the distal part enlarged (Fig. 4B). Dorsal nectar organ medially located on the abdominal segment 8; surrounded by PCOs and specialized dendritic setae (Fig. 4C). Opening of spiracles aligned on the prothorax and A1-A8 segments; the pattern is semi elliptical with an elevated margin (Fig. 4D). Prolegs with apically pointed setae on the sclerotized plate that support the crochets that have a uniserial mesoseries and triordinal crochets, interrupted near center by a fleshy pad (Fig. 4F).

Pupa

Duration 10 – 13 days (n = 4). Maximum length 8.93 mm, width at A3 3.76 mm. Color brown with a light brown spot dorsally on the A2 segment. Several yellow setae on the margin of the head and near the abdominal spiracles (Fig. 2G). Silk-girdle crossing the pupa on the 2A segment. Cuticle sculptured with several setae and PCOs (Fig. 4G). Intersegmental area between A5-6 and A6-7 abdominal segments with files and plates (Fig. 4H) that act as a functional stridulatory mechanism. The abdominal segment A10 with a ventrally flat cremaster, constituted by several short hooked setae (Fig. 4I).

Discussion

Morphology of immature stages

In general the immature stages of *Allosmaitia strophius* resemble those of other species of Eumaeini, with the egg adorned by conspicuous ribs, onisciform larvae, and pupae without tubercles and presenting a silk girdle. By contrast, the egg diverges in some aspects from those described for other Neotropical Eumaeini (see examples in Downey & Allyn 1981, 1984a; Duarte et al. 2005). For example, the egg of *A. strophius* has fewer cells in relation to other Eumaeini genera. Another remarkable difference is the lack of spine-like protuberances at the intersections of the ribs. These structures give an echinoid appearance to the egg of some Neotropical Eumaeini, such as *Calycopis* Scudder, 1876, *Cyanophrys* Clench, 1961, *Pseudolycaena* Wallengren, 1858, among others (see Downey & Allyn 1981, 1984a, Duarte et al. 2005). The micropylar area, depressed inside a well delimited cell, also differs from other Eumaeini, where the limits are not so clearly demarked (see Downey & Allyn 1981, 1984a). Last instar larvae have a smooth cuticula and a few groups of long setae on the dorsal and lateral areas, resembling larvae of *Laothus* Johnson, Kruse & Kroenlein, 1997 and *Rekoa* Kaye, 1904 (see Robbins 1991; Monteiro 1991; Janzen & Hallwachs 2009).

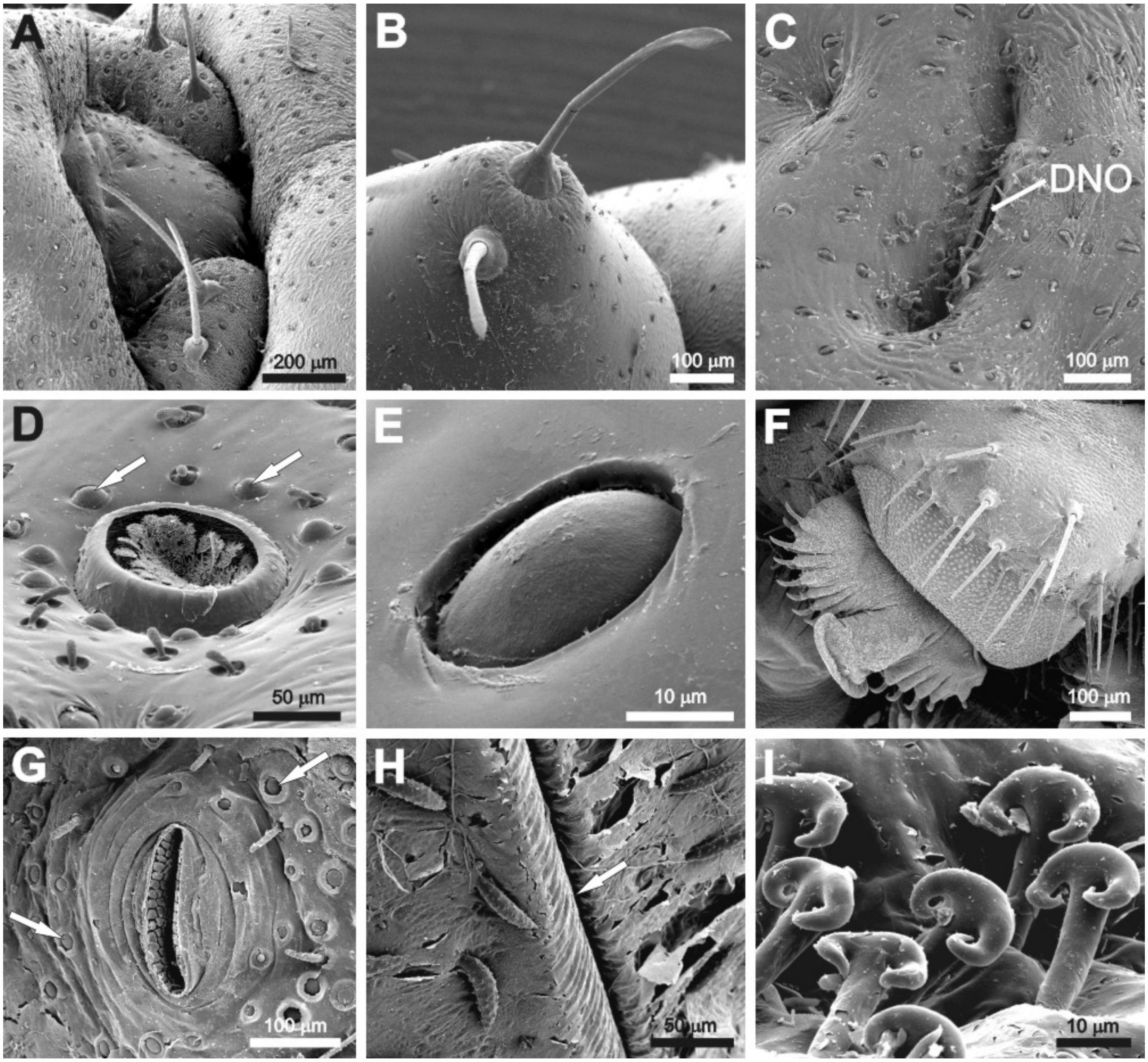

Fig. 4. SEM micrographs of fourth (last) instar larvae (A-F) and pupae (G-I) of *Allosmaitia strophius*. A, prothoracic shield; B, detail of abdominal setae in the dorso-lateral view; C, opening of the dorsal nectar organ (DNO); D, abdominal spiracle, arrows point at perforated cupola organs; E, perforated cupola organ; F, proleg in lateral view; G, abdominal spiracle, note the perforated cupola organs (arrows); H, detail of stridulating area between A5-A6 segments (arrow); I, detail of cremaster crochets.

Host plant use

All available host plant records for *Allosmaitia* larvae suggest their specialized feeding on reproductive tissues (buds and flowers) of Malpighiaceae (Table 1), a pattern also noted by Fiedler (1991). If confirmed, this pattern differs from what is usually observed for some other flower bud feeding Eumaeini that have broader host plant ranges, using two or more plant families (Robbins & Aiello 1982; Chew & Robbins 1984). It seems clear that flowers of Malpighiaceae are an interesting food resource for florivores, since they provide nectar, pollen and floral oils produced by elaiophores (Anderson 1979). This concentration of nutritive resources in the reproductive parts of Malpighiaceae could explain the relative abundance of lycaenid larvae in this plant family (L.A.K. personal observation). Moreover, different Malpighiaceae species may exhibit sequential flowering periods throughout the year (Torezan-Silingardi 2007, and Table 1), providing support for a year round occurrence of florivorous species with a diet specialized to this plant family.

On the other hand, many species of Malpighiaceae are frequently visited by ants since they produce a variety of liquid rewards such as extrafloral nectar and oil, and frequently house honeydew producing hemipterans (Del-Claro et al. 1997; Del-Claro 1998; Fernandes et al. 2005; Torezan-Silingardi 2007; Machado et al. 2008). However, even with abundant secretion-harvesting ants on Malpighiaceae, no symbiotic interactions between larvae of *A. strophius* and ants were observed in the field. This lack of interaction with ants was also confirmed by our observations in the laboratory. Larvae of *A. strophius* maintain several behavioral and morphological traits related to myrmecophily (see Malicky 1970); such as absence of a “beat reflex”, presence of dendritic setae, a thick cuticle, perforated cupola organs, and a dorsal nectar organ (apparently non-functional). The presence of these traits may confer advantage on plants frequently visited by ants, because larvae would have access to ecological niches where predatory ants limit occurrence of other insect herbivores (Atsatt 1981).

Based on the present data it is not possible to know if myrmecophily in *A. strophius* was lost, or if the characters discussed above are plesiomorphic for all Lycaenidae. If such morphological traits were inherited from myrmecophilous ancestors, we could think that the maintenance of these traits could potentially allow a return to the myrmecophilous habit, depending of the ecological context. The understanding of how these characters evolved in the whole family Lycaenidae should be a promising topic for future research.

Acknowledgments

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ANEXO II

NATURAL HISTORY, NEW RECORDS, AND NOTES ON THE CONSERVATION STATUS OF CYANOPHRYS BERTHA (JONES) (LEPIDOPTERA: LYCAENIDAE)[*]

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Abstract.—The natural history and general morphology of the penultimate and last instar larvae, and pupa of the potentially threatened hairstreak butterfly *Cyanophrys bertha* (Jones) are described. New distribution records from southern Brazil are provided. Based on morphological and ecological traits of immatures and adults, the current conservation status of this species is discussed and compared with other sympatric Eumaeini.

Key Words: Atlantic rainforest, *Conura,* florivory, host plant, immature stages, IUCN Red List, myrmecophily, Neotropical, parasitoids.

The Brazilian Atlantic rainforest has been considered a "hotspot" of diversity, characterized by high levels of endemism (about 50% overall, and more than 95% in some groups) (Brown and Brown 1992). Despite this fact, the last remaining forests are still under severe anthropogenic pressure (Morellato and Haddad 2000). For butterflies, the highest species richness accounted for this biome occurs in coastal mountains from 15 to 23°S (Brown and Freitas 2000).

Cyanophrys bertha (Jones), an endemic hairstreak butterfly of the Atlantic rainforest, has been recorded in moist evergreen and seasonal deciduous forests in the coastal mountains of southern Brazil from 800 to 1,400 m high (Robbins and Duarte 2005). This species has been listed in the "vulnerable" category in the IUCN Red List (see Brown 1993, Brown 1996, Gimenez Dixon 1996, Mielke and Casagrande 2004). Recently, Robbins and Duarte (2005) published a phylogenetic analysis and a synopsis for the genus *Cyanophrys*, including some comments on the conservation status of *C. bertha*, and on its relatively basal position in the corresponding phylogeny.

Only 13 museum specimens of *C. bertha* are so far known (listed in Robbins and Duarte 2005) from seven localities in four Brazilian States: two from Minas Gerais, one from São Paulo, three from Paraná and one from Santa Catarina. Two published records from the States of Rio de Janeiro (Brown 1993) and Rio Grande do Sul (Draudt 1919-1920), are not confirmed by voucher specimens (Robbins and Duarte 2005). Excepting for the records above mentioned, and a few notes on adult reproductive and feeding behavior in Brown (1993), no more information is available about the natural history of this species.

Considering that such information are essential for better understanding the population dynamics of any species, and can be used as important subsidies for developing strategies for conservation of endangered butterflies as *C. bertha* (*e.g.* Otero and Brown 1986, Francini et al. 2005), we provide for the first time information about the natural history and general morphology of the early stages of *C. bertha*. Moreover, we report four new distribution records of this species from southern Brazil (confirming its occurrence in the Rio Grande do Sul state), and discuss the current conservation status of this species by comparing it with other sympatric Eumaeini species.

MATERIAL AND METHODS

Collection and rearing of *Cyanophrys bertha*.—The observation and collection of immature stages were carried out from July to August 2008, in an small secondary forest fragment (≈24 ha.) inside the campus of Universidade Regional do Noroeste do Estado do Rio Grande do Sul (27°51'S, 54°29'W; 312 m a.s.l.), Santa Rosa, Rio Grande do Sul State, Brazil. This fragment is close to the Santa Rosa County (≈3 km) and surrounded by a mosaic of pastures and plantation land, but originality all area was covered by Subtropical Atlantic Forest (seasonal deciduous Atlantic Forest).

In order to collect Eumaeini larvae, all *Pyrostegia venusta* (Ker-Gawl.) Miers (Bignoniaceae) (Figs. 1-2) inflorescences present in the area were inspected. Two penultimate instar and one last instar larvae of *C. bertha* were found and then taken to the laboratory for measurements and analyses. These larvae were reared in plastic containers of about 400 ml with fresh branchs of *P. venusta* presenting floral buds. Containers were cleaned daily, and food was offered *ad libitum*. Shed head capsules and pupal exuvia were preserved for measurement purposes. Voucher material of the immature stages and parasitoids were deposited at the Museu de Zoologia (ZUEC), Universidade Estadual de Campinas, Campinas, São Paulo State, Brazil.

Morphology.—Measurements and general aspects of external morphology were taken under a Leica® MZ7.5 stereomicroscope, equipped with a micrometric scale. The head capsule width of larvae is the distance between the most external stemmata (as described in Freitas 2007). Larval and pupal lengths were taken by measuring in dorsal view. Color patterns *in vivo* of immature stages were taken using a Samsung® L100 digital camera. Terminology for early stage descriptions follows Stehr (1987) for general morphology of larvae; Duarte et al. (2005) for pupae; Fiedler (1991) for ant-organs.

RESULTS

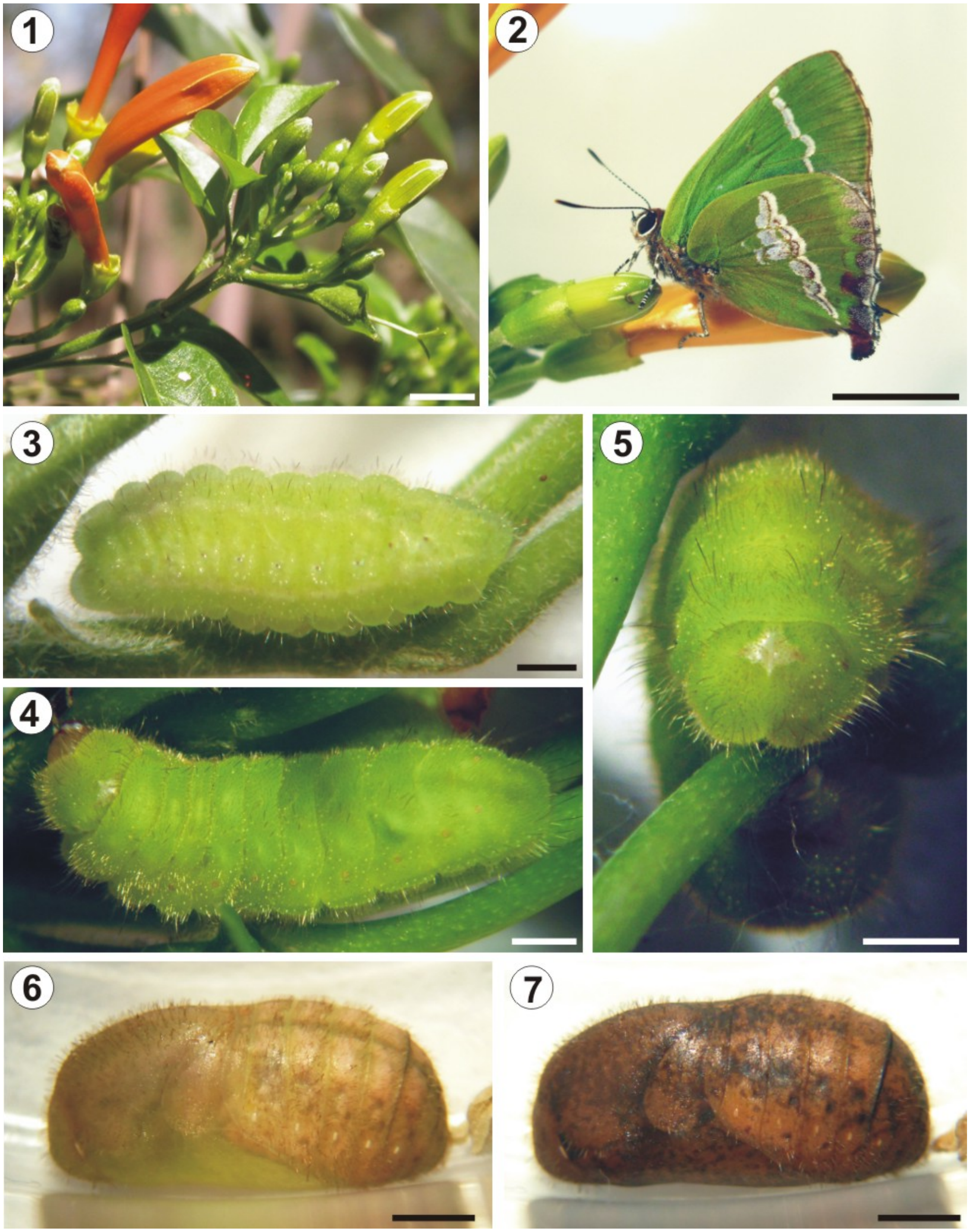
Natural history of *Cyanophrys bertha*.—Larvae are solitary and florivorous, feeding on reproductive tissue of *P. venusta* – a common Neotropical vine which blooms from the beginning of May to September in southern and southeastern Brazil (Gobatto-Rodrigues and Stort 1992). The larva fed on the bud with its retracted head outspread into plant tissue. We did not observe symbiotic interactions with ants. Two larvae were parasitized by wasps of the genus *Conura* Spinola (Hymenoptera: Chalcidoidea), that emerged in the pupal stage.

Immature stages of *Cyanophrys bertha.—Penultimate instar larva* (Fig. 3): Head capsule width 0.76 mm (n = 2); maximum total body length 7.09 mm. Larvae onisciform, with a hypognathous projected head that can be retracted to the thorax. Body little sliced without dorsal projections. Head light brown and body whitish green with two cream bands in the lateral and sub-dorsal areas. Tegument covered by short translucid setae and some conspicuous dark setae in the dorsal area. Prothoracic shield white, and spiracles brown.

Last instar larva (Figs. 4-5): Duration 7 days (n = 2). Head capsule width 1.36 – 1.40 mm (n = 3), maximum total length 1.18 cm. General morphology similar to penultimate instar, with body without dorsal projections. Head light brown and body uniformly light green. Tegument covered by short yellowish setae and some groups of black setae in the dorsal area. Prothoracic shield white; spiracles light brown. Dorsal nectary organ present in the 8A abdominal segment, but untested on the functionality.

Pupa (Figs. 6-7): Total duration 18 days (n = 1). Total body length 0.93 – 1.19 cm (n = 3), width at A1 0.41 – 0.54 cm (n = 3). Color initially translucent light green. Finally, brown, with some irregular dark brown areas. Tegument covered by several black setae. Spiracles aligned, with elliptical format and white in color. Silk-girdle crossing the pupa on the 2A abdominal segment. Intersegmental area between A5-6 and A6-7 abdominal segments, that acts as a functional stridulatory mechanism. The abdominal segment A10 with a ventrally flat cremaster, this is constituted by several short hooked setae.

New records.—PARANÁ: 1 ♀, Piên, Trigolândia, 900 m, 22 March 2007, I. Rank & A. Moser *leg*. (DZUP, Universidade Federal do Paraná, UFPR, Curitiba, PR, Brazil). RIO GRANDE DO SUL: 1 ♂, Derrubadas, Turvo State Park, 350 m, January 2006, C. A. Iserhard *leg*. (CLDZ, Coleção de Lepidoptera do Departamento de Zoologia, Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brazil); 1 ♂, Nova Petrópolis, 750 m, 16 January 2005, A. S. Prestes & A. Moser *leg*. (CLAM, Collection of Lepidoptera Alfred Moser, São Leopoldo, RS, Brazil); 1 ♂, Santa Rosa, Campus UNIJUI, 312 m, 31 August 2008 (ex-larva), S. C. Thiele *leg*. (MZSP, Museu de Zoologia, Universidade Estadual de São Paulo, São Paulo, SP, Brazil).



Figs. 1–7. Some of the life phases of *Cyanophrys bertha* on its host plant *Pyrostegia venusta*. (1) inflorescence of *P. venusta*, scale bar = 1.0 cm; (2) freshly emerged adult, scale bar = 1.0 cm; (3) penultimate instar, scale bar = 0.8 mm; (4) last instar in lateral view, scale bar = 1.4 mm; (5) last instar in frontal view, scale bar = 1.4 mm; (6) pupa immediately after molting, scale bar = 0.8 mm; (7) pupa one day after pupation, scale bar = 0.8 mm.

DISCUSSION

In general, the early stages of *Cyanophrys bertha* are similar to those known from other Eumaeini in terms of general morphology and biology. The larva is a typical florivorous with a onisciform body and a retracted head; pupae without tubercles and presenting a silk girdle. The last instar larvae of *C. bertha* have a body smoother than the other more derived and common *Cyanophrys* species (according to phylogeny proposed in Robbins and Duarte 2005). The latter have developed dorsal tubercles and setae, such as *Cyanophrys acaste* (Prittwitz) (L. A. Kaminski unpbl.) and *Cyanophrys miserabilis* (Clench) (see Ballmer and Pratt 1992: 44, Fig. 18). In Lycaenidae and Riodinidae, the presence of developed tubercles or scoli is probably related to defense against natural enemies in nonmyrmecophilous species (see Kaminski 2008a, Kaminski et al. 2009). Moreover, in some Eumaeini larvae, the loss of myrmecophily seems to be accompanied by the development of dorsal tubercles and appearance of scolus (L. A. Kaminski unpbl.). It would be interesting to record whether this pattern occurs in *Cyanophrys* and related genera, since there is no record of symbiotic interactions with ants in these lineages of Eumaeini. Additionally, experiments examining ant-organ function on the presence or absence of ants, as well as studies on larval sound production to attract ants are necessary to clarify whether myrmecophily is indeed an issue for these Eumaeini lineages (see DeVries 1990, Kaminski 2008b).

Pyrostegia venusta is a very abundant plant in both southern and southeastern Brazil, occurring mainly at the edges of primary and secondary forest fragments, and road edges (Lorenzi 2000). Besides, the host plants records for *Cyanophrys* and other Eumaeini lead to a polyphagous pattern (Chew and Robbins 1984, Robbins and Duarte 2005, Beccaloni et al. 2008, L. A. Kaminski unpbl.). Thus, it is not expected that host plant constraints are identified as the main cause of *C. bertha* rarity. On the other hand, larval competition with other florivorous Eumaeini species, as well as susceptibility to natural enemies may be plausible hypotheses. For example, inflorescences of *P. venusta* are used by at least two other common widespread species of Eumaeini in the study site – *Parrhasius polibetes* (Stoll) and *Michaelus thordesa* (Hewitson) (S. C. Thiele and L. A. Kaminski unpbl.). Larvae of both *P. polibetes* (n=19) and *M. thordesa* (n=5), were recorded during the same period, on the same host plants where larvae of *C. bertha* were observed, and none were infested with parasitoids. The symbiotic interactions with ants could explain the lower level of parasitism in the first two species as compared to *C. bertha*. Moreover, *M. thordesa* larvae developed inside the inflorescences, a possible defense strategy under current investigation (S. C. Thiele and L. A. Kaminski unpbl.). These data suggest that characteristics related to the natural history of immature stages of *C. bertha*, as for example susceptibility to parasitoids may be related to their rarity. However, further studies are necessary to evaluate whether the pattern observed locally is consistent in other both temporal and spatial scales.

The Red List of Threatened Species of Rio Grande do Sul (Specht et al. 2003) did not include any butterfly species. The present finding showing that *C. bertha* is present but rare in the RS illustrates the need of more sampling of butterflies in the state. Upon future revision of the Red List of Threatened Species of Rio Grande do Sul we recommend that *C. bertha* should be added to this list. The RS is located in a transition zone between tropical and subtropical climates, with some ecosystems that deserve conservation priorities (see Morais et al. 2007). Previous *C. bertha* records in southeast Brazil were in mountain habitats over 800 m high (see Robbins and Duarte 2005). The occurrence of *C. bertha* in lower altitudes in the RS is related to higher latitudes, which in turn may promote a subtropical climate and consequently the occurrence of mountains species at lower altitudes. The same principle may explain a new *C. bertha* record in the province of Misiones, Argentina (Bustos 2008). Such southern Brazil ecosystems also present a peculiar fauna of butterflies with diverse potentially endangered endemic species which that need to be considered for conservation. This is particularly important for the Lycaenidae and Riodinidae given that many species in these butterfly families are rare, seasonal, and might have remained undetected to collectors (Brown 1993).

More than twenty years of Lycaenidae observation by us indicated that *C. bertha* is a rare species, up to date only observed in the mountain slopes of Atlantic Rainforest. Recently, this butterfly has been recorded with some frequency from sites in the mountainous region of the south of Minas Gerais to the boundary of São Paulo and Rio de Janeiro state (O. H. H. Mielke and A. Moser unpbl., K. S. Brown Jr. pers. comm). In these localities *C. bertha* was observed at the edges of primary and secondary forest fragments, often surrounded by sparse pasture and plantation land. Several factors, such as susceptibility to natural enemies, geographical distribution restricted to mountainous areas, flight restricted to forest canopy, and the lack of local inventories, may explain in part the scarcity of this species in collections. More information about the general biology of this species, as well as a distribution model for *C. bertha* is needed to support the conservation status and aid in selecting areas for protection (*e.g.* Uehara-Prado and Fonseca 2007). Although *C. bertha* has recently been excluded from the official list of endangered species of Brazil (Machado et al. 2008), its rarity and limited distribution across gradients between tropical and subtropical environments turns it into a potential indicator of other locally endemic species that are threatened by habitat loss in this transition zone.

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