

Phengaris (Maculinea) teleius butterflies select host plants close to *Myrmica* ants for oviposition, but *P. nausithous* do not

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Abstract

Many lycaenid butterfly species have interactions with ants, with 12% obligatorily depending on two sequential sources of larval food, namely host plants and host ants. When host plants are abundant but the density of host ant nests is relatively low, most host plants have no host ant nest in their vicinity and are thus unsuitable for larval survival. Obligatorily myrmecophilous female butterflies, whose caterpillars feed on ant larvae, would have a comparative advantage if they deposit eggs on host plants in the proximity of a host ant nest. However, this ant-mediated oviposition has been hotly debated. In an open-air insectory experiment, we tested whether oviposition is ant-mediated or not for two obligatory myrmecophilous butterfly species, *Phengaris (Maculinea) nausithous* Bergsträsser and *Phengaris teleius* Bergsträsser (Lepidoptera: Lycaenidae). Female butterflies could select host plants close to either no ant nest or a nest of one of the two *Myrmica* species (Hymenoptera: Formicidae) that are thought to be their host ant. Our results support ant-mediated oviposition in *P. teleius*, but also indicate that there is no preference for either of the *Myrmica* species. More eggs were deposited and more caterpillars were found on long flowerheads close to *Myrmica* ant nests than on those without nests. Our findings suggest that ant presence is more important than flowerhead phenology for females of *P. teleius*. In contrast, *P. nausithous* females were not attracted by ants but preferred long flowerheads with a low percentage of green coloration. With these findings, this study contributes to better understanding of the habitat requirements of two highly specialized butterfly species.

Introduction

In insect species, the location where a female deposits her eggs has a huge impact on the survival of the offspring. In most butterfly species, for example, females choose to lay their eggs on or near the plants the caterpillar will feed on, preferably with microclimatic conditions for optimal development (Garcia Barros & Fartmann, 2009). Many lycaenid butterfly species have interactions with ants, with 12% obligatorily depending on two sequential sources of larval food, namely host plants and host ants. For some obligatorily myrmecophilous butterfly species, which have mutualistic associations with ants, females are known to

deposit their eggs within the limited foraging ranges of host ants, i.e., ant-mediated oviposition (Henning, 1983; Pierce & Elgar, 1985; Smiley et al., 1988; Jordano et al., 1992; Fraser et al., 2002). In these species, butterfly larval secretions provide food for the ants while the larvae benefit from protection from parasitoids and predators including the ants themselves. In facultatively myrmecophilous butterfly species, which can also survive without attendance of ants, ant-mediated oviposition is also found to occur (Atsatt, 1981; Wagner & Kurina, 1997).

Unlike most Western Palearctic lycaenid species, *Phengaris (Maculinea)* spp. butterflies are obligate parasites of ants. After a short period of feeding on the host plant, the caterpillar develops as a predator or competitor of ant brood in the host ant nest (Thomas, 1984; Thomas et al., 1989; Nash et al., 2008). It drops or walks to the ground and waits for a *Myrmica* spec. (Hymenoptera: Formicidae) worker ant to be found. Using chemical and acoustical

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deception (Akino et al., 1999; Schönrogge et al., 2004; Nash et al., 2008; Barbero et al., 2009, 2012; Sala et al., 2014), the caterpillars induce workers of any *Myrmica* species to adopt them, but their survival until the adult stage will depend largely on which ant species has found the parasite larva (Thomas et al., 1989; Elmes et al., 1991, 2002; Akino et al., 1999). In order to exploit the ant nest as efficiently as possible, specific adaptations are needed to mimic the ant larvae in their behaviour and communication signals (Akino et al., 1999; Schönrogge et al., 2004; Barbero et al., 2009; Sala et al., 2014). The better the adaptation of a parasite to a specific host ant species, the better this specific ant nest can be invaded, but also the lower is the chance to survive in other ant species' nests (Nash et al., 2008). In *Phengaris* butterflies, this results in local host ant specificity, with some butterfly species being adapted to different host ant species on different locations. To date, 16 species of *Myrmica* have been described as host ants for *Phengaris* in Europe (Als et al., 2001; Steiner et al., 2003; Tartally et al., 2008; Witek et al., 2010a,b, 2014; Arnaldo et al., 2011). To reduce the costs of such an expensive way of raising larvae, strong selection on oviposition site choice is expected, and this choice should include the host plants that are located within the spatial distribution of host ant nests. Especially in species with obligate myrmecophilous relationships, survival of caterpillars would increase if their mothers would be able to select for oviposition sites close to host ant nests rather than spreading the eggs randomly.

The two large blue butterfly species *Phengaris nausithous* Bergsträsser and *Phengaris teleius* Bergsträsser (Lepidoptera: Lycaenidae) often occur syntopically, with *P. teleius* usually being rarer. Both species oviposit on flowerheads of *Sanguisorba officinalis* L. (Rosaceae) (Figurny & Woyciechowski, 1998). The early-instar caterpillars feed on developing seeds. After 2–3 weeks, fourth-instar caterpillars leave their host plant to be found by *Myrmica* spec. worker ants and taken to the underground ant nests. The caterpillars feed mainly on ant larvae; however, *P. nausithous* is also fed by worker ants though to a lesser extent than the truly predatory species *Phengaris alcon* (Denis & Schiffermüller) and *Phengaris rebeli* (Hirschke) (Thomas & Elmes, 1998). In the populations used in our study, the main host ant species for *P. nausithous* is *Myrmica rubra* (L.), whereas *P. teleius* is mainly found in nests of *Myrmica scabrinodis* Nylander (Wynhoff et al., 2008). In most habitats of the two investigated species, the single shared host plant *S. officinalis* is abundant, whereas the nest density of the relevant host ant species is relatively low and butterfly population size is directly dependent on host ant nest density (Anton et al., 2008; Dierks & Fischer, 2009). Hence, the majority of these host plants are sinks

for the caterpillars. A low ant nest density compared to host plant density also suggests a possible advantage of ant-mediated oviposition in these two butterfly species, because when eggs are randomly distributed over the host plants of which most are sinks, the fraction of instars with low survival rate due to lack of host ant nests will be high.

Some studies found that females of *Phengaris* spp. oviposit on suitable host plants regardless whether nests of *Myrmica* ants are in close proximity of the plant, i.e., they oviposit randomly with respect to (host) ant nests (Thomas et al., 1989; Thomas & Elmes, 2001; Nowicki et al., 2005; Musche et al., 2006), whereas others found support for ant-mediated oviposition (Van Dyck et al., 2000; van Langevelde & Wynhoff, 2009; Van Dyck & Regniers, 2010; Patricelli et al., 2011; Wynhoff et al., 2015). Most of these studies were based on field observations, where patterns of egg distribution were compared with patterns of ant nest distribution. The detected correlations provide information on the probability that a caterpillar is found by worker ants of *Myrmica* spp., but they cannot clarify whether female butterflies truly have used the presence of ants as an oviposition cue or whether they have reacted to other cues. For example, ant species may occupy microhabitats with subtle differences in phenology of plants, or female butterflies show only limited dispersal after leaving the host ant nest, both resulting in the same correlation of spatial patterns.

In this study, we conducted an experiment in which the arrangement of plants and ants is controlled such that they are independent of each other and from the distribution of females. Whether or not oviposition in two *Phengaris* butterfly species is ant-mediated is tested in an open-air insectary experiment to better control for confounding factors – for example, the co-occurrence of host ant nests with host plants or correlation between plant characteristics and host ant nest presence – as often found in field studies. This experimental approach allowed a test of the cause–effect relationship: does the presence of host ant nests increase the probability that a female deposits eggs on the nearby host plant? In the experiment, females of both species were free to choose between host plants in plots with the host ant species *M. rubra* or *M. scabrinodis* or without ants, whereas vegetation characteristics were kept as similar as possible between the plots. If females deposit their eggs independent of the presence of *Myrmica* ants, the distribution of observed ovipositions should not be related to the absence or presence of ants. However, if females are able to respond to the presence of ants, then this should be revealed in the oviposition distribution. Ovipositions were studied directly by observations of females and indirectly by capturing the caterpillars when they leave the host plant.

Material and methods

Insectory experiment

The experiment consisted of six plots with *S. officinalis* plants in an open-air gauze insectory, measuring 4×12 m (Figure 1). In early spring, before the experiment, all ant nests in the entire insectory were removed. At the outside edges, PVC-laminated wooden boards with floum were placed to prevent new colonization by *Lasius* or *Myrmica* ant species from outside. In the beginning of June, vegetation sods (40×60 cm) with meadow vegetation including *S. officinalis* plants but free of ant nests were translocated from the nature reserve Moerputten in The Netherlands ($51^{\circ}41'N$, $5^{\circ}15'E$, altitude 2 m above sea level; for further description see Wynhoff, 1998; Wynhoff et al., 2008) to the insectory. The sods were excavated to a depth of 15 cm, leaving the roots of the *S. officinalis* plants untouched. The plots, consisting each of four sods, were separated from each other by 30-cm-wide water-filled ditches to prevent ants from leaving their home plot. Each plot consisted of on average 31 *S. officinalis* plants (range 23–35). At the time of replanting, *S. officinalis* plants had not started flowering yet and were not harmed by the transplantation. All plants and flowerheads were marked and coded with small paper labels of 0.5×0.7 cm. The labels were attached to the plant with a short thin cotton string at the flowerhead stalk, as far away from the flowerhead as possible. Around the plots, the vegetation consisted of various grass species. These grasses were mown regularly.

At the end of July, four nests, two of *M. scabrinodis* and two of *M. rubra*, were excavated from the nature reserve Moerputten and brought to the insectory. The ant nests were placed in the middle of four of the plots and the ants were given the possibility to settle themselves. This resulted in two plots with *M. scabrinodis*, two plots with *M. rubra*, and two control plots. To prevent ant migration to other plots, pit fall traps were placed at the edges of the plots and the ant colonies were fed abundantly every day with wingless *Drosophila* spec. flies (larvae and adults), fruit, and sugar cubes. The short time period between introducing the ant nests and the release of the females (around 6 days) did not allow the ants to affect the plants (as described in Patricelli et al., 2015). Our design provided us with the opportunity to test the effect of ant nest presence rather than the possible indirect effect of plants being affected by ant presence.

In The Netherlands, wet meadows with *S. officinalis* plants which are suitable as habitat for large blue butterflies are rare and protected. Therefore, it was not possible to excavate more vegetation sods and the species were tested one after the other. As females of *P. teleius* use

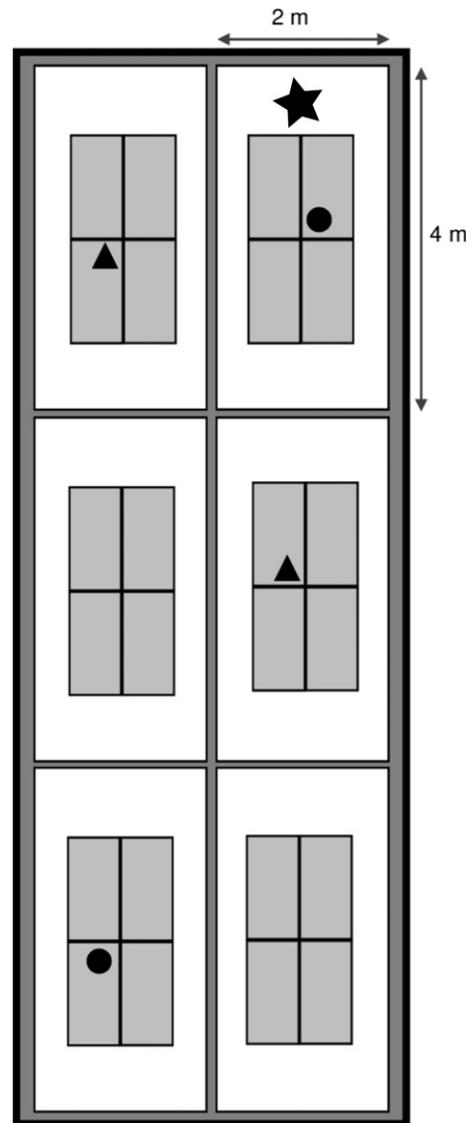


Figure 1 Layout of the open-air insectory. Each plot measures 2×4 m. Black bold line: shelves with floum; dark grey: ditches with water; dot: *Myrmica scabrinodis* nest; triangle: *M. rubra* nest; star: plot invaded by *M. rubra*.

younger flowerheads and, contrary to *P. nausithous*, may choose to avoid occupied flowerheads for oviposition (Figurny & Woyciechowski, 1998; Sielezniew & Stankiewicz-Fiedurek, 2013), this species was tested first. In the early morning of 4 August, 16 young mated females and two males of *P. teleius* were released into the insectory. They were distributed equally over the plots and were then left undisturbed. For oviposition, the females could freely choose between 1 452 flowerheads over the six plots. After a short time, the first females started to lay eggs. During

the first 2 days, ovipositions were continuously recorded (between 09:00 and 19:00 hours) together with the ID of the flowerheads. Observation time per plot was kept equal. On 8 August, the last surviving individuals of *P. teleius* were removed from the insectory. The same day, 16 young mated females and three males of *P. nausithous* were released and during 2 days 132 ovipositions were recorded.

Data collection

The impact of the presence of host ant species on the oviposition behaviour of the females was studied using the observed ovipositions during the first 2 days and the caterpillars by capturing them after leaving the flowerhead. As we could have missed oviposition events during the observation time, we also analysed the presence and absence of caterpillars. The two species differ in egg load and hence in the expected number of caterpillars. *Phengaris teleius* deposits usually just one egg at a time, whereas *P. nausithous* often lays several eggs sequentially on the same flowerhead. For the latter species, the egg load per flowerhead can be in excess of 20 (Figurny & Woyciechowski, 1998).

One day before the release of the butterflies, we measured the height and the size of all flowerheads; the size was measured from the flower stalk to the tip of the flowerhead. We also estimated the percentage of flowers enclosed by green sepals per flowerhead (% green). The females of *P. teleius* prefer very young flowerheads without open flowers and a high percentage of flowers still enclosed by green sepals. In the case of *P. nausithous*, flowerheads selected for oviposition are generally older and longer (Figurny & Woyciechowski, 1998).

In the middle of August, when the butterflies stopped flying in the insectory, all flowerheads larger than 6 mm and a subset of the smaller ones (in total 995 flowerheads) were encapsulated in small gauze bags to prevent the caterpillars from leaving the flowerhead they had lived on. All encapsulated flowerheads were examined daily for caterpillars. Caterpillars were recorded and collected for other purposes, after which the flowerhead was encapsulated again. From all caterpillars, we recorded the species and the ID of the flowerhead from which it dropped. Five gauzed flowerheads were broken during handling and these were discarded from the analysis.

Data analysis

We analysed the effect of the ant species presence on oviposition and caterpillar presence/absence as dependent variables with a generalized linear mixed model (GLMM) (binomial error distribution with logit link). The differences in presence/absence of an oviposition event between the treatments were tested for 1 452 flowerheads. For the

analysis of presence/absence of a caterpillar, we used 990 encapsulated flowerheads. We included the explanatory variables 'ant species presence' together with 'size of the flowerheads' and '% green coloration' as covariates. The size of the flowerheads and the percentage of green coloration were negatively correlated ($r = -0.649$, $P < 0.001$; $n = 1\,452$); hence, we tested their effect separately. As there were on average 31 plants per plot and each plant had on average 7.8 flowerheads, we included 'plot', 'plant', and 'flowerhead' as random factors. We nested 'flowerhead' within 'plant' and 'plant' within 'treatment' to control for the potential non-independence of the data associated with repeated measures of the same flowerhead or of the same plant. We tested the differences in flowerhead size, percentage of green coloration, and number of flowerheads between the treatments with GLMMs (distribution is normal and link function is identity for flowerhead size and number of flowerheads, gamma error distribution with log link for the percentage of green coloration). Random factors and nested design were the same as for the oviposition and caterpillar analyses, except for the number of flowerheads as one value per plant was measured (the residuals met the model assumptions). When analysing oviposition and caterpillar presence of *P. nausithous*, we included the presence of prior oviposition events of *P. teleius* into the models, using the presence/absence of caterpillars (as the *P. teleius* females or the eggs could have left pheromones or the caterpillars could have changed the phenology of the flowerhead). Interactions between the treatments and the plant variables were not significant and therefore not included. For all GLMMs, differences between the treatments were tested with the post hoc least significant difference (LSD) test. To further analyse the differences between treatments, we calculated Cohen's d to estimate effect size (Cohen, 1988). We selected the random covariance matrix in the GLMMs using the Akaike's Information Criterion (AIC). Data analyses were carried out with IBM SPSS Statistics v.22 (IBM, Armonk, NY, USA).

Results

We recorded 166 oviposition events of *P. teleius* and 132 of *P. nausithous*, and found in total 603 caterpillars of *P. teleius* (37.8 per released female) and 118 caterpillars of *P. nausithous* (7.4 per released female). Caterpillars of *P. teleius* were found on 388 flowerheads, caterpillars of *P. nausithous* on only 78 flowerheads. The differences between the numbers of oviposition events and flowerheads with caterpillars suggest that we missed ovipositions during or outside the observations. Flowerhead size did not differ between treatments ($F_{2,1448} = 1.91$, $P = 0.15$); the plots with *M. scabrinodis* had a lower

percentage of green coloration ($F_{2,952} = 6.89$, $P = 0.001$), the plots with *M. rubra* contained on average more flowerheads per plant ($F_{2,182} = 5.66$, $P = 0.004$; number of flowerheads per plant was ln-transformed) (Figure 2). More detailed information

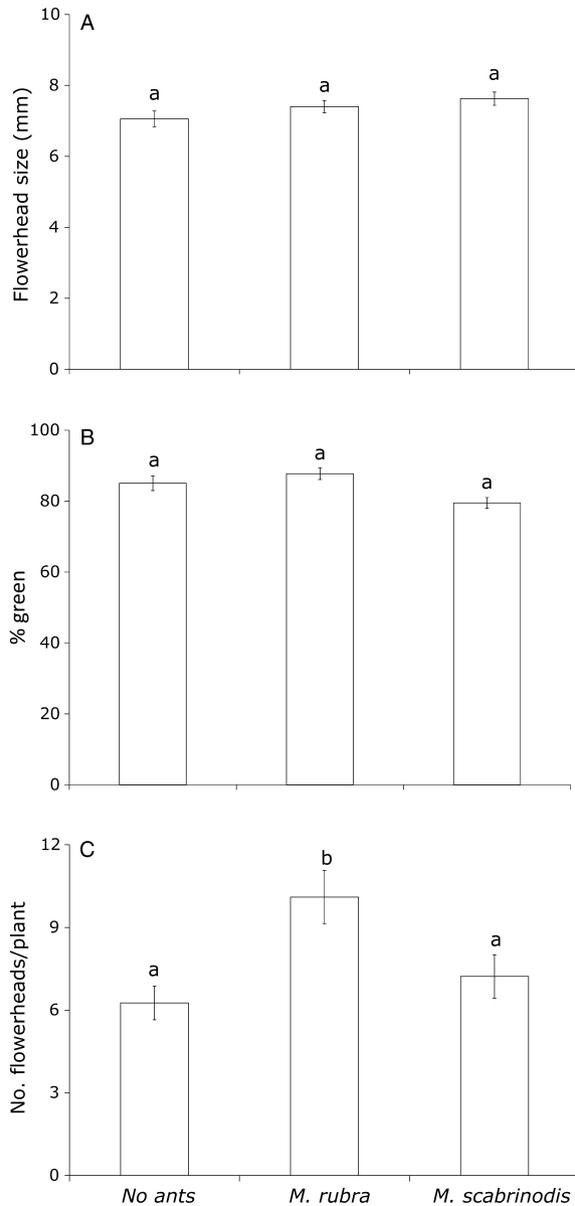


Figure 2 Mean (\pm SE) (A) size (mm) and (B) percentage of green coloration of *Sanguisorba officinalis* flowerheads, and (C) density of flowerheads (number per plant) per treatment: with *Myrmica rubra* or *M. scabrinodis* ant nests nearby, or with no ant nests. Means within a panel capped with different letters are significantly different (LSD test: $P < 0.05$). The numbers of flowerheads per treatment were as follows: no ants, $n = 357$; *M. rubra*, $n = 596$; *M. scabrinodis*, $n = 499$.

on numbers of ovipositions and caterpillars, and on the characteristics of plants and flowerheads of *S. officinalis* is given in Tables S1–S3.

The probability of oviposition for females of *P. teleius* was higher in the vicinity of one of the two ant species (with a borderline significantly higher probability for *M. scabrinodis*; LSD test after the model with flowerhead size: $P = 0.059$), whereas the size of the flowerheads had a positive effect on their oviposition (Table 1, Figure 3A). The percentage of green coloration did not have an effect on oviposition in *P. teleius*. Oviposition probability of

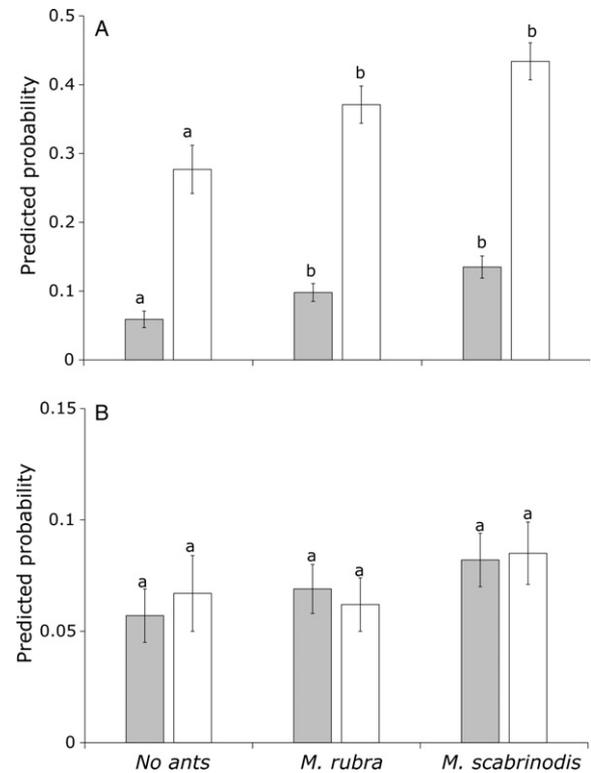


Figure 3 Mean (\pm SE; $n = 1452$) predicted oviposition probability (grey bars) and probability to detect a caterpillar (white bars) of (A) *Phengaris teleius* and (B) *P. nausithous* per treatment: with *Myrmica rubra* (*Mr*) or *M. scabrinodis* (*Ms*) ant nests nearby, or with no ant nests (con). Predicted probabilities are given for the mean flowerhead size (= 7.4 mm). Means within a panel and within a column colour capped with different letters are significantly different (LSD test: $P < 0.05$; see Tables 1 and 2 for details). For *P. teleius* ovipositions, Cohen's $d = 0.06$ for con vs. *Mr*, $d = 0.10$ for con vs. *Ms*, $d = 0.05$ for *Mr* vs. *Ms*. For *P. teleius* caterpillars, Cohen's $d = 0.07$ for con vs. *Mr*, $d = 0.11$ for con vs. *Ms*, $d = 0.05$ for *Mr* vs. *Ms*. For *P. nausithous* ovipositions, Cohen's $d = 0.09$ for absence vs. presence of *P. teleius* caterpillars. For *P. nausithous* caterpillars, Cohen's $d = 0.06$ for absence vs. presence of *P. teleius* caterpillars.

Table 1 Results of the generalized linear mixed models for the effect of presence of ant species (ant treatment) and flowerhead size (model 1) or percentage of flowers enclosed by green sepals per flowerhead (% green, model 2) of the host plant *Sanguisorba officinalis* on the oviposition probability of the two butterfly species *Phengaris teleius* and *P. nausithous*. In the models for *P. nausithous*, also the presence of *P. teleius* caterpillars was added as explanatory variable

Model ¹		<i>P. teleius</i>				<i>P. nausithous</i>			
		Coeff.	F	d.f.	P	Coeff.	F	d.f.	P
1	Ant treatment		6.58	2,1447	0.001		0.50	2,1444	0.61
	Flowerhead size	0.125	46.75	2,1447	<0.001	0.214	110.13	1,1444	<0.001
	Caterpillars <i>P. teleius</i>					+	19.49	1,1444	<0.001
2	Ant treatment		3.22	2,1065	0.040		0.65	2,1062	0.52
	% green		2.59	1,1065	0.11	-0.028	128.17	1,1062	<0.001
	Caterpillars <i>P. teleius</i>					+	27.51	1,1062	<0.001

Coeff., coefficient for the covariates for each variable in the model (if significant).

¹Model accuracy – i.e., % correctly predicted presences and absences – was 85–88% (for *P. teleius*) and 97–90% (*P. nausithous*). Models were built using the Diagonal covariance type (for *P. teleius*) and the Scaled Identity covariance type (for *P. nausithous*). Akaike's Information Criterion (AIC) for *P. teleius* = 7415.7 (model 1), 7188.6 (model 2); AIC for *P. nausithous* = 7627.8 (model 1), 5586.6 (model 2).

Table 2 Results of the generalized linear mixed models for the effect of presence of ant species (ant treatment) and flowerhead size (model 1) or percentage of flowers enclosed by green sepals per flowerhead (% green, model 2) of the host plant *Sanguisorba officinalis* on the probability of detecting caterpillars of the two butterfly species *Phengaris teleius* and *Phengaris nausithous*. In the models for *P. nausithous*, also the presence of *P. teleius* caterpillars was added as explanatory variable

Model ¹		<i>P. teleius</i>				<i>P. nausithous</i>			
		Coeff.	F	d.f.	P	Coeff.	F	d.f.	P
1	Ant treatment		5.77	2,986	0.003		0.80	2,983	0.45
	Flowerhead size	0.180	97.90	1,986	<0.001	0.164	47.54	1,983	<0.001
	Caterpillar <i>P. teleius</i>					+	4.31	1,983	0.038
2	Ant treatment						0.33	2,971	0.72
	% green	-0.015	54.4	1,976	<0.001	-0.025	78.81	1,971	<0.001
	Caterpillar <i>P. teleius</i>					+	3.90	1,971	0.049

Coeff., coefficient for the covariates for each variable in the model (if significant).

¹Model accuracy – i.e., % correctly predicted presences and absences – was 65–69% (for *P. teleius*) and 92% (*P. nausithous*). Models were built using the Diagonal covariance type (for *P. teleius*) and the Scaled Identity covariance type (for *P. nausithous*). Akaike's Information Criterion (AIC) for *P. teleius* = 4392.5 (model 1), 4263.3 (model 2); AIC for *P. nausithous* = 5243.5 (model 1), 5186.1 (model 2).

P. nausithous did not differ in preference between the ant species, whereas the size of the flowerheads and the presence of a previous oviposition of *P. teleius* had a positive effect and the percentage of green coloration had a negative effect. Overall, effect sizes were relatively small, based on Cohen's *d* as estimate for the effect sizes (Figure 3).

The probability of finding a caterpillar of *P. teleius* followed the same patterns as the probability of oviposition. There was a difference between the control vs. the two ant species, but not between the ant species (*M. scabrinodis* vs. *M. rubra*, LSD test after the model with flowerhead size: $P = 0.097$) (Table 2, Figure 3). The size of the flowerheads had a positive effect on this probability, whereas the effect of the percentage of green coloration was negative. Presence of the ant species alone had a significant effect, but when combined with the percentage of green

coloration its effect was non-significant, probably as a consequence of the differences in percentage of green coloration between the ant treatments (Figure 2).

The probability of detecting a caterpillar of *P. nausithous* did not differ between treatments, whereas the effect of flowerhead size was positive and the percentage of green coloration was negative. Also the presence of caterpillars of *P. teleius* had a positive effect on detecting a caterpillar of *P. nausithous*. We replaced the treatments (control – *M. scabrinodis* – *M. rubra*) by analysing the controls vs. the *Myrmica* ants, but did not find qualitative differences in any of the models for *P. teleius* and *P. nausithous*, confirming that *P. teleius* females select plants close to *Myrmica* ant nests, without preference for ant species, and *P. nausithous* females do not react to the presence of ant nests. Moreover, the probability of finding a caterpillar of

P. teleius did not differ between ant treatments when selecting only the small flowerheads (<6 mm; GLMM: $P = 0.28$). This may be explained by the low number of flowerheads with *P. teleius* caterpillars: only 29 of 388 caterpillars were found on these small flowerheads. When selecting the small flowerheads, the ant treatments in *P. nausithous* did not differ either.

Just before the butterflies were released, on 3 August, one *M. rubra* nest split up into two colonies and one of the sister colonies managed to enter a plot that was meant to be a plot with *M. scabrinodis* alone. The *M. rubra* colony was removed immediately, but nonetheless it may have stayed in the *M. scabrinodis* plot for maximally 1 day. We tested the effect of this migration by using an alternative design – i.e., two control plots, one plot with *M. scabrinodis*, two plots with *M. rubra*, and one plot with both *Myrmica* species – but the results were not qualitatively different from those of the original design (i.e., for *P. teleius*: difference between presence and absence of *Myrmica* ants, no difference between the two *Myrmica* species; for *P. nausithous*: no difference between presence and absence of *Myrmica* ants), so we decided to present the results of the original design.

Discussion

The results of our experimental study on two obligate myrmecophilous butterfly species indicate that *Myrmica* ant presence impacts the oviposition site choice of *P. teleius* females, but not that of *P. nausithous* females. Ant-mediated oviposition was also found in field studies of *P. teleius* and *P. nausithous* in the Dutch Moerputten population (Wynhoff et al., 2008), in an Italian *P. arion* population (Patricelli et al., 2011), and in a Portuguese *P. alcon* population (Wynhoff et al., 2015). Females of *P. teleius* seem to prefer the *Myrmica* ant species in general rather than the host ant *M. scabrinodis*, which seems to limit the distribution of the source population for this experiment, i.e., the *P. teleius* population in Moerputten nature reserve (Wynhoff et al., 2008; van Langevelde & Wynhoff, 2009). Other controlled experiments support our findings, as usually oviposition is found close to host *Myrmica* nests, whereas *Phengaris* butterflies do not distinguish between host and non-host *Myrmica* ants. For example, Fürst & Nash (2010) compared oviposition on plants in areas where host and non-host *Myrmica* ants were found (but not in absence of *Myrmica*). Patricelli et al. (2011) found that *P. arion* selected plants close to ant nests of five *Myrmica* species without preference for a single *Myrmica* species.

Longer flowerheads were more likely to receive an egg of *P. teleius* than shorter ones, regardless of the

percentage of green coloration. *Phengaris teleius* females deposited eggs on small flowerheads (<6 mm) regardless of the vicinity of a *Myrmica* ant nest. This suggests that low-quality flowerheads were selected by *P. teleius* because many high-quality flowerheads close to *Myrmica* ant nests were already occupied. *Phengaris nausithous* females oviposited equally on all plots regardless of the presence of *Myrmica* ants. However, they preferred long flowerheads and avoided the green ones. Also, prior oviposition of *P. teleius* affected the oviposition of *P. nausithous*; *P. nausithous* caterpillars were found more often on the flowerheads where caterpillars of *P. teleius* were also found. This suggests that oviposition by *P. nausithous* follows the selection of flowerheads by *P. teleius*. Females of both butterfly species deposit their eggs on large flowerheads with low percentage of green coloration.

Similar effects of the ants were found when analysing caterpillar captures from encapsulated flowerheads. Caterpillars of *P. teleius* were more frequently captured on plants in the vicinity of *Myrmica* ants than on plants without ant species present, without preference for its host ant species *M. scabrinodis*, whereas for *P. nausithous* no effect of ants on caterpillar captures could be shown. For both species, the size of the flowerhead had a positive effect, whereas the percentage of green coloration negatively influenced the occurrence of caterpillars. Flowerhead size is thought to be an indicator of the available resources for caterpillars. Larger flowerheads provide more food and may reduce competition with other caterpillars, as has been shown for the related species *P. alcon* (Arnaldo et al., 2014). Caterpillars of *P. nausithous* are mainly found on older flowerheads (Figurny & Woyciechowski, 1998), which are less green.

The flowerheads in the plots with *M. scabrinodis* had the lowest amount of green coloration and a low number per plant. Yet, the females of *P. teleius* preferred flowerheads close to the *Myrmica* ant nests over others with a higher amount of green coloration but without ant nests in the proximity. This is remarkable, as *P. teleius* is known to lay their eggs mainly on short and green flowerheads under field conditions (Figurny & Woyciechowski, 1998; Sielezniew & Stankiewicz-Fiedurek, 2013). Apparently, in our experiment ant presence is more important than flowerhead phenology. The females of *P. nausithous* behaved as expected (Figurny & Woyciechowski, 1998); they preferred long flowerheads with only a small percentage of green coloration.

It has been suggested that females of predatory *Phengaris* species deposit only one or at most a few eggs per host plant to avoid larval scramble competition, first on the host plant and later in the host ant nest (Mouquet et al.,

2005). This feeding behaviour and subsequent scramble competition in the ant nest results in a high density-dependent mortality when the ant nests are overcrowded with caterpillars. For *P. telei* caterpillars that behave as predators in the host ant nest, only one caterpillar per ant nest develops into a butterfly (I Wynhoff, pers. obs. at Moerputten). By combining ant-mediated oviposition, as found in our experiment, and the spread of eggs over as many host plants as possible (Figurny & Woyciechowski, 1998), females can increase the survival rate of their offspring. Although our earlier results on *P. nausithous* in the field pointed at ant-mediated oviposition (Wynhoff et al., 2008), in the current experiment we did not find an effect of ant nest presence on oviposition patterns. Perhaps in the insectory, the *Sanguisorba* plants offered for oviposition were on average much younger than the plants that females would choose in nature. A limiting number of phenologically suitable flowerheads close to *Myrmica* ant nests may have forced the females to deposit their eggs on phenologically acceptable flowerheads without nearby ant nests, rather than not depositing their eggs at all.

How do females of *P. telei* detect whether a particular flowerhead is close to a *Myrmica* ant nest? Parasites are strongly dependent on their hosts – parasites such as these obligate myrmecophilous butterfly species depend on host plants as well as host ants. Because of this strong dependence, many parasites have developed systems to detect the host, thus favouring maximal survival of their offspring (Price, 1980). Behavioural observations show that visual cues, such as plant characteristics, are important in the first phase of searching for a flowerhead to oviposit on (Thomas & Elmes, 2001; Fürst & Nash, 2010). This might be especially true for *Phengaris* species, whose host plant, such as *P. alcon*, has a scattered distribution (Van Dyck & Regniers, 2010). In the second phase of searching for an oviposition site, after a female has found a suitable flowerhead, other cues come into play (Van Dyck & Regniers, 2010). These other cues could be volatile and non-volatile compounds of *Myrmica* host ant nests (the production of volatiles by *Myrmica* ants is demonstrated by Cammaerts et al., 1978, 1981; Henning, 1983; Hölldobler & Wilson, 1990). Another possibility could be the detection of plant volatiles by gravid females, as has been demonstrated for *P. arion* by Patricelli et al. (2015): *Origanum* host plants produce the monoterpenoid carvacrol when ants disturb their roots. *Myrmica* ants are much more resistant to carvacrol than other ant species, giving them an enemy free space in the *Origanum* roots to occupy. Gravid *P. arion* females were attracted to such disturbed *Origanum* plants and deposited the eggs there. However, our experimental design prohibited this indirect effect of plants being affected by ant presence as we released the females shortly

after the introduction of the ant nests (ca. 6 days). Therefore, our results point at a direct effect of ant nest presence on oviposition preference of *Phengaris* butterflies.

Ant-mediated oviposition in *Phengaris* species has been hotly debated (Thomas et al., 1989; Van Dyck et al., 2000; Thomas & Elmes, 2001; Nowicki et al., 2005; Musche et al., 2006; van Langevelde & Wynhoff, 2009; Fürst & Nash, 2010; Van Dyck & Regniers, 2010; Patricelli et al., 2011; Wynhoff et al., 2015). Our experimental study indicates that females of *P. telei* more frequently deposit eggs on flowerheads of *S. officinalis* near *Myrmica* ant species than on flowerheads without these nests nearby. The sympatric species *P. nausithous* did not show a preference for plants close to ant nests. Our experiment is the first in which both host plants and host ants are kept together to test ant-mediated oviposition. These findings contribute to better understanding the habitat requirements of two highly specialized butterfly species.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Numbers of ovipositions and caterpillars of *Phengaris teleius* and *P. nausithous*, and mean values (\pm SD) for *Sanguisorba officinalis* plant and flowerhead characteristics per plot. Treatments are: *Myrmica scabrinodis* in plots 1 and 4, *M. rubra* in plots 2 and 6, no ants (controls) in plots 3 and 5. Plot 1 was accidentally invaded by *M. rubra*.

Table S2. Numbers of *Sanguisorba officinalis* plants and flowerheads and mean values (\pm SD; n in parentheses) for plant and flowerhead characteristics for ovipositions of *Phengaris teleius* and *P. nausithous*, for accepted and rejected flowerheads. Treatments are: *Myrmica scabrinodis* in plots 1 and 4, *M. rubra* in plots 2 and 6, and no ants in plots 3 and 5. Plot 1 was accidentally invaded by *M. rubra*.

Table S3. Numbers of *Sanguisorba officinalis* plants and flowerheads and mean values (\pm SD; n in parentheses) for plant and flowerhead characteristics for caterpillars of *Phengaris teleius* and *P. nausithous*, for accepted and rejected flowerheads. Treatments are: *Myrmica scabrinodis* in plots 1 and 4, *M. rubra* in plots 2 and 6, and no ants in plots 3 and 5. Plot 1 was accidentally invaded by *M. rubra*.