

Choice behaviour of *Myrmica rubra* workers between ant larvae and larvae of their *Phengaris (Maculinea) nausithous* nest parasites

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Abstract Larvae of *Phengaris (Maculinea)* butterflies are adopted by *Myrmica* workers and are obligate myrmecophiles. Brood recognition by *Myrmica rubra* workers was tested for concolonial larvae (*M. rubra*) versus allocolonial larvae (*M. rubra* and *P. nausithous*) to assay the mimetic efficiency of *P. nausithous*. In addition, we tested *M. rubra* ant colonies from different populations with and without the presence of *Phengaris*, to test for potential local adaptation in adoption behaviour. We show that *M. rubra* can distinguish between nest-mate and foreign larvae as well as between *P. nausithous* and their own larvae. Workers from the allopatric population inspected and rejected more *P. nausithous* larvae than workers from the sympatric population. This might reflect a local host adaptation in which the social parasite more efficiently mimics its sympatric host ants than allopatric ones.

Keywords Host–parasite co-evolution · Brood recognition · Adoption behaviour · Local adaptation

Introduction

Myrmica Latreille 1804 ant colonies are notoriously prone to invasion by both socially parasitic ants (reviewed by

Bourke and Franks, 1995) and social parasites from other insect taxa including Diptera, Coleoptera and Lepidoptera (e.g. Donisthorpe, 1927; Hölldobler and Wilson, 1990; Dettner and Liepert, 1994; Thomas and Settele, 2004; Settele and Kühn, 2009). Highly specific recognition mechanisms are essential for the evolution of the intricate interactions that form the basis of stable host–parasite systems in ants (Nash and Boomsma, 2008), particularly for butterflies of the genus *Phengaris* Doherty 1891, which are nest parasites of several *Myrmica* ant species. Females of *Phengaris* lay their eggs on particular food plants, on which the larvae feed and complete their development up to the fourth larval instar. After moulting into the fourth larval instar, the caterpillar leaves its food plant and moves to the ground, where it depends upon being adopted by foraging workers of a suitable *Myrmica* species, which will carry it into the host colony. Once in the colony the caterpillar will complete its development, pupate and leave the nest as an imago. Therefore, adoption and integration of the social parasite in the host colony are crucial moments, which can be regarded as a series of quite dynamic filters (encounter, infection and exploitation) that can determine local host specificity (Nash et al., 2011). Although signals such as size and tactility (Elmes et al., 2001), behaviour and sound (De Vries et al., 1993; Barbero et al., 2009a; Barbero et al., 2009b) apparently play a role in the initial adoption of *Phengaris* larvae, it seems that chemical signals are at the centre of the adoption process. Because chemical signals have been shown to be essential for nest-mate recognition in ants (e.g. Hölldobler and Wilson, 1990), Elmes et al. (1991) suggested that this is also the main mechanism for *Phengaris* adoption, based on chemical signals on the caterpillar body surface.

Variability in chemical signals is common in ant societies (Whitehouse and Jaffé, 1995) and cuticular hydrocarbons

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have been suggested to play an important role in nest-mate recognition (Lahav et al., 1999; Van Zweden and d'Ettorre, 2009). Nest-mate recognition in ants reflects the ability of workers to discriminate conspecific members from other colonies (Vander Meer and Morel, 1998; Van Zweden and d'Ettorre, 2009; Sturgis and Gordon, 2012).

The ability to recognize nest-mates is highly adaptive and particularly important to prevent intrusion of foreign conspecifics and robbing of the colony (Wilson, 1971; Stuart and Herbers, 2000). Colony brood recognition has been demonstrated in several ant species (Brian, 1975; Meudec, 1978; Isingrini et al., 1985; Hare and Alloway, 1987; Carlin and Schwartz, 1989; Bonavita-Cougourdan et al., 1989; Fénéron and Jaisson, 1992, 1995). Parental care of brood is a key factor in the social life of colonies as workers are able to assess the needs of the offspring such as grooming and feeding (Camargo et al., 2006). Concolonial brood is accepted and nourished by workers, whereas allocolonial brood may be rejected (Bonavita-Cougourdan et al., 1989). The brood has chemical cues that enable workers to recognize them as concolonial (Brian, 1975; Araújo et al., 1996; Viana et al., 2001). Nevertheless, ant workers are often more tolerant towards unfamiliar brood (and sometimes even heterospecific brood) than unfamiliar adults and a possible explanation is that they will become integrated into the colony workforce and also increase colony fitness (Haskins and Haskins, 1950; Elmes and Wardlaw, 1983; Errard, 1984; Plateaux, 1985; Goodloe and Topoff, 1987; Fouks et al., 2011). Adoption of unfamiliar brood does not, however, preclude the preference for nest-mate brood, as revealed in choice experiments (Fénéron and Jaisson, 1995).

In contrast, retrieving *Phengaris* larvae to the colony reduces colony fitness, setting the stage for co-evolutionary arms races between host recognition and parasite attractiveness. The high specificity in host and parasite signals may result in local adaptations similar to those reported for *Microdon mutabilis* Linnaeus 1758 (Schönrogge et al., 2006) and *P. alcon* Denis & Schiffermüller 1775 in their interaction with *M. rubra* Linnaeus 1758 (Als et al., 2001; Als et al., 2002; Nash et al., 2008). Further, new local hosts for *P. alcon* in Portugal (Arnaldo et al., 2011) and for *P. teleius* Bergsträsser 1779 in Poland (Witek et al., 2010), suggest host specificity is modulated by local *Myrmica* species availability.

To be adopted by ants, *Phengaris* larvae mimic ant brood (Thomas and Settele, 2004). However, the efficiency of and local adaptation in adoption of *Phengaris* larvae has not been compared to that of host larvae. In this study, we use *P. nausithous* and its host *M. rubra* to screen for local adaptations by analysing the workers' adoption behaviour towards the butterfly larvae. We chose *P. nausithous* as a study species, because it shows high host specificity for *M. rubra* across Europe (Thomas et al., 1989; Elmes et al.,

1998; Stankiewicz and Sielezniew, 2002; Tartally and Varga, 2005; Witek et al., 2008) in spite of occasional occurrences of *P. nausithous* larvae in *M. scabrinodis* (Elmes et al., 1998; Munguria and Martin, 1999; Witek et al., 2008) and *M. ruginodis* nests. Because *P. nausithous* has much higher initial survival in *M. rubra* nests than in those of any other host species (Patricelli et al., 2010), the mimetic efficiency should be exceptionally high among *Phengaris* butterflies. Other *Phengaris* species are more variable in their host use, and often it is not possible at all to identify a primary host especially for *Phengaris* predator feeders (Witek et al., 2008). This makes it difficult at best to screen for a signature of local co-evolution between host and parasite.

In this study, we compared a *M. rubra* population (called sympatric population) coexisting with *P. nausithous* with another *M. rubra* population (called allopatric) not exposed to *P. nausithous* to find out how the parasite affects the brood recognition and the acceptance in *M. rubra* ants. In case of local adaptation to the parasite, sympatric and allopatric workers should present different behavioural responses towards *P. nausithous* similar to those reported for *P. alcon* (Als et al., 2001; Nash et al., 2008) depending on who is leading the arms race: host or parasite. Therefore, our questions are: (i) do *Myrmica* workers recognize nest-mate larvae? (ii) do workers prefer ant larvae over *P. nausithous* larvae at colony and individual level? And (iii) are there differences in *P. nausithous* larvae choice between *Myrmica* ants from sympatric or allopatric parasite populations?

Materials and methods

We quantify the encounter behaviour of *M. rubra* workers with larvae from (i) their own nest, (ii) conspecific foreign nest or (iii) the parasite (*P. nausithous*) and we compare the retrieval of own brood versus parasite larvae into the *M. rubra* colonies.

Sampling and handling of ants

The study was conducted with 40 colonies of *M. rubra* collected from two different sites in Germany, 30 colonies from Halle (allopatric population, N51°30'E11°56') and 10 colonies from Altenburg (sympatric population, N51°01'E12°28'). We collected the ant colonies from the wild, transferred them to artificial nests (gypsum nest 20 × 9.5 × 3 cm at 20 °C). All colonies consisted of 50 workers, a queen and some brood (≈ 15). Colonies were fed in a foraging arena attached to the nest with dry adult *Drosophila* and diluted honey (≈ 5 g in 40 ml water) in a paper ball placed in a small plastic dish (14 mm diameter)

and water ad libitum. We changed water and food twice a week for all colonies. The ant species were identified using the morphometric key of Czechowsky et al. (2002).

Sampling and handling of butterfly larvae

We collected *P. nausithous* larvae from flower heads of *Sanguisorba officinalis* L. (food plant of *P. nausithous*) in Altenburg (N51°01'E12°28'). In the laboratory, we kept flower heads in a petri dish at room temperature until the fourth instar butterfly larvae emerged.

Recognition behaviour experiment

Behavioural experiments were conducted between April 2010 and September 2010 at the Department of Molecular Ecology at the Martin Luther University Halle Wittenberg. For these experiments we used 20 colonies of *M. rubra* from Halle. We performed three kinds of experiments, all based on behavioural observations in a Petri dish arena (7 cm diameter, 1 cm height), in which we presented one or two *M. rubra* larvae (nest-mate or foreign) in a small plastic dish (14 mm diameter). We observed worker behaviour under a binocular microscope with 32× magnification.

For each test, one worker was introduced into the arena to observe the acceptance of a *M. rubra* worker without nest-mate or nest material (which can affect ant tolerance towards foreign individuals; Buczkowski and Silverman, 2005).

Behavioural observations started 10 min after introduction into the arena in order to allow the test worker time to accommodate to the new environment. The trial consisted in an experimental encounter between an ant larva with a nest-mate or with a foreign worker in a fixed observation period. For each experiment we recorded the number of times a selected behaviour was observed (Table 1—modified from Human and Gordon, 1999). Every arena was carefully cleaned with ethanol before each trial.

Table 1 Categories and descriptions of recorded behaviour of *Myrmica* workers (after Human and Gordon 1999)

Behaviour	Description
No contact	Lack of interaction
Antennation	Investigation of the surface of the larva with the antennae
Larva carrying	Transport of the larva between the worker's mandibles
Taking by mandible	Antennation of the larva while held in the worker's mandibles
Bite	Body of the larva is crushed between workers' mandibles
Gaster contact	Tilting and pushing of the worker's gaster against the ant larva

We made 50 replicate tests for each experimental setup (see below), always using a different test worker to avoid habituation of the workers to the ant larvae. The ant larvae were used in five sequential tests, in total 20 *M. rubra* larvae (10 nest-mate and 10 foreign) were assayed in experimental setup 1. In experimental setup 2, 50 nest-mate *M. rubra* and 50 foreign *M. rubra* were used.

Experimental setup

1. Individual worker behaviour towards a single larva.
We observed the behaviour of *Myrmica* workers either towards conspecific nest-mate or foreign larvae for 3 min to assess the worker behavioural repertoire (Fig. 1a).
2. Individual worker behaviour towards nest-mate and foreign larvae in a simultaneous choice setting.
By modifying experimental setup 1, we tested the preference of a *M. rubra* worker when given the choice between a nest-mate and a foreign conspecific larva. The test worker was observed for 10 min (Fig. 1b) to reveal any behavioural differences towards conspecific nest-mate and foreign brood.

Both experiments 1 and 2 were necessary to establish the feasibility of the arena bioassay to quantify the recognition of *P. nausithous* larva by *M. rubra* workers.

Choice behaviour of individual *Myrmica* workers between ant and butterfly larvae

We used a dual choice experiment to assess the discrimination of *M. rubra* workers between *M. rubra* and *P. nausithous* larvae (Fig. 1b). We used 20 randomly chosen workers (2 from 10 colonies each) from both the sympatric population of Altenburg and from the allopatric population of Halle. We simultaneously placed a *M. rubra*, a *M. rubra* larva, and a *P. nausithous* larva into the arena. Based upon the behavioural repertoire from the previous *Myrmica* recognition experiments, we performed our observations for 10 min. *M. rubra* workers and larvae of both *P. nausithous* and *M. rubra* were only used once to avoid habituation of the workers and chemical contamination of larva body surface via contact of *M. rubra* workers. Both the arena and plastic dish were cleaned with ethanol between the trials (20 trials per population).

Local host–parasite adaptation

We tested for local host–parasite adaptation by comparing worker recognition and adoption behaviour of ten *M. rubra* colonies each from Halle (allopatric with *Phengaris*) and from Altenburg (sympatric with *Phengaris*) using complete

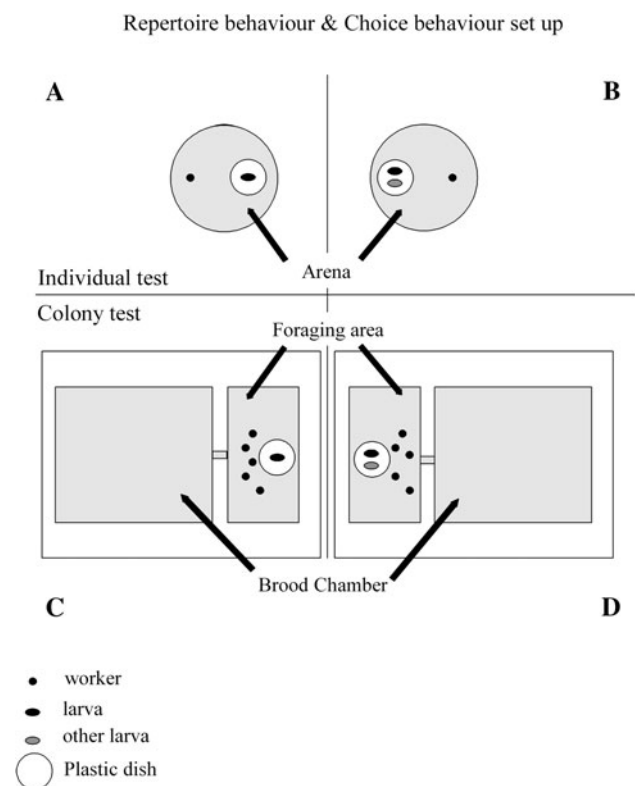


Fig. 1 Experimental set up for: **a** individual worker behaviour towards a single larva; **b** individual worker behaviour towards nest-mate and foreign larvae in a simultaneous choice setting and choice behaviour of individual *M. rubra* workers between ant and butterfly larvae; **c** local host–parasite adaptation—adoption of *P. nausithous* larvae by *M. rubra* colonies from the sympatric and allopatric populations; **d** Local host–parasite adaptation—choice behaviour between ant and butterfly larvae by *M. rubra* colonies from the sympatric and allopatric populations

nests composed of 50 workers, a single queen and ≈ 15 larvae. *M. rubra* and *P. nausithous* larvae were used only once for each trial, to prevent potential effects of compound contamination resulting from contacts with *M. rubra* workers.

All interactions were recorded for 1 h with a digital camera (1,280 \times 1,024 pixel) and the video sequences analysed with VirtualDub[®] (Lee 1998–2009). Since the maximum recorded adoption time for *P. teleius* was 20 h and for *P. alcon* was 23 h for the host and 47 h for the non-host, our observation period was arguably short (Als et al. 2001, Witek et al. 2011). Nevertheless, the chosen time window was sufficient to compare *P. nausithous* larvae adoption time with the much more quickly adopted *M. rubra* larvae, thereby reducing experimental artefacts resulting from extensive lab exposure.

1. Adoption of *Phengaris*

We screened for differential adoption behaviour of *M. rubra* workers coming from the sympatric or the allopatric

population (Fig. 1c). We determined the number of trials with *P. nausithous* adoption, and larval survival within 1 h after adoption. We tested 10 colonies from each population with a total of 23 and 36 *P. nausithous* larvae, respectively.

2. Choice behaviour of colonies

For this second experiment we presented both a nest-mate and a *P. nausithous* larva simultaneously to the colony (Fig. 1d). Again we recorded the interactions between ants and both kind of larvae and noted the number of times and duration of adoption to assess possible differences between ant populations. We tested 10 colonies from the sympatric and 10 colonies from allopatric populations, respectively, with 40 *P. nausithous*/*M. rubra* larvae pairs. The number of adoptions were analysed with the χ^2 test and the means of adoption time with Mann–Whitney *U* test (STATISTICA[®] version 8).

Results

Recognition behaviour of individual ants

The behavioural repertoire of ants were identical in the experiments 1 and 2 and we grouped the behavioural traits into two categories: “inspection” (antennation, larva carrying and taking by mandibles) or “aggression” (biting and gaster contact). *M. rubra* workers show a different behavioural approach with the different kind of larvae (nest-mate, foreign and social parasite) resulting in statistically significant differences ($\chi^2 = 26.30$, $df = 2$, $P < 0.001$, $N = 127$ nest-mate *M. rubra*, $N = 40$ *P. nausithous* and 130 foreign *M. rubra* larvae) (Fig. 2).

Choice behaviour of individual ants

Antennation was the only behaviour of ants observed towards *P. nausithous* larvae. Workers from the allopatric population did not contact the social parasite in one out of 20 trials performed, in comparison the sympatric population did not contact *P. nausithous* 8 times out of 20. The sympatric population antennated mainly towards their own brood. The difference between populations in antennation behaviour displayed is statistically significant ($\chi^2 = 10.04$, $df = 2$, $P = 0.01$, $N = 40$) (Fig. 3).

Colony-level experiments

Adoption of *P. nausithous*

The social parasite *P. nausithous* was adopted 10 times out of 23 trials by workers from the sympatric population.

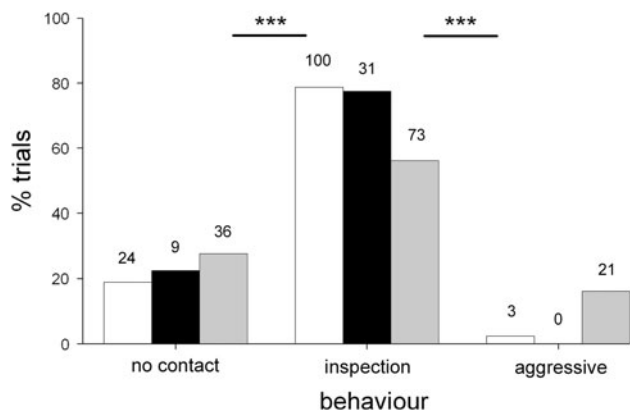


Fig. 2 Behavioural trials of *M. rubra* workers towards: nest-mate *M. rubra* larva (white bar), *P. nausithous* larva (black bar) and foreign *M. rubra* larva (grey bar). Percentage (y-axis) and number of trials (above the bars) are given for each behavioural group; ***difference statistically significant; ($\chi^2 = 26.30$, $df = 2$, $P < 0.001$, $N = 128$ nest-mate *M. rubra* $N = 40$ *P. nausithous* and 130 foreign *M. rubra* larvae)

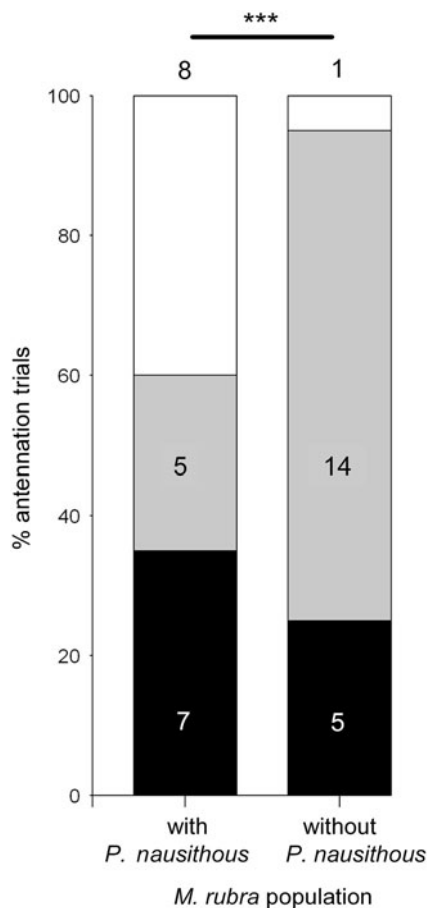


Fig. 3 Choice behaviour of individual *Myrmica* workers between ant and butterfly larvae. Percentage (y-axis) and number of trials (above the bars) for *M. rubra* larva (white bar), *P. nausithous* larva (black bar) and both larvae (grey bar) relatively to the difference (***) difference statistically significant) in antennation between the populations ($\chi^2 = 10.04$, $df = 2$, $P = 0.01$, $N = 40$)

Hence, non-adoption was not significantly different from adoption ($\chi^2 = 0.39$, $df = 1$, $P = 0.53$, $N = 23$; Fig. 4). In contrast, workers from the allopatric population adopted significantly less (10) times and left 26 larvae in the foraging area ($\chi^2 = 7.11$, $df = 1$, $P = 0.01$, $N = 36$; Fig. 4). After adoption into the colony not all larvae survived. The rate of survival was similar in colonies of both populations. In the allopatric colonies 7 (out of 10) larvae survived after the adoption and 6 (out of 10) in the sympatric colonies ($\chi^2 = 0.22$, $df = 1$, $P = 0.64$, $N = 20$; Fig. 4). The observed “adoption behaviour” is therefore not a guarantee for survival of *P. nausithous* in either host population because the ants kill the larvae once in the nest in this case at a rate of 40 %.

Choice behaviour of colonies at population level

Colonies from both sympatric and allopatric populations preferred to adopt their own larvae rather than *P. nausithous* larvae in the simultaneous choice experiments (Fig. 5). The sympatric population colonies adopted 24 *M. rubra* larvae and 10 *P. nausithous* larvae ($\chi^2 = 5.76$, $df = 1$, $P = 0.02$, $N = 34$). The preference for the own larvae was even higher in the allopatric population (33 *M. rubra* larvae vs. 8 *P. nausithous* larvae) ($\chi^2 = 15.24$, $df = 1$, $P < 0.001$, $N = 41$) but this difference was not significantly different between the two host populations ($\chi^2 = 1.00$, $df = 1$, $P = 0.32$, $N = 75$). The mean of adoption times of *M. rubra* (7.31 ± 2.56 min) were significantly shorter than those of *P. nausithous* larvae (16.83 ± 4.29 min) in the sympatric population (Mann–Whitney *U* test, $P < 0.01$, $N = 32$). This difference was not significant in the allopatric population (*M. rubra* 11.93 ± 1.87 , and *P. nausithous* larvae 19.71 ± 5.41 min) (Mann–Whitney *U* test, $P = 0.14$, $N = 39$), but the differences in adoption times between populations (7.77 vs. 8.52 min, respectively) were not statistically significant (Mann–Whitney *U* test, $P = 0.75$, $N = 18$).

Discussion

Our results confirm previous findings from several ant genera that workers can distinguish between nest-mate and foreign conspecific larvae (Brian, 1975; Meudec, 1978; Isingrini et al., 1985; Hare and Alloway, 1987; Carlin and Schwartz, 1989; Bonavita-Cougourdan et al., 1989; Féron and Jaisson, 1992). Since the complete adoption of *Phengaris* larvae can take excessive time under laboratory conditions (>8 h Als et al., 2001; Witek et al., 2011), our low overall adoption rates might just be a reflection of the short observation interval. Nevertheless, excluding adoptions that took more than 60 min: the average of adoption time for *P. nausithous* in the sympatric population

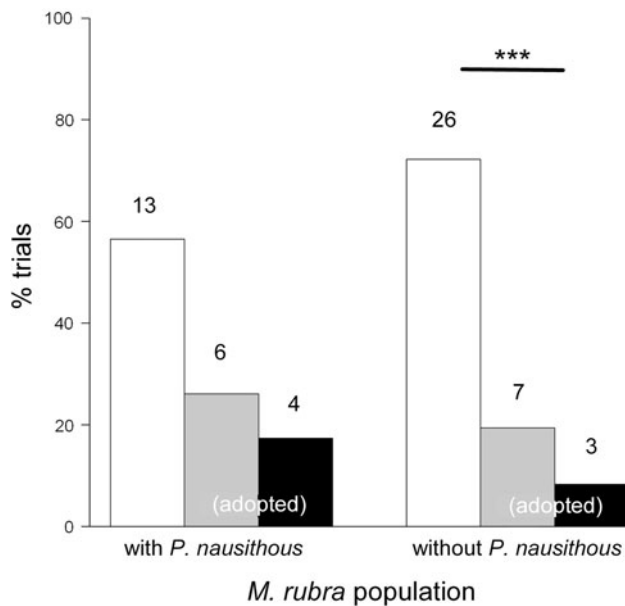


Fig. 4 Percentage (y-axis) and number of trials (above the bars) for *P. nausithous* larvae not adopted (white bar), surviving (grey bar) and killed (black bar) relatively to the difference (***) difference statistically significant) within population with and without *P. nausithous*. Difference in adoption rate in the sympatric population not statistically significant ($\chi^2 = 0.39$, $df = 1$, $P = 0.53$, $N = 23$); in the allopatric population statistically significant ($\chi^2 = 7.11$, $df = 1$, $P = 0.01$, $N = 36$). Difference in surviving after adoption between the populations not statistically significant ($\chi^2 = 0.22$, $df = 1$, $P = 0.64$, $N = 20$)

(16.83 min) was higher than for the cuckoo *P. alcon* in Denmark exploiting *M. rubra* (9.7 min, Als et al., 2001) and much less than for the predator *P. teleius* in Poland exploiting *M. rubra* (43.33 min, *P. teleius* adopted within 1 h Witek pers. comm.). The similarity in adoption times of *P. alcon* and *P. nausithous* adds to previous results about host specificity and integration of *P. nausithous* by Patricelli et al. (2010).

As expected, *Myrmica* workers are more aggressive towards foreign larvae—a trait typically observed within encounter experiments between workers (Dahbi et al., 1996; Errard and Hefetz, 1997; Lahav et al., 1999; Lucas et al., 2005). This behavioural assay can, however, also be used to quantify the interactions between *P. nausithous* and the ants. *M. rubra* workers are well able to distinguish between *P. nausithous* and *M. rubra* larvae in individual and colony encounters. *P. nausithous* larvae were more often inspected than foreign *M. rubra* larva, but antennation behaviour was less often released in the ants of the sympatric than in those of the allopatric population. These results can be interpreted in two ways:

1. Local adaptation of the allopatric host ants which avoid infestation by the parasite because they scrutinize the parasites more carefully and have a reduced adoption rate. This may have caused the parasite to temporarily

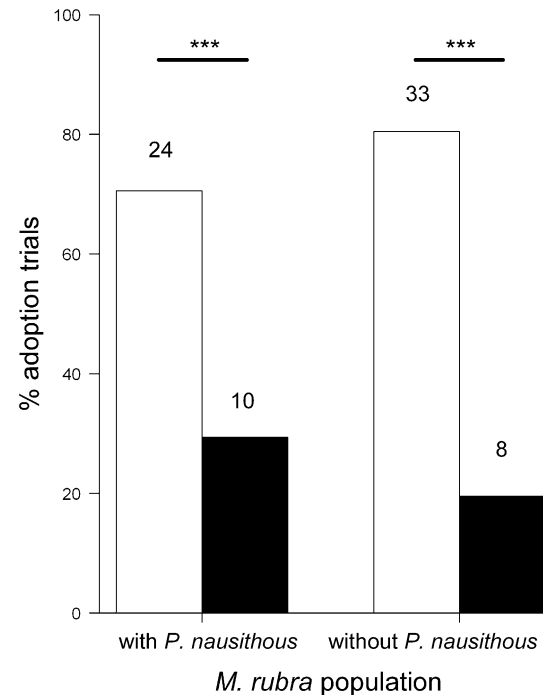


Fig. 5 Choice behaviour of colonies (number of adoption) Percentage (y-axis) and number of trials (given above the bars) for *M. rubra* larva (white bar) and *P. nausithous* larva (black bar) relatively to the difference (***) difference statistically significant) in adoption within and between populations (sympatric population: $\chi^2 = 5.76$, $df = 1$, $P = 0.02$, $N = 34$; allopatric population: $\chi^2 = 15.24$, $df = 1$, $P < 0.001$, $N = 41$; $\chi^2 = 1.00$, $df = 1$, $P = 0.32$, $N = 75$)

disappear from the populations (host is ahead of parasite).

2. The higher adoption rate of the sympatric ants reflects a local adaptation of the *P. nausithous* larvae to better mime the sympatric host population (parasite is ahead of host), as already found for *P. alcon* (Als et al., 2002; Nash et al., 2008).

Foitzik et al. (2001; 2003) also found geographic variation in the response of *Lepthorax longispinosus* and *L. acervorum* host to two slave-making ant species, *Protomognathus americanus* and *Harpagoxenus sublaevis*. The social parasites were shown to induce behavioural differences in different host populations. *L. acervorum* host workers attacked *H. sublaevis* slavemakers from the same population less often than allopatric slavemakers (Foitzik et al., 2003). This may reflect a similar stage of a host–parasite arms race than in our case.

In conclusion, our data suggests that a local co-evolutionary arms race between *P. nausithous* and *M. rubra* resulted in a local adaptation. Given *P. nausithous* is the most specialized *Phengaris* species in terms of number of host ant species used, with a very strong preference for *M. rubra* (Thomas et al., 1989; Elmes et al., 1998; Stanekiewicz and Sielezniew, 2002; Tartally and Varga, 2005;

Witek et al., 2008; Patricelli et al., 2010) it would not be that surprising to find evidence for local adaptation. Although our results give support for local adaptation of adoption behaviour the co-evolution of host–parasite system are highly dynamic oscillating processes. Therefore, other populations might reveal alternative outcomes with the host or parasite having the lead and our results only represent a snapshot in the arms race between *P. nausithous* and *M. rubra*.

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