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Differences in the development of the closely related myrmecophilous butterflies *Maculinea alcon* and *M. rebeli* (Lepidoptera: Lycaenidae)

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Abstract. The initially phytophagous caterpillars of *Maculinea alcon* and *M. rebeli* complete their development in *Myrmica* ant colonies as social parasites. Recent genetic studies show no differences at the species level among various populations of each butterfly taxa. Usually *M. alcon* and *M. rebeli* are identified by habitat and larval food plants (Gentianaceae) and host ant specificity is also considered to be an important feature. However most of the ecological characteristics overlap at least in some parts of their distributions. The developmental and survival characteristics of caterpillars reared by different *Myrmica* species were compared in laboratory experiments and in the field. Morphologically indistinguishable *M. alcon* and *M. rebeli,* which originated from Polish populations, are very similar in terms of host specificity i.e. larvae survived both with *M. scabrinodis* and *M. sabuleti.* However they showed different growth characteristics. The earlier flight period of *M. rebeli,* which is synchronized with the phenology of *Gentiana cruciata*, resulted from the quick growth of caterpillars in *Myrmica* nests in the pre-winter phase, when they gained about half of their final body biomass. After the end of winter they recommenced growth almost immediately. *M. alcon* larvae entered diapause shortly after adoption by ants and began to increase in weight significantly just one month after the onset of spring, which synchronized their development with that of their larval food plant, *G. pneumonanthe*. Therefore neither population group is transferable between habitats and should still be regarded, at least, as distinct conservational units.

INTRODUCTION

The obligatorily myrmecophilous butterflies, *Maculinea alcon* (Denis & Schiffermüller, 1775) and *M. rebeli* Hirschke, 1904 are endangered in Europe (van Swaay & Warren, 1999). Because of their complicated life-history and status representatives of the genus *Maculinea* van Eecke, 1915, are among the most intensively studied butterfly species in the world (Thomas & Settele, 2004; Settele et al., 2005).

The separation of the species *M. alcon* and *M. rebeli,* always controversial (see Steiner et al., 2006 for a review), is strongly challenged by the results of the latest genetic (Als et al., 2004; Bereczki et al., 2005) and cladistic studies (Pech et al., 2004). Recently Steiner et al. (2006) did not find any differences in egg morphology and cuticular compounds of larvae from the type localities of *M. alcon* and *M. rebeli* in Austria.

For a long time, habitat and larval food plants rather than morphological characteristics, were used as the main criteria for attributing specimens to one species or the other. *M. alcon* was considered to be a hygrophil or mesophil using mainly *Gentiana pneumonanthe* and *M. rebeli* as a xerothermophil using mainly *G. cruciata* (Munguira & Martin, 1999). However preferences for larval food plants are not clear-cut. *M. alcon* and *M. rebeli* oviposit on six and seven species of *Gentiana* or *Gentianella*, respectively (Jutzeler, 1988; Munguira & Martin, 1999; Kolev, 2002; Stankiewicz et al., 2005b; Tartally & Varga, 2005; Steiner et al., 2006). The most important conclusion of these observations is that *G. pneumonanthe*, the traditional larval food-plant of *M. alcon*, might also be used additionally by *M. rebeli* and vice versa. Females oviposit mostly on gentian buds and are probably guided by the physiological and chemical characteristics of a plant rather than preferences for a particular species (Sielezniew & Stankiewicz, 2004a).

Besides larval foodplants, the presence of specific ants is another vital factor essential for the existence of both *M. alcon* and *M. rebeli.* During the first three stages, lasting 2–3 weeks, caterpillars are endophytic and eat developing flowers and seeds, but grow very little, gaining about 1.5% of their final biomass. Just after the fourth (final) moult they drop to the ground and await *Myrmica* ants. If a worker comes across a caterpillar within 48 h it is immediately taken to a nest in the same way as ant brood is carried. Retrieved caterpillars spend 10–22 months in colonies being fed by ants with regurgitations and insect prey, and they also eat host larvae. Caterpillars finally pupate in chambers close to the soil

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surface and emerge as adults 3 weeks later (Elmes et al., 1991a, b).

This advanced myrmecophily is associated with chemical mimicry of ants (Akino et al., 1999). The adoption of the larvae and the first phase of their integration into an ant colony is triggered by the presence of a relatively simple set of hydrocarbon compounds in the cuticle. However, after some time caterpillars start to synthesize additional chemicals, which enable them to achieve a high social status in the nests of specific ants. If they are adopted by non-host ants the survival rate is poor and depends, among other factors, on the physiological state of the colony, e.g. food stress may increase mortality (Elmes et al., 2002, 2004; Schönrogge et al., 2004).

The first data on the host ants of *Maculinea* butterflies indicated a high level of specificity. *M. alcon* was found mainly in nests of *M. ruginodis* Nylander, 1846, while *M. rebeli* almost exclusively in *M. schencki* Emery, 1895 colonies (Thomas et al., 1989). Further studies revealed a much more complicated pattern of butterfly-ant relationships. First Elmes et al. (1994) discovered geographical variation in the host specificity of *M. alcon*, which used three different species along a north-south gradient: *M. rubra* (Linnaeus, 1758), *M. ruginodis* and *M. scabrinodis* Nylander, 1846. Next Als et al. (2002) revealed that in Denmark both *M. rubra* and *M. ruginodis* were parasitized at some sites. However in eastern parts of the European range *M. alcon* has never been recorded in colonies of these two species. *M. scabrinodis* is reported as a host of this butterfly in Poland (Sielezniew & Stankiewicz, 2002) and Hungary (Tartally & Varga, 2005) and additionally in some localities it develops successfully in nests of the closely related *M. vandeli* (Sielezniew & Stankiewicz, 2004b) or *M. salina* (Tartally, 2005).

As far as *M. rebeli* is concerned *M. schencki* is not the major host in the east, except at the most northern, highly isolated sites in Lithuania (Stankiewicz et al., 2005c). In Austria and Hungary this butterfly is recorded associated with five species: *M. sabuleti* Meinert, 1846*, M. scabrinodis, M. specioides* Bondroit 1918, *M. lonae* Finzi, 1926 and *M. schencki* (Steiner et al., 2003; Tartally & Varga, 2005). However in Poland *M. schencki* is not exploited, although it can be quite abundant and *M. rebeli* populations are supported by *M. sabuleti* and *M. scabrinodis* (Steiner et al., 2003; Stankiewicz & Sielezniew, unpubl.). There is also an anecdotal observation of pupae in a *M. rugulosa* Nylander, 1846 nest at a site in the south-east of the country (Stankiewicz et al., 2005a).

One of the most important conclusions of field studies on the ant relationships of *M. alcon* and *M. rebeli* is overlapping host specificity in Eastern Europe. In Poland (Stankiewicz et al., 2005b) and Hungary (Tartally & Varga, 2005) both species are found in colonies of *M. scabrinodis,* which is a widespread and quite tolerant ant encountered in various open and sunny grassland habitats except very dry ones (Elmes et al., 1998). Other *Myrmica* species, used by *M. alcon* or *M. rebeli*, usually have narrower niches and are more limited as potential hosts for both *M. alcon* and *M. rebeli*. For example *M. sabuleti,* the major host of *M. rebeli*, at many sites is confined to xerothermic meadows and absent from the typical wetter *M. alcon* habitats. Interestingly all the important hosts of "cuckoo" *Maculinea* in Eastern Europe are closely related and belong to the "*scabrinodis*" group. Therefore it is likely that the differences in host-ant specificity of *M. alcon* and *M. rebeli* in the region reflect only differences in ant species composition in their habitats.

To test this hypothesis and look for other possible characteristics, laboratory experiments were performed. The Polish populations of *M. alcon* and *M. rebeli* are particularly suitable for such studies because: (1) previous field data suggest that they are very similar to each other in terms of host ant specificity; (2) imagines do not differ morphologically but live in typical habitats i.e. wet or xerothermic meadows, respectively. The survival and growth of caterpillars were compared in artificial nests of a few *Myrmica* species, from both *M. alcon* and *M. rebeli* sites. The results of these studies combined with field observations are presented in this paper.

MATERIAL AND METHODS

Collection of *Maculinea*

Pre-adoption larvae of *M. alcon* were obtained from two populations. Most of the material was collected at "Jasiów" (51°01´N, 20°39´E; 350 m a.s.l.) in the Świętokrzyskie region (southern Poland), where the butterfly develops in *M. scabrinodis* and *M. vandeli* nests. Details are given in Sielezniew & Stankiewicz (2004b). The rest of the *M. alcon* stock originated from "Augustówka" near Warsaw (51°59´N, 21°29´E, 100 m a.s.l.), where *M. scabrinodis* is the host (Sielezniew & Stankiewicz, 2002). However, all *M. rebeli* caterpillars originated from the biggest Polish population in Przemyśl (49°46´N, 22°46´E, 320–350 m a.s.l) in south-eastern Poland, where both *M. sabuleti* and *M. scabrinodis* are hosts (Sielezniew et al., 2003).

Samples were collected in late July and early August 2003 and 2004. Twenty or thirty gentian shoots, with eggshells visible, which suggests the presence of caterpillars in flowerheads, were collected at each site. To avoid harming the populations plants were collected only from patches where caterpillars were unlikely to survive after dropping to the ground. Appropriate gentians were identified, i.e. those growing outside the foraging zone of host ants, by placing sugar baits close to them. If only non-host ants were attracted, shoots were considered for sampling. Cut shoots were immediately transported to the laboratory and put in plastic cups with water. The immersed parts of the stalks were wrapped with tissue to prevent caterpillars from drowning when leaving shoots. These bunches of shoots were kept in large plastic containers covered with a net to prevent caterpillars escaping.

Twice a day the gentians and containers were checked for the presence of just-emerged fourth instar caterpillars, which were then used for experiments, i.e., introduced into *Myrmica* ant colonies. The plants remained fresh for about two weeks, which enabled most of the caterpillars present to moult successfully to the fourth instar. Preliminary studies, like those of Elmes et al. (1991b), showed that small larvae had little chance of survival. Thus, only pre-adoption larvae with a weight of not less than 1 mg were used. A few small caterpillars, which left the shoots at an earlier instar were also rejected.

Collection of *Myrmica*

All ant nests for the laboratory experiments originated from *Maculinea* sites or adjacent areas. In the butterfly habitats colonies situated in the vicinity of larval food plants were never chosen. In June and July 2003 and 2004, a total of 72 nests of seven *Myrmica* species were fully excavated with turf and quickly transported to the laboratory in big containers or bags. Colonies of *M. scabrinodis, M. vandeli, M. gallienii, M. ruginodis* and *M. rubra* were excavated on wet *M. alcon* meadows. From the dry *M. rebeli* habitat: *M. sabuleti, M. schencki* and *M. scabrinodis* colonies were obtained. Ants were identified according to Czechowski et al. (2002) and Radchenko et al. (2003). In the laboratory, nests were carefully dissected and all specimens were caught, counted and then put into artificial nests. If excavated colonies were very large, then they were divided into subcolonies of about 200–250 workers, one queen and a proportional number of brood. In this way a total of 145 laboratory colonies was formed (Table 1).

Experimental design

The techniques used to rear ants and caterpillars were similar to those described by Wardlaw (1991) and Wardlaw et al. (1998). Experimental colonies were maintained in covered plastic boxes, 24 × 15 × 5 cm, with ventilation in the form of two openings (1 cm in diameter) in the lid, each covered with fine netting. The upper parts of the sides of each box were coated with a thin layer of Fluon to prevent ants from escaping during manipulations that involved opening boxes. Two thin, moist rectangular sponges were placed in the bottom of each box. Two walnut shells, which served as shelters, were placed on the bigger sponge (7 × 5 cm). The smaller sponge (5 × 3 cm) at the opposite end was used as a watering place and kept a little wetter. Food was also provided every 2–3 days in the form of sugar and *Drosophila* flies (adults and larvae which originated from a permanent standard laboratory culture).

Boxes were cleaned once a week and walnut shells and sponges replaced if they became mouldy. Carbon dioxide was used to anaesthetise ants during major manipulations.

Originally five caterpillars of *M. alcon* or *M. rebeli* were to be introduced into every ant culture. However, because of variation in the availability of pre-adoption larvae and the necessity to complete the set within 1–2 days, the numbers occasionally differed slightly (3–6). *M. alcon* caterpillars were placed in the nests of seven *Myrmica* species. Five of them were native to butterfly habitats in Poland (*M. scabrinodis, M. vandeli, M. gallienii, M. ruginodis* and *M. rubra*) and the remaining two originated from the *M. rebeli* site. As far as *M. rebeli* is concerned only three species (*M. sabuleti, M. scabrinodis* and *M. schencki)* were tested. There were insufficient number of caterpillars and further collecting could have affected the population. *M. scabrinodis* occurred at both the *M. alcon* and *M. rebeli* sites. As it is thought that there are two ecotypes of the species, i.e., xerophilous and hygrophilous ecotypes (Elmes et al., 1998) cross-over experiments were performed with both *Maculinea* species.

TABLE 1. Summary of the laboratory colonies of different species of ants that hosted *Maculinea* caterpillars.

Myrmica species	No. of nests excavated	No. of laboratory colonies	No. of colonies with introduced caterpillars of	
			M. alcon	*M. rebeli*
M. scabrinodis (from *M.* alcon sites)	8	25	16	9
M. scabrinodis (from *M. rebeli* sites)	7	34	20	14
M. scabrinodis (all)	15	59	36	23
M. sabuleti	15	22	12	10
M. vandeli	4	6	6	–
M. gallienii	6	25	25	–
M. rubra	4	7	7	–
M. ruginodis	4	12	12	–
M. schencki	10	14	7	7
Total	58	145	105	40

All caterpillars were weighed just before placing them in artificial nests and if they survived, measurements were continued regularly at 7 (±1) day intervals. Simultaneously, in most cases, length and diameter of caterpillars were measured to calculate the volume of each individual using the formula used by Als et al. (2002) for *M. alcon*, i.e. volume of cylinder with hemispherical ends:

$$V = \pi r^2(l-2r) + 4\pi r^3/3$$

where r is the radius and l is the total length. A regression between weight and volume was used to estimate the weight of larvae in the field, where direct measurements were impossible.

Boxes with ants and larvae were kept in a climatic chamber at a constant temperature of 20°C and a 14L : 10D photoperiod. The main aim was to compare the survival and growth rates of *M. alcon* and *M. rebeli* over a period of eight weeks, i.e., the approximate duration of the pre-winter phase. After that most of the boxes were kept at a lower temperature (6–10°C) and under short day conditions (8L : 16D) for about 4 months (overwintering) and the others remained at 20°C. Because of the high mortality of both ant workers and caterpillars during the winter period, due to disease rather than unsuitability of the host, it was not possible to continue observations. Very few individuals were reared to adulthood and therefore results from the post-winter phase were analyzed only in terms of growth rate.

Field studies

The growth of caterpillars in the field was studied at *M. alcon* sites in the Świętokrzyskie region and a *M. rebeli* site in Przemyśl. We looked for larvae and pupae in ant nests in late September/early October 2003, late April/early May, late May/early June and late June 2004. Areas within 1m of gentians were searched for *Myrmica* colonies. At *M. alcon* sites larval food plants were difficult to find in spring because of the delayed development of *G. pneumonanthe* shoots from rhizomes and the much more delicate structure of these plants compared to *G. cruciata*. To overcome this difficulty, gentians loaded with eggs were marked using GPS in the previous season and only those patches were investigated next year.

Nests were not fully excavated and the search limited to higher chambers, especially in seasons when there were mainly small larvae. Destruction of nests before completion of larval growth would make development impossible and such invasive activities were avoided for reasons of conservation. Moreover the main intention of the studies was to follow the growth of the *Maculinea* caterpillars during their stay in *Myrmica* colonies. Studies were performed on warm sunny days when caterpillars were usually carried by workers into the upper parts of nests. All individuals found were recorded using a digital video camera, which allowed the later measurement of length and width (diameter) by comparison with a standard frame. We did not manage to find all the larvae and after examination the nests were immediately covered.

Ants were identified in the field using a magnifying glass and a sample of about 10 workers was collected each time to check

TABLE 2. Summary of the results for 564 *M. alcon* caterpillars reared in laboratory colonies of seven different *Myrmica* species for eight weeks after introduction (week 0), N – number of caterpillars alive, S – percent surviving, W ± S.D. – mean body weight (mg) and standard deviation.

Myrmica species		Week								
		0	1	2	3	4	5	6	7	8
M. scabrinodis from *M. alcon* habitat	N	105	101	98	97	96	93	93	91	90
	S	100	96	93	92	91	89	89	87	86
	W ±	1.6	3.2	3.9	4.2	4.3	4.5	4.5	4.5	4.5
	S.D.	0.4	1.2	1.8	2.3	2.2	2.4	2.4	2.3	2.3
M. scabrinodis from *M. rebeli* habitat	N	81	76	75	75	75	74	72	70	70
	S	100	94	93	93	93	92	89	86	86
	W ±	1.5	3.4	4.0	4.4	4.6	4.7	4.8	4.8	4.7
	S.D.	0.3	1.2	1.6	2.1	2.2	2.4	2.6	2.8	2.6
M. scabrinodis all cultures	N	186	177	173	172	171	167	165	161	160
	S	100	95	93	93	92	90	89	87	86
	W ±	1.5	3.2	3.9	4.3	4.5	4.6	4.6	4.6	4.6
	S.D.	0.4	1.2	1.7	2.2	2.2	2.4	2.5	2.5	2.4
M. vandeli	N	30	26	26	26	25	25	25	25	24
	S	100	87	87	87	83	83	83	83	80
	W ±	1.5	2.8	3.3	3.4	3.8	3.9	4.2	4.4	4.5
	S.D.	0.3	0.7	1.0	1.1	1.7	2.0	3.0	3.5	3.7
M. sabuleti	N	61	46	44	43	41	40	39	38	38
	S	100	75	72	71	67	66	64	62	62
	W ±	1.6	3.3	4.2	4.7	4.7	4.8	4.6	4.8	4.7
	S.D.	0.4	1.4	1.7	2.3	2.1	2.0	1.7	1.9	1.8
M. gallienii	N	120	103	100	100	97	95	94	89	85
	S	100	86	83	83	81	79	78	74	71
	W ±	1.4	2.6	3.1	3.2	3.4	3.5	3.5	3.7	4.0
	S.D.	0.3	0.8	1.3	1.6	1.8	1.8	1.8	1.9	1.9
M. rubra	N	41	7	7	7	6	6	6	5	4
	S	100	17	17	17	15	15	15	12	10
	W ±	1.5	2.4	2.8	2.6	3.1	2.9	3.1	3.1	3.0
	S.D.	0.3	0.6	0.7	0.5	0.5	0.6	1.0	0.6	0.5
M. ruginodis	N	83	12	8	5	4	4	4	4	4
	S	100	15	10	6	5	5	5	5	5
	W ±	1.4	2.6	2.9	3.3	3.7	3.9	4.0	3.7	4.2
	S.D.	0.3	1.2	0.7	1.3	1.6	1.5	1.8	1.8	2.1
M. schencki	N	43	2	2	2	2	2	2	2	2
	S	100	5	5	5	5	5	5	5	5
	W ±	1.3	2.5	2.8	2.9	3.2	3.4	3.5	3.8	3.8
	S.D.	0.2	0.4	1.1	1.3	1.3	1.7	1.8	2.2	1.8

the accuracy of the determination using a stereoscope microscope in the laboratory.

Statistical analyses

Differences in body weights and body volumes of *Maculinea* individuals reared in the laboratory or recorded in the field were tested using Wilcoxon tests. For the analyses of survival of different groups of larvae in experimental colonies the method of Cox Proportional Hazards along with the Wald test was applied. However, the growth was described using simple regression. Of the alternative models fitted, the model which yielded the highest r^2 value was selected in every case.

The survival analysis was performed using Statistica 6.0 software. All other statistics were carried using Statgraphics Plus 5.0 or Excel spreadsheets.

RESULTS

Survival of *M. alcon* and *M. rebeli* in the laboratory

The mean initial weight of 564 fourth instar *M. alcon* caterpillars (1.55 ± 0.34 mg) introduced into *Myrmica* laboratory colonies was significantly lower (W = 19210, $p < 0.001$) than that of the 192 *M. rebeli* caterpillars (1.86 ± 0.40 mg). There were no significant differences in ini-

TABLE 3. Summary of the results for 192 *M. rebeli* caterpillars reared in laboratory colonies of three different *Myrmica* species for eight weeks after introduction (week 0), N – number of caterpillars alive, S – percent surviving, W ± S.D. – mean body weight (mg) and standard deviation.

Myrmica species		Week								
		0	1	2	3	4	5	6	7	8
M. scabrinodis from *M. rebeli* habitat	N	66	61	58	57	57	57	56	55	54
	S	100	92	88	86	86	86	85	83	82
	W ±	1.9	6.0	9.1	11.7	14.9	18.1	21.0	24.7	27.2
	S.D.	0.4	2.2	4.6	6.6	9.5	12.7	15.5	18.5	20.7
M. scabrinodis from *M. alcon* habitat	N	42	33	33	33	33	33	33	33	33
	S	100	79	79	79	79	79	79	79	79
	W ±	1.7	6.3	8.4	10.6	12.9	15.7	17.3	19.5	21.0
	S.D.	0.4	3.1	5.4	7.2	9.8	13.7	16.3	19.8	22.5
M. scabrinodis all cultures	N	108	94	91	90	90	90	89	88	87
	S	100	87	84	83	83	83	82	81	81
	W ±	1.8	6.1	8.7	11.1	13.9	16.8	18.9	21.5	23.5
	S.D.	0.4	2.5	4.9	6.8	9.6	13.1	15.8	18.9	21.4
M. sabuleti	N	50	47	45	45	45	45	45	43	41
	S	100	94	90	90	90	90	90	86	82
	W ±	1.9	6.5	8.9	10.9	13.4	16.1	18.3	19.4	20.5
	S.D.	0.4	2.4	4.3	7.0	10.2	14.1	17.3	20.1	21.9
M. schencki	N	34	3	1	1	0				
	S	100	9	3	3	0				
	W ±	1.5	6.3	10.5	7.0					
	S.D.	0.5	3.7							

tial body weights among caterpillars, whether *M. alcon* or *M. rebeli*, tested with different *Myrmica* species.

M. alcon caterpillars survived best in colonies of *M. scabrinodis*. After one week 95% of the individuals were still alive and after eight weeks dropped to 86% (Table 2). Comparison of mortality of *M. alcon* in *M. scabrinodis* colonies originating from different habitats did not reveal significant differences (Wald = 0.017, p = 0.90). Hence, in subsequent analyses characteristics of all caterpillars reared by *M. scabrinodis* were pooled.

Survival of *M. alcon* caterpillars in *M. vandeli* (80% after eight weeks), *M. gallienii* (69%) and *M. sabuleti* colonies (62%) was lower than in *M. scabrinodis* colonies, but there were significant differences only between survival in *M. scabrinodis* and *M. sabuleti* (Wald = 15.8, $p < 0.0001$) and *M. scabrinodis* and *M. gallienii* colonies (Wald = 9.68, $p < 0.01$). *M. rubra, M. ruginodis* and *M. schencki* were considerably worse hosts compared to all other ant species ($p < 0.001$). The first and eighth week were survived by, respectively 17 and 10%, 15 and 5% and 5 and 5% of all introduced caterpillars.

As far as *M. rebeli* is concerned the highest mortality was recorded for caterpillars introduced into *M. schencki* colonies. Only 9% were alive after 7 days and none survived until the fourth week. Survival in colonies of *M. sabuleti* and *M. scabrinodis* was similar i.e. 94 and 82% after one week and 87 and 82% after eight weeks, respectively (Wald = 0.075, p = 0.78). As with *M. alcon*, a comparison of the number of survivors in *M. scabrinodis* cultures from different habitats did not reveal significant differences (Wald = 0.213, p = 0.65).

A summary of the results for survival and changes in body weight of *M. alcon* caterpillars reared for eight weeks are given in Table 2 and for *M. rebeli* in Table 3.

Most of the *Myrmica* and/or *Maculine*a stock did not survive the winter i.e. a period of chilling lasting for about four months which coincided with high ant mortality. Thirteen individuals of *M. rebeli* (6 with *M. scabrinodis* and 7 with *M. sabuleti*) and only 8 of *M. alcon* (5 with *M. scabrinodis*, one each with *M. vandeli, M. sabuleti* and *M gallienii*) were reared to adulthood.

Growth of *M. alcon* in the laboratory

M. alcon caterpillars grew slightly during the first three weeks after introduction into ant cultures, achieving 4.4 ± 2.3 mg. Then their growth stopped and the average body weight remained stable (Fig. 1). In the eighth week caterpillars weighed 4.6 ± 2.4 mg and the distribution was right-skewed (Fig. 2). Regression analysis fitted the reciprocal-X model to describe the relationship between mean weight (y) and time (x) over the eight weeks ($y = 5.14–3.57/x$, $r^2 = 0.990$, $F_{1,7} = 721.38$, $p < 0.001$).

Thirty one *M. alcon* larvae were kept at 20°C for 20 weeks in colonies. After that time their mean body weight (6.4 ± 3.3 mg) did not differ significantly from that at eight weeks (6.8 ± 4.0 mg). Then they were kept at low temperature with all the other caterpillars.

Eight caterpillars of *M. alcon*, which successfully overwintered and were reared to adult stage, pupated at 82.9 ±

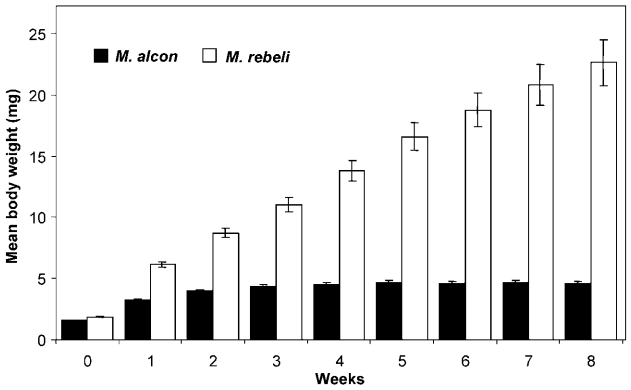

Fig. 1. Trends in the mean body weights of *M. alcon* (N = 236) and *M. rebeli* caterpillars (N = 137) reared in laboratory colonies of *Myrmica* for 8 weeks (week 0 – introduction into a nest). The error bars represent ± 1 SE of the mean. All differences between the species are significant (Mann Whitney Test, $p < 0.001$).

11.3 mg (Table 4). Before winter they attained about 6% of the pupal biomass and lost 29% of it during the period of chilling. When the temperature increased they did not start to grow immediately but after about four weeks (Fig. 3) and finally pupated 68.3 ± 13.4 days after the end of overwintering. Duration of the pupal stage was 20.1 ± 2.0 days.

Growth of *M. rebeli* in the laboratory

The mean weights of fourth instar *M. rebeli* caterpillars were significantly higher than that of *M. alcon* over the whole period (8 weeks) of rearing at a constant temperature of 20°C (Fig. 1). However, infraspecific differences among larvae reared with different ant species were not significant and the comparison was of all survivors.

Average body weight of *M. rebeli* caterpillars increased significantly up to 22.6 ± 21.4 mg in the eighth week. The relationship between weight (y) and time (x) over the 8 weeks can be fitted by the square root-X model ($y = 10.40*\sqrt{(x)} - 8.93$, $r^2 = 0.996$, $F_{1,7} = 1693.78$, $p < 0.001$).

However the growth of individuals differed and generally it was possible to distinguish two groups from the third week (Fig. 4). About 41% of the 137 caterpillars grew fast. The frequency distribution of larval weights in the eighth week is presented in Fig. 2. Clear differences in weight and regular measurements at weekly intervals enabled us to follow the trajectories of every individual in this group. Regression analysis reveal a linear relationship between weight (y) of "fast" developers and time (x) over the period weeks 3–8 ($y = 5.42*x - 3.89$, $r^2 = 0.997$, $F_{1,5} = 1993.36$, $p < 0.001$). After 56 days their mean body weight was: 44.5 ± 18.5 mg. The remaining caterpillars

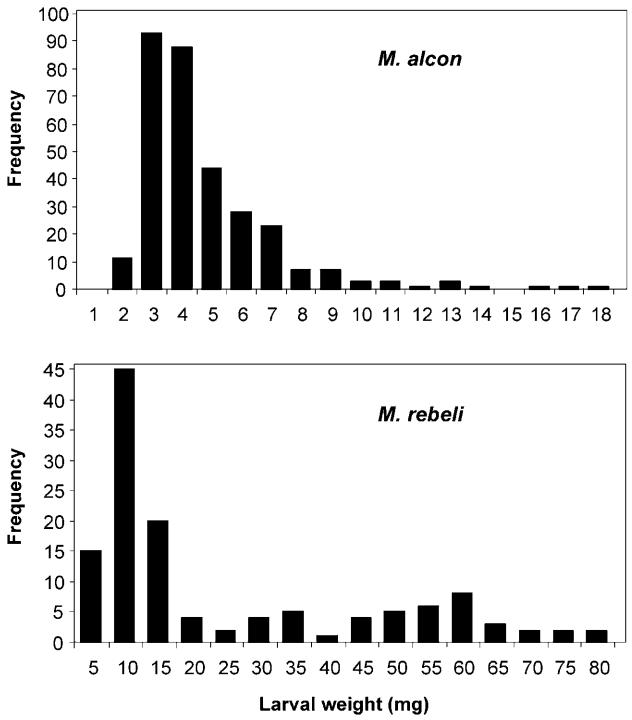

Fig. 2. Frequency distributions of the body weights of *Maculinea alcon* and *M. rebeli* caterpillars reared for 8 weeks in laboratory colonies of *Myrmica* ants.

grew only until the third week and after 56 days had achieved an average weight of 8.2 ± 3.3 mg. Nevertheless they were twice as heavy as *M. alcon* caterpillars after the same period. A double reciprocal regression model describes the average growth in weight of the "slow" developers ($y = 1/(0.088 + 0.221/x$, $r^2 = 0.995$, $F_{1,5} = 1018.15$, $p < 0.001$).

Twenty seven *M. rebeli* caterpillars were kept for 30 weeks at a constant temperature of 20°C. It was possible to follow the growth trajectories of individuals only after the third week when considerable variation in growth was detected (Fig. 5). Only one caterpillar had pupated after 160 days (at 92 mg) and a female emerged 20 days later. Body weights of 12 caterpillars increased over the period 12–21 weeks but then remained stable or started to decrease slightly and five of them died. As far as small caterpillars are concerned, six started to grow after a period of quiescence (12 to 18 weeks). Body weights of the other caterpillars remained stable and five of them died after 22–27 weeks. After 30 weeks all survivors were kept at a lower temperature but died within the next 20 weeks.

All other caterpillars after the end of the eighth week were exposed to a period of low temperature (3–4

TABLE 4. Summary of the results for 8 individuals of *M. alcon* and 13 of *M. rebeli* reared successfully to adulthood.

	Pre-winter body weight (mg)		Post-winter body weight (mg)		Duration of post-winter phase prior to pupation (days)		Pupal weight (mg)		Duration of pupal stage (days)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
M. alcon	5.1	1.7	3.6	1.3	68.3	13.4	82.9	11.3	20.1	2.0
M. rebeli	58.3	16.6	49.1	15.5	38.6	4.7	104.0	19.8	20.1	2.6

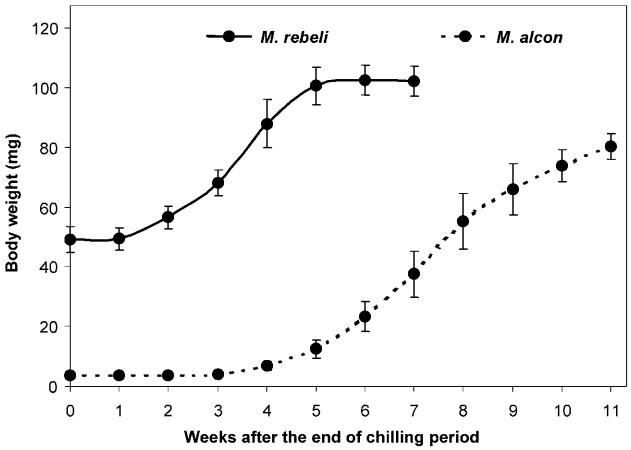

Fig. 3. Trends in the body weight (mean and S.E.) of the *Maculinea alcon* (N = 8) and *M. rebeli* (N = 13) individuals that were successfully reared throughout the post-winter phase in the laboratory. *M. alcon* caterpillars pupated in weeks 6–13 and those of *M. rebeli* in weeks 5–7 after the end of chilling. Adults emerged in weeks 10–16 and 8–10, respectively.

months) and then returned to 20°C. As for *M. alcon* most died because the ants died. However, 13 caterpillars of *M. rebeli* successfully completed development. The mean weight of pupae was 104.0 ± 19.8 mg. They were larger than those of *M. alcon* but not significantly so (W = 21.0, $p < 0.05$). Caterpillars attained about 56% of their final weight in the pre-winter phase and then lost 16% of it during the period of chilling. Caterpillars of *M. alcon* and *M. rebeli*, which successfully completed development, attained respectively about 6% and 57% of pupal weight before winter. Weight loss during the period of chilling was about 16%. In contrast to *M. alcon*, *M. rebeli* caterpillars needed only about one week to resume growth, when the temperature was raised (Fig. 3) and pupation occurred after 38.6 ± 4.7 days from the end of the overwintering period. Adults emerged from pupae after 20.1 ± 2.6 days, i.e., the duration of this stage was exactly the same in both species (Table 4).

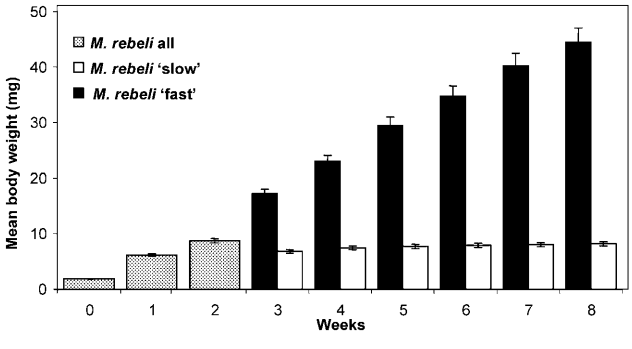

Fig. 4. Trends in the mean body weights of 137 *M. rebeli* caterpillars reared in laboratory colonies of *Myrmica* for eight weeks (week 0 – introduction to a nest). From the third week it is possible to distinguish two groups: "fast" developers, which showed a linear increase in body weight and "slow" developers, which entered a quiescent period. The error bars represent ±1 SE of the mean.

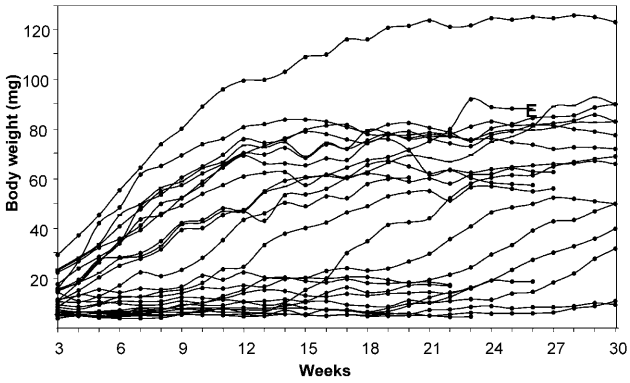

Fig. 5. Changes in body weight of 27 *M. rebeli* caterpillars reared for 30 weeks in six laboratory *Myrmica* nests at a constant temperature of 20°C. It was possible to follow the growth trajectory of individuals only after the third week. One adult emerged (E) and 11 caterpillars died in weeks 21–27 (earlier end of a line).

There is a clear relationship between the simultaneous measurements of size and weight of caterpillars of *M. alcon* and *M. rebeli* (Fig. 6). As expected, it is described by a linear regression model ($y = 0.88x - 0.65$, $r^2 = 0.984$, $F = 82902$, $p < 0.001$), which was used to roughly estimate of larval body weights in the field.

Growth of *M. alcon* in the field

In late September about 60 nests of host ants near *G. pneumonanthe* plants with visible eggshells were opened. However, only ten small caterpillars were observed in the peripheral chambers of four of the *M. scabrinodis* nests. Their mean body volume was 3.2 ± 1.3 mm^3 (1.4–5.7 mm^3) with a mean estimated weight of about 2.2 mg. Larvae were not looked for in the deeper parts of colonies as it would have been too destructive.

In early May, in a total of 17 nests (15 of *M. scabrinodis* and 2 of *M. vandeli*), 41 caterpillars were found and measured. The mean body volume was 8.6 ± 13.3 mm^3 (mean weight of about 6.9 mg), but the distribution of the body volumes was bimodal. Most caterpillars (35) were

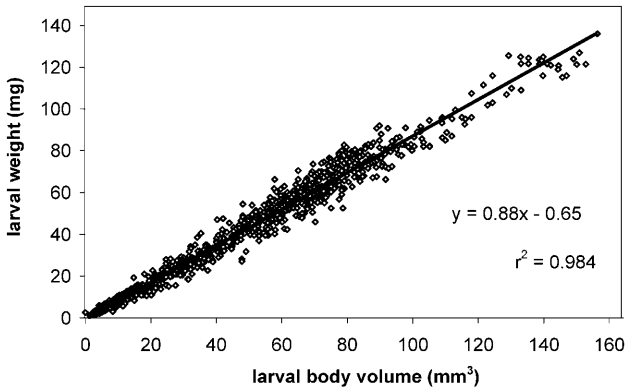

Fig. 6. The relationship between the larval weight and body volume of *M. alcon* and *M. rebeli* caterpillars (1384 measurements) reared in the laboratory. Equation of the linear regression can be used to roughly estimate the mean weight of larvae found in the field.

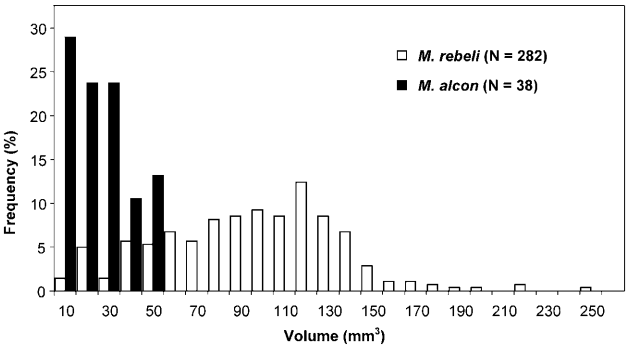

Fig. 7. Frequency distributions of the volumes of *M. alcon* and *M. rebeli* caterpillars measured in the field in late May (*M. rebeli* site) and early June (*M. alcon* site).

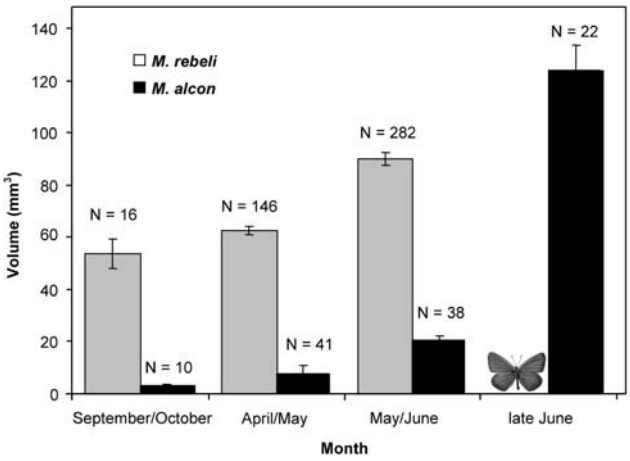

Fig. 8. Trends in the volumes of *M. alcon* and *M. rebeli* caterpillars/pupae from September/October to late June the following year at two field sites. The error bars represent ±1 SE of the mean. Peak of flight activity occurred at the end of June when only the occasional pupae can be found.

small (2.0–6.2 mm^3), with a mean volume of 3.9 ± 0.9 mm^3 (estimated mean weight 2.7 mg), and the remainders were large, 36.0 ± 18.7 mm^3 (16.2–65.4 mm^3). The large caterpillars were found only in nests with very few workers. The mean volume of larvae was higher than the previous autumn ($W = 95.5$, $p < 0.05$), but for particular larvae (35 individuals) the difference was insignificant ($p > 0.05$).

About one month later, in early June, a total of 38 *M. alcon* caterpillars in 13 *Myrmica* nests (9 of *M. scabrinodis*, 2 of *M. vandeli* and 2 mixed colonies of both species) were measured (Fig. 7). Their mean body volume was 20.4 ± 11.9 mm^3 (4.5–43.8 mm^3), twice that recorded one month previously ($W = 1373.5$, $p < 0.001$). The mean body weight at that time was estimated at about 17 mg.

In late June a total of 28 individuals (22 larvae and 6 pupae) were found in 12 *Myrmica* colonies (7 of *M. scabrinodis* and 5 of *M. vandeli*). Volumes were variable (25.1–219.7 mm^3) with a mean of 123.8 ± 51.5 mm^3 and a mean estimated weight of 108 mg, i.e., six times greater than that recorded one month previously ($W = 12.0$, $p < 0.001$).

Growth of *M. rebeli* in the field

In late September caterpillars were very difficult to find without destroying nests and only 16 caterpillars from 4 nests (2 of *M. sabuleti* and 2 of *M. scabrinodis*) were measured. The sizes were variable (19.0–99.4 mm^3) with a mean body volume of 53.7 ± 22.8 mm^3 (mean body weight of 47 mg), which was significantly greater than that of *M. alcon* ($W = 144.0$, $p < 0.001$).

In late April the following year 146 caterpillars were found in 29 nests (19 of *M. sabuleti* and 10 of *M. scabrinodis*). Their mean body volume was 62.6 ± 19.6 mm^3 (25.7–124.0 mm^3) with an estimated average weight of 54 mg. They were bigger than the caterpillars recorded in autumn but not significantly so ($t = -1.70$, $p = 0.09$). However their mean body volume was about seven times greater than that of *M. alcon* larvae measured at the same time ($W = 5854.5$, $p < 0.001$).

One month later, i.e. at the end of May, 282 *M. rebeli* prematures were measured (Fig. 7). They originated from 19 nests of *M. sabuleti* and 12 of *M. scabrinodis*. Considerable variation was noticed (5.1–245.6 mm^3) and in addition to larvae three prepupae and two pupae were measured. The mean body volume of all individuals was 89.8 ± 39.5 mm^3 with a mean estimated body weight of 78 mg, which is about 50% greater than the previous measurements at this site ($W = 29986$, $p < 0.001$).

The end of June was the peak flight period of *M. rebeli* in Przemyśl in 2004. Three of about 30 nests searched contained pupae slightly variable in volume (187.0–244.7 mm^3), with a mean value of 210.1 ± 17.8 mm^3.

The trends in the mean sizes of *M. alcon* and *M. rebeli* from September to late June the following year are illustrated in Fig. 8.

DISCUSSION

Survival in the laboratory nests

The laboratory experiments with *M. rebeli* generally confirmed field observations. Caterpillars survived well in nests of *M. sabuleti* and *M. scabrinodis* but quickly died in *M. schencki* colonies (Table 3). However, in the field a higher proportion of *M. sabuleti* nests was infested than of *M. scabrinodis* (Sielezniew et al., 2003; Sielezniew & Stankiewicz, unpubl.). Interestingly the proportion of infested *M. scabrinodis* nests (about one third of all colonies found near gentians) is similar throughout Poland at *M. alcon* and *M. rebeli* sites (Sielezniew & Stankiewicz, unpubl.). *M. sabuleti* inhabits warmer patches of turf with shorter and sparser vegetation, where it might be easier for foraging ants to find caterpillars that have dropped from plants. Other possible explanations include differences in the population structure and behaviour of workers. In spring the large polygynous nests of *M. rubra* tend to divide into smaller colonies (Czechowski, 1984). The frequent occurrence of *M. scabrinodis* colonies close to each other might also suggest colony fission in this species, but detailed studies are needed to test this hypothesis.

Finally it is not possible to exclude that the compounds synthesized by *M. rebeli* caterpillars during their integration into a colony best mimic the odour of *M. sabuleti.* Different *Myrmica* species can be successfully parasitised if they are chemically similar to *M. sabuleti*, and some other unknown conditions are met, e.g. a favourable colony structure. Comparison of our data with ongoing extensive studies of the chemical profiles of *Maculinea* butterflies and *Myrmica* ants (Everett et al., 2005) will give new insights into the host ant specificity of *M. rebeli*. However, both field and laboratory studies indicate it is rather unlikely that *M. rebeli* caterpillars from Polish sites can be reared successfully by *M. schencki,* unlike some Austrian populations, which seem to show multi-host mimicry (Schlick-Steiner et al., 2004).

As far as *M. alcon* is concerned seven different *Myrmica* species, including five that originated from butterfly habitats, were tested. Of the "native" ants, *M. scabrinodis, M. vandeli* and *M. gallienii* proved to be suitable hosts for eight weeks, but the mortality rate was very high in colonies of *M. rubra* and *M. ruginodis* (Table 2). This is consistent with field data, except for *M. gallienii*, which is not recorded as a suitable host, i.e. enabling complete development. In autumn or early spring a few small caterpillars were observed in wild *M. gallienii* nests (Sielezniew & Stankiewicz, unpubl.).

It is commonly known that *Maculinea* butterflies are less host specific in artificial conditions (Elmes et al., 2004). Therefore, although *M. alcon* caterpillars might be initially well integrated into *M. gallienii* colonies, it is likely they are unable to integrate fully, i.e. to achieve a high hierarchical status. Elmes et al. (2004) found that host specificity in *M. rebeli*, in stressful conditions, e.g. starvation, is more clear cut. In preliminary behavioural experiments, *M. alcon* caterpillars removed from walnut shelters were retrieved much slower by workers in *M. gallienii* than in *M. scabrinodis* laboratory colonies (Stankiewicz, unpubl.). Relatively large *M. gallienii* colonies nesting mainly in tussocks of grass or sedges in fen habitats very likely suffer a food shortage in spring when surrounding areas are often flooded. Moreover weakly integrated caterpillars are more prone to diseases, e.g. fungal infections.

The greater availability of *M. alcon* enabled us to include *M. sabuleti* and *M. schencki* in our experiments, ant species native to xerothermic habitats (e.g. *M. rebeli* ones), which are encountered only exceptionally at *M. alcon* sites. Few caterpillars introduced into *M. schencki* colonies survived, as is also the case for *M. rebeli* in colonies of this species. However *M. sabuleti* was a relatively good host for *M. alcon*, although survival was significantly lower than when hosted by *M. scabrinodis*. On the other hand mortality of *M. alcon* larvae hosted by *M. sabuleti* was similar to that recorded in *M. vandeli* colonies. *M. vandeli* and *M. scabrinodis* are about equally suitable as hosts in the field (Sielezniew & Stankiewicz, 2004b).

Because of the shortage of *M. rebeli* stock all possible combinations were not tested, e.g. larvae reared with *M. vandeli.* Nevertheless our data indicate close similarities in the host ant specificity of Polish populations of *M. alcon* and *M. rebeli.* Therefore, host ant specificity cannot be used as an argument for separating these questioned species as in the past (Elmes et al., 1994). However, both groups of populations are distinct from *M. alcon* populations hosted by *M. rubra* and *M. ruginodis* (Elmes et al., 1994; Als et al., 2002) as well as from *M. schencki* dependent *M. rebeli* populations (Thomas et al., 1989; Stankiewicz et al., 2005b).

Growth of caterpillars

Growth in the pre-winter phase is the most important, visible and measurable characteristic distinguishing *M. alcon* and *M. rebeli.* If, in the field, differences can be attributed to different climatic conditions in the habitats of the species, laboratory observations strongly reject this hypothesis.

We tried to collect both *M. alcon* and *M. rebeli* almost simultaneously. Because of the difference in their flight periods we tried to collect the last available *M. rebeli* and the first available *M. alcon* larvae. However, we could not avoid a time-lag and therefore we can not exclude that the larvae of both taxa experienced different abiotic conditions in the field during the phytophagous phase of their development. Whether photoperiod and temperature experienced by larvae during the first three instars, by eggs or even by ovipositing females, influence their growth and diapause remains an open question, which can only be answered by rearing these species through their entire life cycle in the laboratory.

The mean pupal weight of the few individuals that completed their development made it possible to estimate the proportion of biomass achieved in the pre-winter phase. After 8 weeks *M. alcon* larvae had gained less than 5% of their final weight, which is very similar to that recorded for individuals from a Spanish population with the same host ant specificity. However *M. alcon* caterpillars from the Netherlands and Denmark reared by *M. ruginodis* stopped growing at about one fourth of the pupal weight (Schönrogge et al., 2000).

In our experiments the mean weight of *M. rebeli* caterpillars increased in the pre-winter phase to up to 22% of their final biomass. Fast developers and slow developers achieved 44 and 8%, respectively. This is consistent with the results of Elmes et al. (1991b) and Thomas et al. (1998) who studied *M. rebeli* from populations associated mainly with *M. schencki.*

Differences in the growth of caterpillars of *M. alcon* and *M. rebeli* in Poland reflect differences in the phenology of their larval food plants. *G. cruciata* shoots are available for on average about one month earlier than those of *G. pneumonanthe,* which grow in wetter and cooler habitats. Therefore the first period of rapid growth of *M. rebeli* caterpillars in late summer/early autumn is an adaptation that enables adults to synchronize their emergence with the development of the larval host plant. However, *M. alcon* caterpillars mainly increase in body biomass in spring, i.e. in June, in the field (Fig. 8).

The bimodal distribution in the body weight of *M. rebeli* at the end of the pre-winter growth phase (Fig. 2) indicates a growth polymorphism, a phenomenon observed in *M. rebeli* and *M. alcon* in Western Europe. The bigger larvae pupate in one year while the smaller ones complete development in two years (Elmes et al., 1991b; Schönrogge et al., 2000). However, our laboratory observations on *M. rebeli* are not consistent with our field records where cases of growth polymorphism were much rarer than expected from the laboratory study. This is puzzling. The possibility that our non destructive technique of nest searching simply resulted in an underestimate of the number of slow developers, which may have been present in the deepest chambers, cannot be excluded. Another explanation is that a biennial life cycle is not an obligatory feature of some individuals but an adaptation induced by special conditions. In *M. alcon,* it is impossible to distinguish classes of caterpillars after 8 weeks. Differentiation of larval sizes in the field seems to be more the result of starvation in colonies with very few workers (see also Sielezniew & Stankiewicz, 2004b), but the sampling method may have missed the slow developers.

The inhibition of the growth of *M. alcon* caterpillars observed in the third week after introduction persists if the period for which they are exposed to high temperature is prolonged. In *M. rebeli* the lack of a period of low temperature also prevents the development of almost all larvae. Of 24 larvae only one pupated after 24 weeks, others showed asynchronized growth, which sooner or later stopped (Fig. 5). Some larvae started to grow after weeks of quiescence but were unable to pupate. Only a 3-month period of cooling triggered the resumption of development, which indicates an obligatory diapause. There are records of caterpillars growing fast, and pupating within a few weeks of adoption (Tartally, 2004). To check if this occurs in Polish populations, they need to be reared under different conditions i.e. higher temperatures and an abundance of ant larvae.

Differences in diapause and growth of the caterpillars of these taxa are likely to be a mechanism for synchronizing their flight periods with the availability of host plants. This is very important because of the short life expectancy of adult butterflies (see Meyer-Hozak, 2000). This may have consequences for *M. alcon* and *M. rebeli* evolution. Steiner et al. (2006) suggest that their present questionable taxonomic status results from incomplete allopatric speciation, or ongoing ecological speciation. Both scenarios are plausible and not mutually exclusive over the whole species range. Moreover, similar ecological features, such as host ant specificity, could evolve simultaneously in allopatry. As far as we know, *M. alcon* and *M. rebeli* do not occur sympatrically anywhere, although their most typical larval food plants occasionally do in particular biotope complexes (Sielezniew & Stankiewicz, 2004a). It would be interesting to find and investigate populations of both taxa present in close proximity. Differences in the flight periods of *M. alcon* and *M. rebeli* would reduce gene flow significantly, but would not in reproductive isolation in the absence of pre- or post-mating mechanisms. Nowadays the probability of finding a fine mosaic of dry and wet gentian meadows in the western and central European landscape is rather low. Therefore, ecological studies should be continued further east, in the hypothetical region of origin of *Maculinea* butterflies (Fiedler, 1998). In the absence of geographical barriers the existence of polymorphic populations of butterflies of the *M. alcon* group, or biotypes adapted to particular Gentianaceae plants and/or *Myrmica* ants, are expected. The latter feature would suggest ongoing sympatric speciation. Examples of host races in some phytophagous insects support such a theoretical possibility (Berlocher & Feder, 2002; Drès & Mallet, 2002). Assuming a continuation in the present rapid increase in knowledge of *Maculinea* butterflies, then in the near future a comparison of the results of genetic, biochemical and ecological studies is likely to provide answers about the phylogeography of *M. alcon* and *M. rebeli.*

CONCLUSIONS

Differences in the phenology of *M. alcon* and *M. rebeli* result from differences in the pattern of growth of the caterpillars in *Myrmica* nests. Our laboratory studies indicate that these differences are independent of environmental factors, which suggests a genetic basis for this phenomenon. Therefore, even if *M. alcon* and *M. rebeli* are finally judged to be only ecotypes of *M. alcon,* as some authors have already suggested (Als et al., 2004, Bereczki et al., 2005), they are in some important features quite distinct. This knowledge is important for conservation, especially future reintroduction programmes, i.e. individuals from one type of habitat or even region might not be able to adjust to another. Populations of the *M. alcon* species group show a complicated multidimensional pattern of physiological and ecological diversity associated with both larval food plants and host ants, and in the absence of detailed studies every population should be considered as a potentially distinct "conservational unit".

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