

## Different host plant utilization ability of two closely related *Melitaea* species (Lepidoptera: Nymphalidae)

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**Abstract.** In natural systems plant-insect interactions are influenced by several factors. For instance plants could be characterised by the presence of defensive chemicals and herbivores are forced to evolve resistance against them. There are several studies on induced plant defence, which indicate it can mediate competition between herbivorous insects, shaping host plant choice and community structure. Therefore, realized host plant use can be much narrower than the potential one. A laboratory experiment was done to clear up the possible difference in host plant utilization ability of *Melitaea phoebe* and *Melitaea ornata*, especially their utilization of *Cirsium pannonicum*. The caterpillars were reared individually on three different species of host plant (*Ci. pannonicum*, *Ci. arvense* and *Centaurea scabiosa*). The weights of the larvae were measured every second day and the data analysed using ANOVA. *M. phoebe* caterpillars developed well and all pupated when fed on *Cirsium arvense* and *Centaurea scabiosa*, whereas those fed on *Cirsium pannonicum* developed poorly and 10% died. In contrast, *M. ornata* developed well on *Ci. pannonicum* and the other two host plants. Despite this *M. ornata* was only found on *Ci. pannonicum* in Hungary.

### INTRODUCTION

In natural systems plant-insect interactions are influenced by several factors. On the one hand, many plants are characterised by the presence of defensive chemicals (Ehrlich & Raven, 1964; Häggström & Larsson, 1995; Dobler et al., 1996; Monique, 2001; Wahlberg, 2001). These substances form part of the plant's defence system and affect herbivores in different ways, e.g. they can attract predators or parasitoids of herbivores (Vet & Dicke, 1992), as well as having direct effects, which may be toxic, anti-digestive, anti-nutritive and deterrent (Bernays & Graham, 1988; Jaenike, 1990; Wittstock & Gershenson, 2002; Kessler & Halitschke, 2007). Up to now, several studies have dealt with induced plant defence, which can mediate competition between herbivorous insects (Ohgushi, 2005; Denno & Kaplan, 2006; Kessler & Halitschke, 2007) shaping host plant choice and community structure (Jaenike, 1990; Kaplan & Denno, 2007). Therefore, the realized host plant use could be much narrower than the potential one (Jaenike, 1990).

On the other hand, herbivores are forced to evolve resistance to plants' defensive chemicals, for instance by means of detoxifying mechanisms. When a novel detoxifying mechanism arises, it will open up a new array of potential host plants, consisting of all those that produce the now less harmful chemical. These food plants constitute a biochemical group, but need not be related phylogenetically as unrelated plants can also have the same defensive chemicals. It follows that herbivores are often not adapted to a

single plant species but to a particular type of secondary metabolite, as is the case in *Blepharida* beetles (Becerra, 1997) and pierid butterflies (Wheat et al., 2007).

The biochemistry of host plant specialization in Melitaeini butterflies is well studied and their adaptation to food plant chemistry is more conservative than the taxonomic relations between *Melitaea* species and their host plants (Wahlberg, 2001). Most of these food plants contain irido-glycosides with a few exceptions, including the family Asteraceae, the host plants of the *Melitaea phoebe* species group.

The most well-known species in this group is *Melitaea phoebe* ([Denis & Schiffermüller], 1775) (Knapweed fritillary), which occupies an almost continuous area from North Africa across southern and central Europe to north-eastern China. *M. phoebe* and its subspecies are generally bivoltine and oligophagous, and feed on host plants belonging to the family Asteraceae (Table 1).

In Hungary, *M. phoebe* is a widely distributed, common species. In natural and semi-natural habitats it feeds on various *Centaurea* species. Caterpillars were also collected from *Cirsium pannonicum* in the Aggtelek Karst area (North-East Hungary). In ruderal habitats, caterpillars mostly feed on *Cirsium arvense*, *Cirsium vulgare* and were also found on various *Carduus* species.

In general, host plant use by herbivores may differ greatly in different parts of its distribution as it is not uncommon that an oligophagous species become specialist, especially

TABLE 1. Known host plants of *Melitaea phoebe*.

Region	Host plant species	Citation
Europe	<i>Centaurea</i> spp. (in most of the literature is <i>C. scabiosa</i> )	(Ebert & Rennwald, 1991; Settele et al., 2005; Russell et al., 2007; Varga, 2007; Tolman & Lewington, 2008)
Russia	<i>Arctium</i> spp., <i>Cirsium</i> spp., <i>Centaurea</i> spp., <i>Inula</i> spp., <i>Rhapticum</i> spp., <i>Serratula</i> spp.	(Gorbunov & Kosterin, 2007; Kuznetsov, 2011)
Siberia (Russian Republic of Buryatia)	<i>Stemmacantha uniflora</i>	(Wahlberg et al., 2001)
Hungary	<i>Centaurea scabiosa</i> , <i>Centaurea sadleriana</i> , <i>Cirsium arvense</i> , <i>Cirsium vulgare</i> , <i>Carduus</i> spp.	pers. obs.

in marginal parts of its distribution (Fielding & Coulson, 1995; Martin & Pullin, 2004a, b). This is the case in *Melitaea ornata* Christoph, 1893 (Eastern knapweed fritillary), whose known area is disjunct ranging from the Levant across Asia Minor, the Balkans to southern Italy and Sicily as well as the Carpathian Basin, but recently it has been indicated from Northern Iran, southern Russia (Volgograd region, South-Urals) and Eastern Kazakhstan (Tóth et al., 2013). It feeds on Asteraceae but its food plants differ regionally (Table 2).

Based on a review of museum specimens and field surveys, *M. phoebe* and *M. ornata* can co-occur in the same habitat, but the ratio of these two species is very variable. In the eastern part of the Mediterranean region *Melitaea ornata* is usually a more frequent species than *M. phoebe*. In the Carpathian Basin, on the edge of the distribution area, *M. ornata* has become a more localised species than *M. phoebe*, which can colonise more northerly parts of the western Palearctic region.

In Hungary, the ratio of the two species in the same habitats has been surveyed (Tóth et al., 2011). The results show that *M. ornata* occurs in higher numbers only in those habitats where its only known food plant (*Cirsium pannonicum*) is abundant, despite the fact that there are several Asteraceae (*Carduus* sp., *Centaurea* sp.) there that are used as food plants in the Mediterranean area. In these habitats *M. phoebe* has relatively low abundance although caterpillars were recorded feeding on *Ci. pannonicum*.

The aim of the study was to clear up the possible difference in host plant utilization ability of *Melitaea phoebe* and *Melitaea ornata*, especially that of *Cirsium pannonicum*.

Based on Tóth et al. (2011) we assumed that *Melitaea phoebe* would develop less well on *Cirsium pannonicum* than *M. ornata*. This hypothesis was tested using a laboratory experiment.

## MATERIAL AND METHODS

Two mated *Melitaea phoebe* females were collected from Eastern Hungary in spring 2011. Females were kept in a net-cage in

the field in which there were nectar sources and different food plants. In total, the females laid ~300 eggs on the same *Cirsium arvense* plant. One hundred caterpillars were randomly chosen for the experiment.

*M. ornata* caterpillars in the second larval stage were collected in field from a single nest. Since this species is localized and vulnerable in Hungary only 30 larvae were used in the experiment.

The caterpillars were reared individually to pupation in 100 ml plastic cups kept under standard laboratory conditions (25°C, 18L : 6D). Three different species of food plants were used: *Cirsium arvense* (main host of *Melitaea phoebe* in lowland areas), *Centaurea scabiosa* (main food plant of *M. phoebe* in hilly regions) and *Ci. pannonicum*, the only known food plant of *Melitaea ornata* in Hungary. Only leaves were used for feeding. The cups were checked and provided with fresh leaves every day.

The weight of the larvae was measured every second day with an assay scale. Larval duration was defined as the period from the day when the experience started until the pupation. Pupae were collected and weighted 24 h after ecdysis and then replaced in the plastic cups until adult emergence. Sexes of the individuals were determined based on the emerged imagoes. Larval survival was calculated for each treatment (food plant). Relative growth rate (RGR, in milligrams per milligram per day), which quantifies mass gained per unit time, was calculated based on an exponential growth model (Lederhouse et al., 1992; Nitao et al., 1991):  $RGR = (\ln(W_p) - \ln(W_l)) / D$  where  $W_p$  is pupal mass,  $W_l$  is initial larval mass, and  $D$  is larval duration up to the pupal stage. The weights and the growth rates were analysed using the ANOVA in R statistical (R-Core-Team, 2013) computing environment.

As polyandry occurs in many nymphalid butterflies (Scot, 1972; Wiklund et al., 2003) and we had no information on the number of males which mated with the females we used an analysis of enzymes to obtain information on the genetic variability of our experimental "population" and to compare it with that in natural populations. We obtained enzyme data from 93 experimental *Melitaea phoebe* imagoes and five natural populations (Table 3). Allozyme polymorphism was studied at 14 loci (aldehyde oxydase (Aox), esterase (Est), glucose-6-phosphate dehydrogenase (G6pdh), glutamate oxalacetate transaminase (Got), a-glycerophosphate dehydrogenase (Gpdh), hexokinase (Hk), isocitrate dehydrogenase (Idh), malate dehydrogenase (Mdh), malic enzyme (Me), phosphoglucose isomerase (Pgi), phosphoglucomutase (Pgm), 6-phosphogluconate dehydrogenase (6pgdh)

TABLE 2. Known host plants of *Melitaea ornata*.

Region	Host plant species	Citation
Sicily	<i>Centaurea deusta</i> , <i>C. busambarensis</i> , <i>C. solstitialis</i>	(Russell et al., 2007)
Greece	<i>Centaurea achaia</i> , <i>C. raphanina</i> , <i>C. salonita</i> , <i>Carduus nutans</i>	(Russell & Pamperis, 2011; Russell et al., 2007)
Hungary	<i>Cirsium pannonicum</i>	(Tóth et al., 2011; Varga, 2007)
Volgograd region (Russia)	<i>Jurinea cretacea</i> , <i>Centaurea ruthenica</i> , <i>C. marschalliana</i>	(Kuznetsov, 2011)

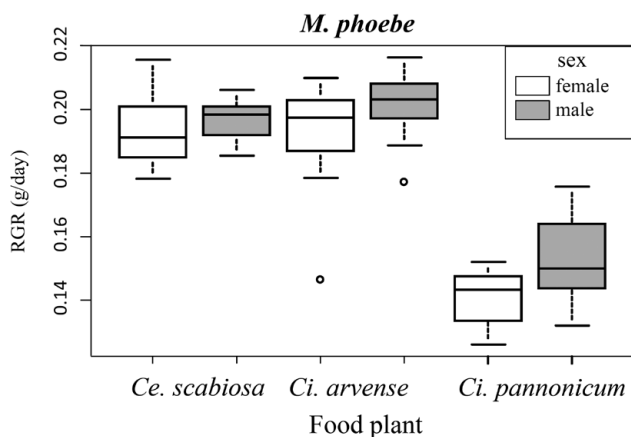


Fig. 1. Relative growth rate (RGR) of *Melitaea phoebe* caterpillars fed on three different host plants. The males developed slightly faster. *Cirsium pannonicum* was the worst food-plant for *M. phoebe* and the difference is significant ( $p < 0.001$ ).

and superoxid dismutase (Sod1, Sod2)) by vertical polyacrylamide gel electrophoresis. Thoraxes homogenized in 350  $\mu$ l of extraction buffer were used to study G6pgdh, Got, Gpdh, Hk, Idh, Mdh, Me, Pgi, Pgm, Sod1 and Sod2. Abdomens homogenized in 400  $\mu$ l of extraction buffer were used to analyse Aox, Est and 6pgdh. The extraction buffer, the electrophoresis buffer systems and running conditions, together with the staining solutions were used according to Bereczki et al. (2005).

Genotypes of the individuals were scored according to their enzyme pattern. Genotype and allele frequencies were calculated on the basis of banding patterns. Measures of genetic variation (Table 3) were calculated for each sample using GenAlEx 6.41 (Peakall & Smouse, 2006) and FSTAT 2.9.3.2 (Goudet, 1995). Since the size of the experimental population exceeded that of the studied natural populations we also calculated two additional parameters, which do not depend on the number of individuals: (i) the effective number of alleles ( $n_e$ ), which is the number of equally frequent alleles that it would take to achieve the given level of effective heterozygosity in the population; (ii) allelic richness ( $A_r$ ) was measured using Hurlbert's rarefaction method (1971) where the expected value of  $n_A$  is the number of alleles that would occur in a subsample of the given sample with  $N_s = N_{min}$  (where  $N_{min}$  is the size of the smallest sample of the study in question; in our case  $N_{min}=14$ ).

Based on the most variable enzyme locus (6pgdh), we calculated the possible minimal number of parents applying a new computational approach as follows. First, we constructed all possible genotypes from the presence of unique alleles. Then based on Punnett's tables we searched all parental genotypes permu-

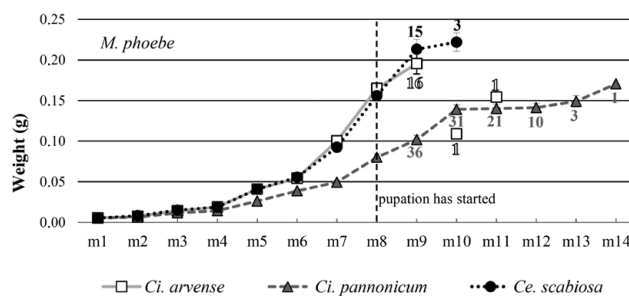


Fig. 2. Average weights of *Melitaea phoebe* caterpillars fed on three different host plants. There were no significant differences between the weights of the different groups at the start of the experiment. After the 8<sup>th</sup> measurement some of the caterpillars started to pupate. The numbers indicate the non-pupated caterpillars. The caterpillars showed slightly better development on *Centaurea scabiosa* than *Cirsium arvense*, while *Cirsium pannonicum* was the worst host plant in this comparison.

tationally. In the next step, we formulated a complete set of all pairs of possible parent types. To find the minimum number of parents that could provide the experimental offspring set, we selected groups of parents starting with  $n = 2$  and increased the number of set elements by one in each further step. In each step we selected  $n$  elements of the complete set of parents and derived all possible offspring types. When the offspring set of the selected set of parents' genotypes included all of the genotypes of the experimental offspring, we considered  $n$  the minimum number of parents necessary for producing the number of unique genotypes in the offspring of our experiment.

## RESULTS

### Enzyme studies

All indices of polymorphism indicated a high level of variation in the *M. phoebe* populations (Table 3). Overall, the average number of alleles per locus was about 2.5, the average frequency of heterozygotes was almost 20% and the percentage of polymorphic loci was over 70%.

Based on the allozyme data, at least 5 parents were necessary to establish our experimental population. The parameters of genetic variability in the experimental population are close to the measures of variation in natural populations and exceed it in only a few cases (e.g. I, GD,  $H_o$  in Table 3). In the experimental population the observed heterozygosity ( $H_o$ ) and the effective number of alleles ( $N_e$ ), which does not depend on the number of individuals,

TABLE 3. The parameters of the genetic diversity of the *M. phoebe* populations. Zab – Zabanyik, 2011/05/25; Egy – Egyek, 2012/05/26-06/07; Szh – Szölöhegy, 2000/05/06; Bor – Borház-tető, 2006/06/01; Mal – Mályvád, 1999/07/21; Exp – experimental "population". N = the number of individuals;  $N_a$  = the number of different alleles; I = Shannon's information index =  $-1 * \sum (\pi_i * \ln(\pi_i))$ ; GD = gene diversity;  $H_o$  = observed heterozygosity = No. of Hets / N; P% = percentage of polymorphic loci;  $N_e$  = the number of effective alleles =  $1 / (\sum \pi_i^2)$ ;  $A_r$  = allelic richness.

Population	N	$N_a$	I	GD	$H_o$	P <sub>95</sub> %	$N_e$	$A_r$
Zab	14.000	2.643	0.424	0.233	0.143	85.71	1.375	2.643
Bor	15.000	2.429	0.400	0.222	0.195	64.29	1.375	2.399
Szh	20.000	2.429	0.384	0.213	0.175	71.43	1.385	2.247
Mal	15.000	2.500	0.445	0.253	0.176	78.57	1.450	2.462
Egy	20.857	2.929	0.539	0.301	0.217	100.00	1.663	2.654
Exp	90.643	2.286	0.422	0.241	0.250	57.14	1.647	1.990
Average	29.250	2.536	0.436	0.244	0.193	76.190	1.483	2.399



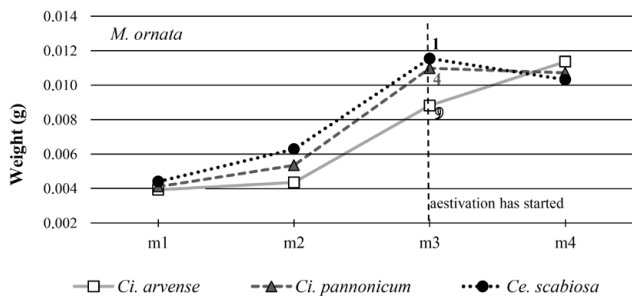


Fig. 3. Average weights of *Melitaea ornata* caterpillars fed on three different host plants. There were no significant differences between the weights of the different groups at the start of the experiment. At the time of the third measurement some of the caterpillars had started aestivating. It seems that *Centaurea scabiosa* and *Cirsium pannonicum* are slightly better food plants than *Ci. arvense*.

are higher than in natural populations (except for  $N_e$  in the Egyek population, although  $N_e$  is only slightly higher in the Egyek population than the experimental population) and higher than the average.

### Initial weights

Based on the first measurements there were no significant differences between the initial weights of the caterpillars ( $p = 0.2$ ,  $F = 1.4$ ) (see: Figs 2, 3). The *M. phoebe* caterpillars weighed 0.0050 g on average (min. 0.0030, max. 0.0060) when we started the experiment, whereas those of *M. ornata* were 0.0042 g on average (min. 0.0033, max. 0.0064).

Of the *Melitaea phoebe* caterpillars that pupated and emerged as butterflies 42.8% were females and 57.2% males. The females took slightly longer to complete their development (in average 2 days more) than the males since the females pupated with slightly larger weight than males. The results of the ANOVA indicated that the growth rate was significantly affected by host plant (host plant,  $p < 0.001$ ,  $F = 181.16$ ) in both sexes (host plant  $\times$  sex,  $p < 0.001$ ,  $F = 5.649$ ) (Fig. 1).

The caterpillars developed well on *Cirsium arvense* and *Centaurea scabiosa* and all of them pupated, whereas those that fed on *Cirsium pannonicum* showed a much worse development. All of the caterpillars survived on all of the food plants except *Ci. pannonicum* on which 10% mortality was recorded. One “outlier” individual was detected in the *Cirsium arvense* group developed much worse than any of the others (Fig. 2).

Unfortunately, we were not able to prevent the aestivation of the larvae of *Melitaea ornata*. When the caterpillars reached a critical weight they entered aestivation. Although we only measured the weights four times, we obtained some information on the development of the caterpillars. The larvae fed on *Centaurea scabiosa* developed faster than those fed on other food plants and the development was the slowest on *Cirsium arvense*. Between the third and fourth measurements 90% of the individuals fed on *Centaurea scabiosa* aestivated, while only 50% of those fed on *Cirsium pannonicum* and 10% of those fed on *Cirsium arvense* (Fig. 3).

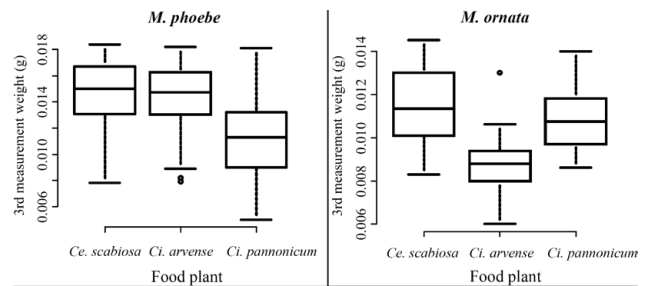


Fig. 4. The weights of the caterpillars at the 3rd measurement. *Melitaea phoebe* developed much better on *Centaurea scabiosa* and *Cirsium arvense* than on *Cirsium pannonicum* ( $p < 0.05$ ,  $F = 5.57$ ). In this comparison, the best food plant for *M. ornata* was *Centaurea scabiosa* followed by *Cirsium pannonicum*, while the worst was *Cirsium arvense* ( $p < 0.05$ ,  $F = 15.57$ ).

Since the initial weights of *M. phoebe* and *M. ornata* caterpillars were very similar we could compare the weights at the third measurement (Fig. 4). The two species showed very different patterns of host plant utilization. *M. phoebe* caterpillars reached the lowest weights on *Ci. pannonicum*, while *M. ornata* developed worst on *Ci. arvense*.

During aestivation *M. ornata* caterpillars woke up and moulted once more, after which their head capsule turned red. In autumn the caterpillars were replaced to field for overwintering but only two survived, probably due to extreme fluctuations in temperature in winter 2011.

### DISCUSSION

In this study the performance of *M. phoebe* and *M. ornata* were tested on *Centaurea scabiosa*, *Cirsium arvense* and *Ci. pannonicum*.

Despite rearing them under standard laboratory conditions all the *M. ornata* caterpillars aestivated on reaching a critical weight. Thus, the monovoltinism of *M. ornata* is probably a genetically determined adaptive trait, which could be advantageous in a Mediterranean climate, in which most of the annual precipitation falls in winter and spring, and animals and plants have to adapt to summer aridity. In lepidopterans, larval aestivation in summer could be a good strategy as it is obvious in some closely related Mediterranean species, such as *Melitaea aethrie* or *M. arduinna*. *M. ornata* caterpillars are highly resilient when they aestivate. They can survive not only the lack of food and humidity but even the cold winters, which are regular in Hungary and Russia (e.g. Volgograd region), where this species also occurs.

Although only the caterpillars of *M. phoebe* completed their development we obtained valuable information on both species' biology and their food plant utilization ability. The *M. phoebe* “population” studied was very diverse genetically and very similar in this respect to natural populations. Therefore, the genetic variability of the laboratory stock proved to be high enough to draw general conclusions regarding the different host plant utilization ability of *M. phoebe*.

Based on our results we can conclude that *Ci. pannonicum* was the worst food plant for *M. phoebe* in our study. The development takes longer time, the final weights were

significantly lower and 10% mortality was recorded, thus feeding on *Ci. pannonicum* has negative consequences for *M. phoebe* caterpillars. The negative effect of lethal toxicity is evident but sub-lethal effects like slower development rate and lower pupal weight could also exert negative effects on the fitness of herbivorous insects (Clancy & Price, 1987; Benrey & Denno, 1997; Awmack & Leather, 2002). Surprisingly, *Ci. pannonicum* is the only known food plant of *M. ornata* in Hungary, although our results indicate that the population of *M. ornata* studied developed well on *Ce. scabiosa* and less so on *Ci. arvensis*. Additionally, it is known that *M. ornata* uses several *Centaurea* species in the Mediterranean region (see: Table 2).

Based on the distribution data and the results of species distribution modelling (Tóth et al., 2013) it is clear that *M. ornata* is a Ponto-Mediterranean-Turkestanian faunal element, which became a localized species with an island-like distribution pattern at the northern margins of its distribution surrounded by more or less continuous populations of *M. phoebe*. The opposite situation is reported in Turkey (Hesselbarth et al., 1995) and Southern Greece (Russell et al., 2007), where the climate is usually optimal for *M. ornata* and suboptimal for *M. phoebe*, thus there *M. phoebe* is the more localized species.

Further studies are needed to clear up the physiological adaptation that has enabled *M. ornata* to utilize *Ci. pannonicum* without negative effects. This mechanism could be the key factor in the habitat and food plant specialism of this species in Hungary. According to previous investigations (de Lattin, 1967; Thomas, 1985; Fielding & Coulson, 1995; Bossart, 2003) the restricted food plant use of marginal populations are mostly explained by the limited availability of food (e.g. *Calluna* spp. in Northern Atlantic region). It is obvious that this hypothesis does not fit our case. We assume that the food plant specialism of *Melitaea ornata* in the Carpathian Basin is shaped by suboptimal climate conditions but it also might be influenced by the possible competitive pressure of the more generalist *M. phoebe*.

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