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Host plant preference in the protected myrmecophilous Transylvanian Blue (*Pseudophilotes bavius hungarica*) butterfly (Lepidoptera: Lycaenidae) and its relationship with potential ant partners

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Abstract When selecting specific host plants, caterpillars of many lycaenid butterflies, such as the protected Pseudophilotes bavius hungarica, are known to engage in various interactions with ants, which help them survive. Although P. bavius is a protected species, data about its host plant selection is very scarce, and little information is available on its myrmecophilous relationships. Our aim was to identify the host plant characteristics that determine the occurrence of the caterpillar and to clarify the specificity of its myrmecophily. We conducted a series of field surveys regarding host plant characteristics. Laboratory experiments were carried out to investigate the nature of interactions between the caterpillar and its potential ant partners. Control experiments involving non-visiting ants were also performed. On the basis of our findings, the physical characteristics of host plants do not seem to influence host plant choice, but the absence of aphids and the presence

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³ Department of Taxonomy and Ecology, Babeş–Bolyai University, Clinicilor 5-7, 400006 Cluj-Napoca, Romania of different ant species proved important. According to the results of behavioural assays, neutral reactions to the caterpillars were recorded in the case of ant species that regularly visited the host plant (*Lasius paralienus, Camponotus aethiops*), in contrast to *Tapinoma subboreale*, which was not observed at all on the host plants and which behaved aggressively towards the larvae. Therefore, the caterpillar is expected to show a certain ant host selectivity. The study constitutes an essential contribution to our knowledge of the natural history of a protected butterfly species, which can be used as a basis for more appropriate management strategies, while also shedding light on aspects of myrmecophilous relationships in Lycaenidae in general.

Keywords Butterflies · Conservation · Mutualism · Myrmecophily

Introduction

A wide variety of organisms, including plants, animals, fungi, and even bacteria, are known to engage in either mutualistic or antagonistic relationships with ants that can be either obligatory or facultative (Hölldobler and Wilson 1990; Schmid-Hempel 1998). The specific case of food-for-protection myrmecophily, however, is mostly restricted to arthropods, when the organism requires active feeding by ants or their protection, while the partner offers the ants other benefits, e.g. sugary droplets, which serve as a means of appeasement, making the mutualistic partner more valuable alive than as prey (e.g. Stadler and Dixon 1999; Axén 2000; Stadler 2002; Nyabuga et al. 2012).

In ant-mutualistic butterfly caterpillars, the basis of myrmecophily is also a food-for-protection type of relationship, in which the larvae rewards the ant partner with sugary excretions in exchange for protection (Fiedler 1995; Axén et al. 1996; Axén 2000). Experimental studies confirm that while ant-tended caterpillars weigh less or have prolonged development times probably due to costs related to the production of sugary secretions, at the same time they are exposed to fewer parasitic pressures than untended larvae (Pierce et al. 2002; Kaminski and Rodrigues 2011) and thus, they have better chances of survival (Robbins 1991; Axén 2000; Weeks 2003; Kaminski et al. 2010; Bächtold and Alves-Silva 2012). Ants also remove other herbivores from the plant, thereby providing the myrmecophilous larvae with a considerable advantage in times of food shortages (Ballmer 2003; Kaminski et al. 2010; Bächtold et al. 2014).

In lycaenid butterflies numerous studies have been published concerning both facultative (Fiedler 1991, 1995, 2001; Axén 2000; Weeks 2003; James 2006a; Gilbert et al. 2010; Kaminski et al. 2012; Bächtold and Alves-Silva 2012; Bächtold et al. 2014: Hojo et al. 2014: Thompson et al. 2014: Bury and Savchuk 2015) and obligatory myrmecophily (e.g. Als et al. 2002; Steiner et al. 2003; Tartally et al. 2008; Witek et al. 2014). Despite the fact that the vast majority (>75%) of myrmecophilous lycaenid butterflies are involved in facultative relationships (Fiedler 2006) and many of them are protected, little information is available on host plant selection and on the identity of ant partners compared to the more comprehensively studied obligatory myrmecophylic species. such as *Maculinea* (= *Phengaris*; see e.g. Witek et al. 2014). This information is highly relevant, since scientifically documented knowledge of the ecological demands of a given species could ensure the success of conservation strategies (see evidence based nature conservation sensu Sutherland et al. 2004; see Marttila et al. 1997 specifically). Moreover, a differential approach is required in the case of a protected species for which the larval stage has different ecological requirements than the adult stage, as in the case of butterflies.

Pseudophilotes bavius is a protected European lycaenid butterfly with a wide distribution. The P. bavius caterpillar engages in facultative food-for-protection type myrmecophilous relationships (Tolman 1992; James 2006a, b; Crișan et al. 2011; Dincă et al. 2011; Thompson et al. 2014; Bury and Savchuk 2015), as do other members of its genus (e.g. Obregón et al. 2014). Despite its conservation value, there is very little information available on the host plant selection of the species. Generally, the preference of butterflies for certain plant morphological characteristics during egg laying is well documented (e.g. Wiklund 1984; Czekes et al. 2014), and in addition to details concerning morphology, other specific features, such as presence of aphids and ants, could also be important for the survival of lycaenid caterpillars. Furthermore, the available information on the caterpillar's exact relationship with ants is mostly based on non-systematic field studies (e.g. Jutzeler et al. 1997; Crisan et al. 2011; Bury and Savchuk 2015), whereas for other Pseudophilotes species systematically documented

information is available (James et al. 2003; Thompson and Gilbert 2014; Thompson et al. 2014), as a few studies have been performed to elucidate the extent of the generality of larval host preference e.g. in the case of *Pseudophilotes sinaicus* (James et al. 2003; Thompson et al. 2014) and *P. baton* (Marttila et al. 1997).

The aim of the current study, therefore, is to address the following questions: (1) which host plant characteristics predict the presence of the butterfly larvae; (2) which host plant characteristics predict the presence of potentially caterpillar-tending ants on the host plant; (3) how specific is the myrmecophily of the caterpillar, and are there specific ant species that tend to the caterpillar which might differ in their behaviour towards the caterpillar compared to other non-tending ants? We (1) studied food plant preference of the larvae on the basis of observations made in the field and (2) performed laboratory experiments to characterize the interactions between caterpillars and potential ant partners.

Materials and methods

Study species

Pseudophilotes bavius hungarica (Diószeghy 1913), also known as the Transylvanian Blue, is a protected butterfly subspecies endemic to Transylvania (Romania). The butterfly has a narrow distribution, inhabiting forest-steppe patches of the Transylvanian Plain (Jutzeler et al. 1997; Crişan et al. 2011, 2014). It flies in May, depending on weather conditions (Crişan et al. 2011, 2014). Females lay eggs in the unopened flower of Salvia nutans, a post-glacial relic in the Carpathian Basin, in its early stage of inflorescence (Crisan et al. 2011). Within 7–12 days, the whitish larvae hatch (Crisan et al. 2011), and then, after the first ecdysis, they change colour and purple stripes appear on them resembling the calvces of the host plant. In the second half of June, in the last larval stage, they crawl down to the litter and pupate (Crisan et al. 2011). Crişan et al. (2011) reported that the larvae are visited by the ant Camponotus atricolor (Nylander 1849) in Transylvania, while according to Jutzeler et al. (1997) Crematogaster sordidula (Nylander 1849) could also tend to the caterpillar, at least under laboratory conditions. Formica ant species were also observed with P. bavius in Crimea (Bury and Savchuk 2015). However, these data are based on unsystematic field observations.

Pseudophilotes bavius is included in the annexes of the Council Directive 93/43/EEC¹ on the conservation of natural

¹ Council Directive 92/43/EEC on the conservation of Natural habitats and of wild fauna and flora OUG no 57/2007 approved with amendments by Law 49/2011 on the protected nature reserves, natural habitats, wild flora and fauna.

habitats and wild fauna and flora (Annex II. and IV.). It is also featured in Romanian legislation (OUG no. 57/2007, approved with amendments by Law 49/2011). Altogether, five Natura 2000 sites have been designated for the protection of this species in Romania. Specifically, the Transylvanian endemic *hungarica* subspecies is considered endangered, and it is feared that it will soon be critically endangered if its natural habitats disappear due to changing land use practices (Rákosy et al. 2003). The abandonment of traditional haymaking causes the accumulation of decayed plant material and litter, which is a major threat to the survival of the butterfly's host plant *Salvia nutans* (Jakab and Kapocsi 2005). Furthermore, the subsequent invasion of abandoned areas by shrubs is an important factor that significantly alters the habitats of the butterfly (Crişan et al. 2014).

Study site

The study site is in Transylvania, Romania (N 46.795373°; E 23.956771°; Suatu village, Cluj County), and it is part of the Suatu Natura 2000 Site (ROSCI0238). It is an abandoned vineyard with a high density of Salvia nutans. The hill is terraced with a southwest-facing slope, between 370 and 425 m a.s.l. (Crişan et al. 2014). The climate of the region is temperate continental with an average yearly precipitation of 550-600 mm and a mean annual temperature of 8.5 °C (Kun et al. 2007). The natural vegetation of the area is forest-steppe/forest (Kun et al. 2007). At present, small remnants of oak (Quercus robur, Q. petraea) and oak-hornbeam (Q. petraea, Carpinus betulus) forests can be found on the hilltops and on northern facing slopes. Most of the area is used for agricultural crops, and it is grazed by sheep and cattle, while some parcels are still used as hayfields. The steep, southwest-facing sides of the site were terraced for viticulture in the mid-1960s. The use of the land for viticulture lasted two decades, after which it was used for extensive grazing. The terraces currently house the Transylvanian Blue population. Due to the abandonment of traditional haymaking, shrubs are invading the terraces (Crisan et al. 2014).

Data collection and experimental methods

In order to characterize host plant selection of the caterpillar we randomly selected 215 *Salvia nutans* shoots in the same study site by randomly throwing a marked object by the same person, and choosing the shoot closest to the spot where the object fell. We did this on 16–18 June, 2014. We recorded several characteristics of the plants: height (cm), number of inflorescences, presence of butterfly larvae, presence and abundance of different ant species on the plant and, separately, on the caterpillar, and the presence and abundance of aphids. The abundance of aphids was visually estimated on the basis of the measured stem length occupied by 10 individual aphids (rounded to the closest 10) (see Erős et al. 2009). All characteristics were assessed and measured by the same person. Measuring tape was used to determine plant height.

Interaction assays between the butterfly larvae and different ant species were carried out under laboratory conditions to reveal the differences between tending and non-tending ant species with regards to their relationship to the caterpillar. Due to its protected status, we only used 21 P. bavius larvae collected from the study area, all in the fourth instar and therefore all of the same size. Four species of ants were used during the assays. Two of the ant species that were tested, Lasius paralienus Seifert 1992 and Camponotus aethiops (Latreille 1798), had been recorded during field observations (see above) on the host plant (see Results). Tapinoma subboreale Seifert 2012 was also included in the assays as a control species, since it commonly occurs in the study area, but it has not been observed on the host plant (see Results). In addition, we also included Myrmica scabrinodis Nylander 1846, a species that does not occur at all in the study area but is known to be one of the main host species of other lycaenids in the region, such as Phengaris species (see Witek et al. 2014). All ant species tested, with the exception of *M. scabrinodis*, came from the study site, while M. scabrinodis was collected from Luna de Jos (Cluj County), ca. 35 km from the study site. Queenless colony fragments of each of these ant species were collected from the field 1 day before the experiment and were set up in the lab with food and water ad libitum. For each test one caterpillar was allowed to interact with one ant worker in a $10 \times 10 \times 4$ cm plastic box. The ants were transferred to the test arena with the use of a pooter and were kept in the arena for 5 min. No unusual behavior was observed in the ants during the accommodation period. The observations began with the first contact between the ant and the caterpillar, and the period of observation lasted for 5 min (see Agrawal and Fordyce 2000). All interactions were recorded and categorized as follows: (1) antennation, (2) mandible gaping, (3) biting. The first type of event was taken as neutral and the latter two events were regarded as aggressive behaviour (Atsatt 1981; Pamminger et al. 2011; Maák et al. 2014). Due to the small number of available larvae, the same caterpillar was presented separately to four different ant workers of the four different species in a randomized order. Between tests, the larvae were kept separately for 120 min in order to ensure that they lost most of the chemical cues they might have gained in the course of their contacts with the ants, although due to the brevity of the period of contact, it is unlikely that they accumulated many such cues. Altogether, 84 tests were carried out. After the experiment, the larvae continued to be kept under laboratory conditions and used in other experiments (Crişan et al. in prep.).

Data management

The effect of different host plant characteristics on the presence of P. bavius hungarica larvae was analyzed using the Generalized Linear Model approach (GLM, binomial error, maximum likelihood approximation, N=215). The presence of larvae on the host plant was introduced as a dependent variable, while plant height, number of inflorescences, presence and abundance of aphids, and presence and abundance of ants were introduced as input variables and factors. The effect of host plant characteristics on the presence of ants was also tested separately using GLM (binomial error, maximum likelihood approximation, N=215). In this case, the presence of ants was the output variable, while the presence of larvae, plant height, the number of inflorescences, and the presence and abundance of aphids were introduced as input variables and factors. We also tested the effect of host plant characteristics on the abundance of plant-visiting ants using GLM (Poisson error, maximum likelihood approximation, N=215), with the number of ant individuals as an output variable and the presence of larvae, food plant height, the number of inflorescences, and the presence and the abundance of aphids as input variables and factors (Grueber et al. 2011). Automated model selection was carried out in each case, and the effects of different explanatory factors and variables were averaged across the best models with delta <4 (see Grueber et al. 2011).

An aggression index (AI) for each caterpillar-ant interaction assay was calculated using the formula: AI=number of adverse interactions/total number of interactions (see Pamminger et al. 2011; Maák et al. 2014). In a similar manner, an antennation index (ANI) was also calculated as follows: ANI=number of antennation events/total number of interactions. The resulting AI and ANI values were analyzed using a Generalized Linear Mixed Model approach (GLMM, binomial error, maximum likelihood fit, N=84), with the indices as output and the ant species as fixed input factors, while the ID of the caterpillars was considered a random factor in order to handle dependencies. Non-metric Multidimensional Scaling (NMDS, Bray-Curtis similarity index) was applied to visualize the association between interaction types and the different ant species tested.

All statistical analyses were carried out using the R 3.1.1 statistical package (R Development Core Team 2008). GLMs and GLMMs were performed using the *lme4* R-package (Bates et al. 2013). Automatic model selection procedure was performed using the *dredge* function of the *MuMIn* R-package (Bartoń 2013). *Relevel* function was used in order to carry out sequential comparisons among factor levels when performing GLMM analysis. In this case, we applied table-wide sequential Bonferroni-Holm correction to determine the exact significance levels among different factor levels. NMDS was performed with the *metaMDS* function of the *vegan* R-package (Oksanen et al. 2013).

Results

Host plant choice and ant attendance in the field

Altogether, 28 *P. bavius hungarica* larvae were detected on 215 plants investigated, and each larva was found on a separate host plant. The most stable and also most abundant ant species on *Salvia nutans* and on the aphids were *Lasius paralienus*, *Camponotus aethiops* and *Plagiolepis pygmaea*, while members of the subgenus *Serviformica* were detected in low numbers and at a low degree of stability (Table 1).

The best average GLM model explaining the presence of caterpillars retained all original input variables and factors. The presence of ants had a significant positive influence on the caterpillar's presence (z=2.68, p<0.01), while their abundance (z=-2.27, p<0.05) and also the presence of aphids (z=-2.86, p<0.01) had a significant negative effect. On the other hand, the height of the host plant (z=0.295, p=0.76), the number of flowers (z=0.29, p=0.77), and the abundance of aphids (z=0.05, p=0.95) did not have any observable significant effect.

In the case of the ants, the best average GLM model explaining their presence also retained all original input factors and variables. However, none of the factors proved relevant in this case. Thus, the presence of ants was not influenced significantly by the presence of caterpillars (z=0.73, p=0.46), the presence and abundance of aphids (z=0.01, p=0.99), or even the height of the host plants (z=0.12, p=0.9) or the number of inflorescences (z=0.35, p=0.9)p = 0.72). The best average GLM model regarding the abundance of ants again retained all original input variables and factors. The abundance of ants was significantly influenced positively by the presence of aphids (z=8.43, p<0.0001) and negatively by the presence of the caterpillar (z = -2.32, p < 0.01). The abundance of aphids (z = 0.04, p = 0.98), the height of the food plant (z = 1.84, p = 0.06), and the number of flowers (z=1.66, p=0.09) did not have any observable significant effect.

Table 1 The mean abundance $(\pm SD)$ of ants at aphid colonies and on the food plants, and the stability (%) of their presence (no. of positive obs./no. of all obs.) on the host plant

Species	Aphid	Plant	Stability
Camponotus aethiops (Latr., 1798)	0.21 (±0.66)	0.37 (±0.81)	23.25
Lasius paralienus Seifert, 1992	0.42 (±1.03)	0.5 (±1.07)	25.11
Formica (Serviformica) sp.	0.02 (±0.12)	0.11 (±0.22)	6.5
Plagiolepis pygmaea (Latr., 1798)	0.64 (±2.19)	0.87 (±2.42)	18.6

Interaction assays

Generally, with the exception of *Tapinoma subboreale*, the behavior of all ant species tested was neutral towards the caterpillar during the interaction assays performed under laboratory conditions. Only *T. subboreale* showed increased aggressive behaviour towards the larvae (Fig. 1), as also shown by the output of the NMDS analysis, since all aggressive interactions were associated with this species (Fig. 2). Due to the lack of aggression in other ant species, GLMMs could only be performed for the ANI outputs, and not for the AI values. The GLMM results indicate that *Camponotus*

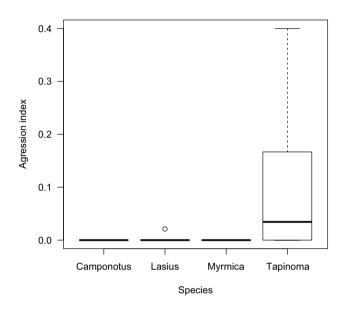
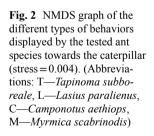
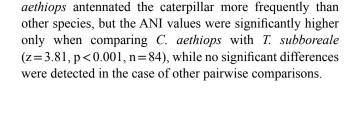


Fig. 1 Aggression indices of tested ant species. (Abbreviations: Tapinoma—*Tapinoma subboreale*, Lasius—*Lasius paralienus*, Camponotus—*Camponotus aethiops*, Myrmica—*Myrmica scabrinodis*)



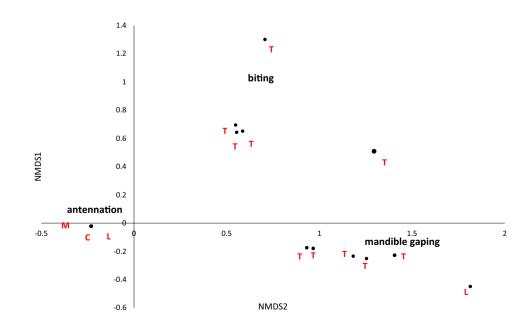


Discussion

Host plant selection in the field

Host plant features usually play a significant role in the oviposition decisions of the female butterflies (e.g. Baylis and Pierce 1991; Webb and Pullin 2000; Pickens and Root 2008; Fürst and Nash 2010; Jansson 2013; Czekes et al. 2014; Thompson et al. 2014), since plant quality has a major influence on the survival of the hatching larvae (e.g. Baylis and Pierce 1991). Thus, adult females prefer larger food plants with a higher number of stems per clump and a higher number of flowers and/or leaves (see James et al. 2003; Arnyas et al. 2006; Czekes et al. 2014; Thompson et al. 2014). Since P. bavius eggs are laid inside an inflorescence, we could only assess female preferences on the basis of the presence of the larva, which is only a proxy for female egg-laving preferences, since eggs can be lost due to predation, parasitism, or harsh climatic conditions, such as heavy rainfall. According to our findings from the field survey, the morphological characteristics of food plants are of no relevance to the presence of caterpillars, while the absence of aphids and the presence of ants seemed important.

Aphids may contribute to the occurrence of fungal infection on the host plant due to the deposition of their sugary secretions, and/or their presence may trigger defensive



reactions from the plant, which could lower the quality of the larval food plants (Stadler and Dixon 2008). Therefore, aphid infestation may have a negative effect on butterfly oviposition (Stadler and Dixon 2008; Árnyas et al. 2009). The negative link between the occurrence of aphids and *P. bavius* caterpillar on *Salvia nutans* supports the conclusions of previous studies, in particular given that our findings refer directly to the presence of larvae.

Egg laying choices could create the spatial link between larvae and host ants because of the lycaenid larvae's limited mobility (e.g. Pierce and Elgar 1985; Fiedler 1991; Seufert and Fiedler 1996). The positive association between the presence of ants and caterpillars is also observable here (Thomson et al. 2014). However, ants are not attracted specifically to plants bearing caterpillars, while their abundance is positively influenced only by the quantity of aphids. The negative link between ant abundance and caterpillars could in fact benefit the caterpillar, since high ant abundance could imply undesired costs for the caterpillar, such as enhanced production of sugary secretions, which could be detrimental to the larval development, as it has been shown to be in other species (e.g. Robbins 1991; Axén et al. 1996). Therefore, the presence of ants is beneficial to the larvae, but only if the ants are low in number.

Potential ant partners

There also seems to be adaptation to a given range of potential host ant species on the part of the larvae, implying the existence of a certain degree of host selection. Thus, ant species that were generally visiting the host plant were not aggressive towards the caterpillar, whereas T. subboreale, which was absent from the plant, behaved more aggressively. The least aggressive species towards the caterpillar was Camponotus aethiops. Presumably, this species of ant could be the preferred host, at least in the wider study region. Crisan et al. (2011) also observed the related C. atricolor with the caterpillar close to our study site, while in the case of the related Iberian Pseudophilotes abencerragus and P. panoptes, ants of the Camponotus genus were observed to tend to the caterpillars, in addition to members of other genera (Obregón et al. 2014; see Table 2). In other butterfly species, such as the riodinid Apodemia paucipuncta and the lycaenid Parrhasius polibetes, evidence was found suggesting that the Camponotus species might be involved in the facultative myrmecophily (De Vries et al. 2004; Kaminski and Rodriguez 2011). In the case of lycaenids, it is generally assumed that Camponotus species are important ant partner (Fiedler 2006; Bächtold et al. 2014), because members of this genus are successful plant guards (Oliveira 1997; Bächtold et al. 2014), and they do not usually attack the larvae (Kaminski et al. 2010; Bächtold et al. 2014). However, the host choice of P. bavius must be quite generalist, since in our case the Lasius and Myrmica species also responded positively or at least neutrally to the larvae.

Conservation perspectives

The myrmecophilous lifestyle requires adaptations on the part of the myrmecophilous species, this being important not only in order to attract ants, but also to decrease the ants'

Table 2 Known ant partners and enemies of different Pseudophilotes species

Pseudophilotes species	Ant partners	Ant enemies	References
P. sinaicus Nakamura 1975	Lepisiota obtuse (Emery 1901) Lepisiota hirsuta (Santschi 1914)	Crematogaster antaris (Forel 1894)	James (2006b); Thompson et al. (2014)
P. abencerragus (Pierret 1837)	Camponotus foreli Emery 1881	-	Obregón and Gil-T (2011)
	Plagiolepis pygmaea (Latreille 1798) Crematogaster auberti Emery 1869		Obregón et al. (2014)
P. panoptes (Hübner 1813)	<i>Camponotus piceus</i> (Leach 1825) <i>Lasius grandis</i> Forel 1909 <i>Crematogaster auberti</i> Emery 1869	-	Obregón and Gil-T (2011)
	Camponotus sp.		Fiedler (2006)
	Camponotus cruentatus (Latreille 1802) Camponotus foreli Emery 1881		Obregón et al. (2014)
P. bavius (Eversman 1832)	Camponotus atricolor (Nylander 1849)	-	Crișan et al. (2011)
	Crematogaster sordidula (Nylander 1849)		Jutzeler et al. (1997)
	Formica sp.		Bury and Savchuk (2015)
P. bavius hungarica (Diószeghy 1913)	Camponotus aethiops (Latreille 1798) Lasius paralienus Seifert 1992		Present study
P. vicrama (Moore 1865)	Camponotus sp.	_	Bury and Savchuk (2015)
P. baton (Bergsträsser 1779)	<i>Myrmica scabrinodis</i> Nylander 1846 <i>Lasius alienus</i> (Foerster 1850)		Fiedler (2006)

aggressiveness towards them (Barbero et al. 2012) and ensure them the protection of the ants (Axén et al. 1996; Axén 2000). Many factors can affect the costs and the benefits of myrmecophilous relationships, including even the absence of third parties such as aphids (Axén et al. 1996). The Transylvanian Blue is a protected endemic butterfly threatened by changing agricultural practices that could reduce the size of its habitats due to the abandonment of traditional hay making followed by afforestation (e.g. Crişan et al. 2011, 2014). Until now, major efforts have concentrated on the management of the areas from the perspective of the persistence of appropriate host plant populations. However, since the larvae are facultatively myrmecophilic, the preservation of appropriate ant communities with a considerable proportion of e.g. ground-dwelling Camponotus ant species, which require a certain amount of bare soil patches and low vegetation, should constitute a non-negligible aspect of the management plans. The findings of the current studies could serve as the basis for further development of appropriate conservation plans for this endangered butterfly species.

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