Ecological and biological aspects of a leaf miner on *Erythroxylum tortuosum* (Mart.) (Erythroxylaceae) in a Cerrado fragment in Southeastern Brazil

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Abstract: Ecological and biological features of a lepidopterous leaf miner and its host plant, *Erythroxylum tortuosum* (Erythroxylaceae), were studied in a Brazilian Cerrado fragment. Foraging traits, the temporal emergence pattern of the leaf miners and the morphology and anatomy of the mines were described. The leaf miner was identified as an *Agnippe* (Chambers) (Gelechiidae) species; the mines were characterized as blotch mines because the larva moved forward and backward in all directions within the mines while feeding. The pupal stage remained in a darkened region of the leaf, under which a pair of semicircular openings were observed on the abaxial external leaf surface. The openings consisted of a resistant, thick layer of silk. A delicate network of silk spun by the larvae was also observed within the mines. On average, the area of mines and the percentage of mined area of leaves reached maximums of 83.174 mm² and 2.750%, respectively. The caterpillars consumed the palisade parenchyma and some small veins. Cells with phenolic compounds near mine cavities were also observed. The mined and unmined leaves had a single-layered epidermis, with mucilaginous cells on the adaxial surface, and papillary cells and stomata on the abaxial surface. Finally, the temporal emergence of the leaf miner and its parasitoids (and their identification) are presented.

Key words: Erythroxylaceae; insect-plant interaction; mine anatomy; mine morphology; parasitoids.

INTRODUCTION

It has been argued that among herbivorous insects the leaf miners are one of the most neglected groups (Sinclair & Hughes, 2010), probably because their immature stages are very cryptic. Larvae of leaf miners are endophagous, feeding and living within leaf tissue and producing channels or mines (thus the popular name) (Hespenheide, 1991). Therefore, studies focused on the ecology and general biology of this insect group have been numerically less representative when compared to studies considering many other ectophagous insects (Sinclair & Hughes, 2010), particularly also because of their small size and because the adults are often difficult to identify. Approximately 10,000 species of leaf miners have been described, distributed over 60 holometabolous families (Eiseman, 2022). Leaf miners occur in Lepidoptera, Diptera, Coleoptera and Hymenoptera (Hering, 1951; Hespenheide, 1991; Sinclair & Hughes, 2008; Elb et al., 2010).

At first glance, it seems that leaf miners experience several disadvantages while feeding, because most of them live within a single leaf which makes them quite dependent on resource choice (e.g., leaf quality and quantity) (Ishino *et al.*, 2011), and vulnerable to natural enemies such as predators and parasitoids (Hawkins *et al.*, 1997). However, there are many species of leaf miners. They probably appeared 275-250 million years ago (Rozefelds, 1988; Labandeira, 1998, 2002), and environmental regulation, predator/parasitoid avoidance and avoidance of

plant defenses, appear as potential hypotheses to explain the adaptive importance of the leaf-mining habit (see Sinclair & Hughes (2010) for details).

Despite their great diversity, leaf miners are one of the least studied groups within Lepidoptera (Davis *et al.*, 2002). In addition, very little is known about the host plants that are inhabited by leaf miners. So far, most of the studies done on leaf miners have been conducted in the northern hemisphere, and, even so, our knowledge of the host plants of many species is far from complete (Sinclair & Hughes, 2008). In the Neotropics, most research attention has been focused on species that have some sort of applied relevance (e.g., crop pests or weed biological control agents) (e.g., Mc Kay *et al.*, 2009; Pantoja-Gomez *et al.*, 2019), which clearly reveals the importance of further studies with leaf miners, especially in tropical regions where very little is known about their fundamental ecology, biology and diversity.

Study system and goals: The Cerrado biome, which comprises various typically Brazilian savanna vegetation formations, is considered one of the world's richest ecosystems (Felfili & Silva Júnior, 2001). However, this important biome has suffered severe fragmentation due to anthropogenic activities. This system is also included in the world list of "hotspots" of biodiversity (Seligmann *et al.*, 2007).

Erythroxylaceae has a wide geographical distribution, occurring in subtropical and pantropical regions, but only one genus occurs in the Neotropics, namely *Erythroxylum* P.

Browne (Wanderley *et al.*, 2002). This plant genus encompasses approximately 180 species, with 130 occurring in Brazil; many species are arboreal and shrubby, distributed across forest and Cerrado areas (Ribeiro *et al.*, 1999). *Erythroxylum tortuosum* (Mart.) is typical of the Cerrado, being a deciduous shrub-arboreal species (Amaral Jr., 1973). There is currently little information available about the ecology and biology of organisms that use *E. tortuosum* as a host plant (specific information can be found in the Natural History Museum's HOSTS database (Robinson *et al.* 2010)), and even less is known about the natural enemies of the herbivores that feed on *E. tortuosum*. One of the few insects associated with *E. tortuosum* that has been reported is a univoltine species of micro-moth whose larvae are leaf miners (Ishino *et al.*, 2011; De Sibio & Rossi, 2012).

Here, we studied some ecological and biological features of the leaf miner mentioned above and its host plant, *E. tortuosum*, in a Brazilian Cerrado fragment. Specifically, we describe the foraging traits and the temporal emergence pattern of the leaf miners as well as the morphology (including mine size throughout the season) and the anatomy of the mines. Finally, the parasitoids of the leaf miner were identified and their temporal emergence pattern is defined.

MATERIALS AND METHODS

Study area: The study was conducted in a Cerrado fragment (176.7 ha) situated in the Palmeira-da-Serra Farm, municipality of Pratânia, State of São Paulo, Brazil (22°48'50"S, 48°44'35"W). Dry summers and wet winters characterize the region (mesothermic climate), where the average annual rainfall and temperature are, respectively, 1,534 mm and 20.3°C; in the rainy and dry seasons the average annual daytime relative humidity is around 80% and 55%, respectively. In the dry season the daily minimum relative humidity can reach 15% (Rodrigues & Machado, 2008). Crops such as soybean, corn, Eucalyptus, Pinus, and sugarcane have been cultivated around the study area, suggesting that disturbance is a common process near the fragment (Ishino et al., 2012). Four transects (100 m apart) were established, each 20 m wide and 150 m long (from the edge to the interior of the fragment), where all *E. tortuosum* plants were located. Numbered metal labels (5 x 5 cm) were used to mark 54 E. tortuosum plants.

Assessing mine morphology and anatomy, and leaf miner foraging: Mined leaves were collected at fifteen-day intervals from November 2005 until June 2006; 15 mined leaves were randomly collected from each of 16 plants distributed over transects, totaling 316 leaves. Under a stereomicroscope (Nikon SMZ 800 – 40X of magnification), these mines were observed externally and internally after dissection and some external and internal structures were described on both abaxial and adaxial mine surfaces.

To measure the area of the mines during leaf collections, a total of 366 mined leaves were randomly collected from 35 *E. tortuosum* individuals distributed over the transects. As above, leaves were collected at fifteen-day intervals from November 2005 until June 2006, and the number of plants used for leaf collections was reduced during the season in order to guarantee

the availability of plant material during the whole period of mine growth (Table 1). After collection, leaves were photographed in the laboratory at a standard distance of 20 cm using a digital camera (resolution: 2048 x 1536 pixels; Canon Powershot A400), and a transparent plate was placed over the leaves to flatten them and minimize distortions (Sinclair & Hoffman, 2003; Ishino *et al.*, 2011; De Sibio & Rossi, 2012). Mined areas of leaves (mm²) were measured on digital photographs using image analysis software (MetaVue, Version 6.3r4). The area of each leaf was also measured and the percentage of leaf area that was mined was calculated.

To study mine anatomy, three plants were randomly selected, and five mined and five unmined leaves were collected at random from each plant. The leaves were taken to the laboratory and fixed in a FAA 50 solution (Johansen, 1940), and then kept in 70% ethanol. After that, the leaves were dehydrated in an ethyl series and infiltrated in methacrylate according to the manufacturer's protocol. The leaves were then sectioned by using a rotating microtome (8µm thickness) and stained with blue toluidine (0.05%; pH 4.7) (O'Brien *et al.*, 1964). Permanent slides were mounted in synthetic resin (Permount), analyzed under light microscopy and photographed with a photomicroscope. The photos were used to characterize the main anatomical features of mined and unmined leaves.

Table 1. Mean values (\pm standard deviation) of mined areas (mm²), and the percentage of leaf area mined, observed on leaves collected throughout the growing season. N_p = Number of plants used per collection; N_r = Number of leaves collected.

Months	Mined area	% of leaf mined	Np	NL
Nov-Dec	4.246 (± 1.671)	0.143 (± 0.052)	17	129
Jan	31.778 (± 9.483)	$1.010 (\pm 0.467)$	17	79
Feb	47.431 (± 9.991)	1.703 (± 0.756)	11	60
Mar	72.086 (± 18.262)	2.750 (±1.318)	10	38
Apr	78.898 (± 41.825)	2.225 (± 1.024)	6	16
May	78.763 (± 35.631)	$2.606 (\pm 2.008)$	8	26
Jun	83.174 (± 20.592)	2.449 (± 0.685)	5	18

Assessing the emergence of the leaf miner and its parasitoids: In order to verify the emergence of leaf miners and their parasitoids, we used the same 366 leaves collected for mine area measurements (see previous section). Collected leaves were first photographed for mine area measurement, and then kept in Petri dishes for insect emergence. To determine the period of leaf miner oviposition, both sides of the leaf surface were carefully examined during collections in a search for leaf miner eggs. Assessment of leaf miner oviposition started in October 2005, one month before the first collection of leaves.

Leaves were individually kept in Petri dishes with a piece of moist cotton attached to the leaf petiole. The emergences of the leaf miner and its parasitoids were recorded monthly. Leaf miners were mounted with wings extended on small triangles fixed with entomological pins and placed in a small cardboard box. The parasitoids were placed in small plastic tubes filled with alcohol (70%). All insects were then sent to specialists, containing all collection information (e.g., collection location, date, collector), as follows: Klaus Sattler (Department of Entomology, Natural History Museum, UK) identified the micro-Lepidoptera; Michael Sharkey (Department of Entomology, University of Kentucky, USA) and Zuleide A. Ramiro (Experimental Center of the Biological Institute, Campinas, SP, Brazil) identified the braconid wasps; Christer Hansson (Biological Museum, Lund University, Sweden) and Valmir A. Costa (Experimental Center of the Biological Institute, Campinas, SP, Brazil) identified the Eulophidae individuals; Valmir A. Costa also identified Eupelmidae parasitoids; John S. Noyes (Department of Life Sciences, Natural History Museum, UK) identified the Encyrtidae. The leaf miner specimens were deposited in the Lepidoptera Collection of the Natural History Museum, London, UK, and the parasitoid specimens were deposited in the Department of Botany, São Paulo State University (Unesp), Botucatu-SP, Brazil.

RESULTS AND DISCUSSION

Mine morphology and anatomy, and leaf miner foraging: Only one species of leaf miner emerged from the leaves and was identified as an undescribed species of the genus *Agnippe* Chambers, 1872 (Lepidoptera, Gelechiidae) (Fig. 1), as already reported in two other studies (Ishino *et al.*, 2011; De Sibio and Rossi, 2012). Table 2 shows the known *Agnippe* species (including junior synonyms *Evippe* Chambers, 1873, *Phaetusa* Chambers, 1875, and *Tholerostola* Meyrick, 1917), including this undescribed species occurring on *E. tortuosum* (hereafter *Agnippe* sp.2; Ishino *et al.*, 2011), with their regions of occurrence and host plants.

Leaf damage caused by *Agnippe* sp.2 was typical of leaf miners, which by definition are larvae that feed internally, in the mesophyll between the upper and lower epidermis (Fig. 2A), where they form galleries (Jolivet, 1998; Labandeira, 2002). Depending on the species, leaf miner larvae can consume tissues like parenchyma, epidermis (leaving the cuticle intact) and veins of different thicknesses (Hering, 1951; Jolivet, 1998; Melo-de-Pinna *et al.*, 2002; Elb *et al.*, 2010; Pereira



Figure 1. Adult individuals of *Agnippe* sp.2: **(A)** dorsal view; **(B)** lateral view. Scale (1.0 mm) is represented by the black bar in (A).



1 2 3 4 5 6 7 8 9 10 11 12





Figure 2. (A) Mines (arrows) of *Agnippe* sp.2 on *Erythroxylum tortuosum* (adaxial surface); **(B)** two mines on the adaxial surface (black arrows) with augmented view: (I) epidermis after larval damage; (II) remains of leaf nervure; (III) darkened region; (IV) thin silk network made by the larva; **(C)** two mines (black arrows) of *Agnippe* sp.2 on *E. tortuosum* (abaxial surface): white arrows show a pair of semicircular openings, indicating the exact location where the first instar larva penetrated the leaf.





Figure 3. Cross-section in the midrib and intervein regions of *Erythroxylum tortuosum* leaves. (A) Unmined leaf (without mine cavity) showing vascular bundles, the adaxial epidermis with mucilaginous cells (arrows above) and the abaxial epidermis with papillary cells (arrow below). (B)-(D) Leaves with mine cavity: (B) a small leaf miner caterpillar within the mine and a soft membrane (empty arrow) right above it; (C) presence of the mine cavity and the vascular bundle; (D) detail of the mine cavity showing the leaf miner caterpillar and the soft membrane inside the cavity (empty arrows). ada = adaxial; aba = abaxial; vb = vascular bundle; la = larva (caterpillar); ca = cavity. Bars: (A) and (B) = 250μ m; (C) = 150μ m; (D) = 100μ m.

et al., 2018), although some leaf miners do not eat leaf veins (Almeida-Cortez & Melo-de–Pinna, 2006). Most *Agnippe* sp.2 mines were characterized as "blotch mines" because elongated and bending corridors, which characterize "serpentine mines", were rarely seen (Elb *et al.*, 2010). The larvae, which had protruding heads, formed blotch mines because they moved forward and backward in all directions within the mines while feeding. Serpentine mines were seen only for new growing larvae (i.e., young larvae from first instars).

On the adaxial surface, each mine presented a rigid darkened region, longitudinally situated right beside the central vein (Fig. 2B). In the laboratory, it was verified that the pupae of the leaf miners remained in the darkened region, and the prepupal stage usually started in June. Because mine expansion was almost complete by April (Table 1), it is possible that larvae spend their last two months of development deepening the area already mined, or that leaf consumption was drastically reduced due to low humidity at this time of the year (winter season), when the leaves are drier. Under the darkened region, a pair of semicircular openings was observed on the abaxial external leaf surface, indicating the exact location where the first instar larva penetrated the leaf (Fig. 2C). The openings consisted of a resistant and thick layer of silk and were also used by the larvae to eject faeces after foraging and by the adult moths to emerge from the mine. A delicate network of silk spun by the larvae was also observed within the mines, mainly on the inferior surface (Fig. 2B); the openings and silk network formed a single structure. Mines never crossed the leaf midrib,



Figure 4. Eggs of *Evippe* sp.2 (indicated by the arrows) deposited on the abaxial leaf surface of *Erythroxylum tortuosum*.

and unification of two mines into one occurred in about 5% of the observed mines. The area of mines and the percentage of leaf area mined reached, on average, a maximum of 83.174 mm² and 2.750%, respectively (Table 1).

Cross-sections in the mine region showed that the caterpillars consumed the palisade parenchyma and some small veins (third or fourth order), leaving the epidermis intact (Fochezato et al., 2018). Cells with phenolic compounds near mine cavities were also observed, which had a soft membrane around the interior (Fig. 3B,D). The mined and unmined leaves had a single-layered epidermis, with mucilaginous cells on the adaxial surface, and papillary cells and stomata on the abaxial surface, as previously noted by Beiras & Sajo (2004) (Fig. 3A). Figure 3C shows the detail of the vascular bundle of the midrib that remained intact. In the mined leaves it was possible to observe a caterpillar within the mine cavity (Fig. 3B,D). As mentioned above, Agnippe sp.2 caterpillars consume parenchymal tissue and the smaller veins (see Melo-de-Pinna et al., 2002 for another leaf miner species) and a soft membrane was observed inside the mine cavity. It is possible that this membrane was formed by silk threads woven by the larva; however, more studies are necessary to confirm the nature of this membrane.

Emergence of the leaf miner and its parasitoids: It has been argued that plant phenology is an important selective force for endophytic parasites such as leaf miners (e.g., Mopper et al., 2000). For example, leaf miner oviposition and larval development must be strictly linked with the production of leaves and leaf abscission (Faeth et al., 1981; Crawley & Akhteruzzaman, 1988; Hunter, 1992; Komatsu & Akimoto, 1995). Agnippe sp.2 females oviposited on the abaxial leaf surface, from October until April, and most eggs were laid in November when leaves were young, presented tender tissues and were generally expanded (Fig. 4 shows some eggs deposited on a leaf); this enables the first instar larva to reach the mesophyll. In the laboratory, six adult leaf miners emerged from August to October (Table 3), indicating that adult emergence and oviposition were synchronized with the peak of production of young leaves (see plant phenology details in Ishino et al., 2012). Because five adult leaf miners emerged in April and May (Table 3), it is possible that the life cycle of some individuals was shortened due to stress after removing

the leaves from the plants. On the other hand, as we did not observe the emergence of adults under field conditions, it is also possible that the life cycle of those individuals that emerged from August to October was lengthened due to suboptimal conditions. However, this second hypothesis is less likely, since many caterpillars in the intermediate stage of development died inside the leaves during March and April (we dissected the leaves when adult leaf miners did not emerge), indicating that the most likely period of emergence is from August to October. We also cannot neglect the possibility that a few leaf miners may emerge before the expected time in the field, resulting in a small partial second generation. Pupation occurred within the mine and adult moths emerged from semicircular openings on the abaxial external leaf surface (see "Mine morphology and anatomy, and leaf miner foraging" section). As leaf abscission occurs from August to September (Ishino et al., 2012), many pupae may drop to the ground within the abscised leaves, from which most moths may emerge.

Because the leaves dried quickly after collection, reducing larval survival, the emergence of adult leaf miners was very low. However, mortality imposed by parasitoids may also explain why few adults emerged (Table 3). Although mines may confer some protection against predators and parasitoids, many parasitoids prefer to attack larvae that are either chemically or physically (e.g. galls and mines) protected against predators (Jolivet, 1998; Gentry & Dyer, 2002). Parasitoid wasps of the families Braconidae, Encyrtidae and Eulophidae emerged from *Agnippe* sp.2 larvae (Table 3). As above, fewer parasitoids emerged, probably due to reduced larval survival (Table 3).

The parasitoids *Bracon* sp. and *Bassus* sp. (Braconidae) emerged mostly in March and April, and only one individual was observed in August. *Closterocerus coffeellae* Ihering, 1914 (Eulophidae), *Copidosoma* sp. (Encyrtidae) and *Chelonus* (*Microchelonus*) sp. (Braconidae) emerged mostly in August and September; however, one individual of *Chelonus* (*Microchelonus*) sp. was also observed emerging in April (Table 3). *Horismenus aeneicollis* Ashmead, 1904 (Eulophidae) presented less seasonal distribution than the other species, emerging in May, July, August and October, and *C. coffeellae* was the most abundant parasitoid (Table 3). Although two other parasitoids emerged, *Brasema* sp. (Eupelmidae) and *Chrysocharis* sp. (Eulophidae), the emergence dates could not be precisely determined.

Conclusion: Although it is a common consensus today that the Brazilian Cerrado has a great biological diversity, there is a scarcity of insect species described. Therefore, many leaf miners and other moth species remain to be discovered as well as their parasitoids. To improve the knowledge about the diversity of small Lepidoptera in this very important ecosystem, biological and ecological information about a Cerrado leaf miner was therefore provided here.

ACKNOWLEDGMENTS

We thank Dr Klaus Sattler (Department of Entomology, Natural History Museum, UK) for the micro-Lepidoptera identification, and Drs Michael Sharkey (Department of **Table 2.** Known species of the genus *Agnippe* Chambers, 1872, their regions of occurrence and host plants. Information about the focal species of this study is shown in bold. Data provided by Klaus Sattler (Department of Entomology, Natural History Museum, UK).

Species of Agnippe	Author	Year	Region of occurrence	Host plant
abdita	Braun	1925	Nearctic	Cercocarpus (Rosaceae)
aequorea	Meyrick	1917	Neotropical (Peru)	-
albidorsella	Snellen	1884	East Palaearctic	Lespedeza (Leguminosae)
aulonota	Meyrick	1917	Neotropical (Equator)	-
Biscolorella (type species of Agnippe)	Chambers	1872	Nearctic	Gleditsia (Leguminosae)
conjugella	Caradja	1920	Palaearctic	-
crinella	Keifer	1927	Nearctic	-
dichotoma	Li	1993	Palaearctic	-
echinulata	Li	1993	Palaearctic	-
evippeella	Busck	1906	Nearctic	-
evippella	Forbes	1931	Neotropical (Porto Rico)	Aeschynomene
				(Leguminosae)
fuscopulvella	Chambers	1872	Nearctic	-
haberlandi	Amsel	1961	Palaearctic	Alhagi (Leguminosae)
kuznetzovi	Lvovsky and Piskunov	1989	Palaearctic	-
laudatella	Walsingham	1907	Nearctic	Quercus (Fagaceae)
leuconota, Syn. plutella Chambers, 1875	Zeller	1873	Nearctic	-
lunaki	Rebel	1940	Palaearctic	-
miniscula	Li	1993	Palaearctic	-
novisyrictis	Li	1993	Palaearctic	-
omphalopa	Meyrick	1917	Neotropical (Ecuador)	-
penicillata	Amsel	1961	Palearctic	-
plumata	Meyrick	1917	Neotropical (Guyana)	-
postpallescens	Walsingham	1897	Neotropical (West Indies)	-
prunifoliella	Chambers	1873	Nearctic	Prunus (Rosaceae)
pseudolella, Syn. cephalella Caradja, 1920	Christoph	1888	Palaearctic	-
scoteropis	Meyrick	1931	Neotropical (Paraguay)	-
syrictis	Meyrick	1936	Palaearctic	Prunus (Rosaceae)
yongdengensis	Li	1993	Palaearctic	-
zhengi	Wang and Li	1994	Palaearctic	-
zhouzhiensis	Li	1993	Palaearctic	-
sp.1 (introduced into Australia (1998-	-	-	Neotropical (Argentina)	Prosopis (Leguminosae)
2000) for biological control of Prosopis			/	/
spp.)				
sp.2	-	-	Neotropical (Brazil)	<i>Erythroxylum tortuosum</i> (Erythroxylaceae)

Table 3. Monthly emergence of adult leaf miners and parasitoids (n° of individuals) from *Erythroxylum tortuosum* leaves throughout the year 2006.

	Months of emergence									
Leaf miner and its parasitoids	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
Agnippe sp.2	0	0	0	2	2	0	0	2	2	1
Bracon and Bassus	0	0	3	1	0	0	0	1	0	0
Microchelonus sp.	0	0	0	1	0	0	0	2	2	0
Closterocerus coffeellae	0	0	0	0	0	0	0	5	3	0
Horismenus aeneicollis	0	0	0	0	1	0	1	1	0	2
Copidosoma sp.	0	0	0	0	0	0	0	1	1	0

Entomology, University of Kentucky, USA), John S. Noyes (Department of Life Sciences, Natural History Museum, UK), Christer Hansson (Biological Museum, Lund University, Sweden), Zuleide A. Ramiro (Experimental Center of the Biological Institute, Campinas, SP, Brazil) and Valmir A. Costa (Experimental Center of the Biological Institute, Campinas, SP, Brazil) for the identification of the parasitoids. M.N. Ishino and M.N. Rossi are particularly grateful to Fapesp (Fundação de Amparo à Pesquisa do Estado de São Paulo – process number 05/52569-0), and P. R. De Sibio acknowledges Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), for financial support.

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