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ORIGINAL PAPER

Influence of host plant phenology and oviposition date on the oviposition pattern and offspring performance of the butterfly *Phengaris alcon*

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Abstract The timing of oviposition and selection of the phenological stage of the host plant can have significant consequences for development and success of offspring, and is particularly important for endangered specialist species with rare habitats, such as *Phengaris alcon* butterflies. Females of this species oviposit on marsh gentians, *Gentiana pneumonanthe*. For the first time, we evaluate the survival of eggs deposited by early and late flyers in relation to the phenological stage of marsh gentian flower buds, as well as caterpillar survival and development. An analysis was conducted on 127 gentian shoots, on which 837 eggs were monitored. We observed more frequent oviposition on the apical and youngest buds, with increased egg load by females during the first one-third of the flight period. Offspring survival of about 55 % was observed, with up to 15 caterpillars per bud. Offspring survival was significantly higher from eggs that were oviposited on larger flower buds and on flower buds in an early developmental stage. Also, early flyers' offspring gave rise to better survival rates and the caterpillar development in flower buds differed significantly according to bud size, with more days required in smaller buds. Finally, the significant differences found across the entire study period illustrate that the understanding of oviposition through time is important to the conservation of this rare European species.

Keywords Alcon blue · Oviposition preferences · Survival · Caterpillar development

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Introduction

Many species of Lepidoptera are affected by anthropogenic and climatic events. *Phengaris* (*Maculinea*) species are affected by one or more of such processes primarily due to the complexity of their life cycle (Van Swaay and Warren 1999). These species start their life cycle being phytophagous on a specific host plant and later develop an obligate myrmecophilous relationship with ants of the genus *Myrmica* Latreille, 1804 (Hymenoptera: Formicidae). One of the *Phengaris* species, *P. alcon* deposits its eggs on flower buds of the marsh gentian, *Gentiana pneumonanthe* (WallisDeVries 2004; Maes and Van Dyck 2005). After hatching, the young caterpillars bore into the bud and feed on the soft seeds. When they reach the fourth larval instar and are about 1–2 % of their final weight, they leave the plant by dropping to the soil and wait to be adopted by the ants (Akino et al. 1999). Sometimes they are forced to leave the host plant before the fourth larval stage due to decreasing food quality as flower buds develop into the next phenological stage. Other authors suggest that competition in the phytophagous stage is important for survival and that *Phengaris* larvae experience a high level of density dependent mortality inside the flower bud (Thomas et al. 1991). Larval phytophagous survival is, therefore, dependent on: (1) the synchrony between egg deposition and flower bud phenology (or food quality), and (2) the egg density per flower bud, reflecting the competition for food quantity. Larvae die if they cannot reach the reproductive tissue of the host plant, and if they subsequently fail to reach the fourth instar before leaving the host plant (Elmes et al. 1991). So, oviposition behaviour is an important aspect of butterfly conservation and understanding oviposition cues is of vital importance for the management of endangered butterflies (Bergman 1999).

In our study area in Portugal, *G. pneumonanthe* flowers 2–3 weeks after the start of the alcon blue flight period (unpublished data). This could have consequences for the oviposition site choice of 'early flyers' and the survival of their offspring, since the flower buds might be undeveloped at the time the eggs are deposited. The amount of food resources present in the very early buds might not be enough to satisfy the larval requirements, especially when several larvae compete in the same bud. Thus, the main research questions studied in the present study were: (1) does the date of oviposition, host plant phenology and number of eggs has an influence on the survival of caterpillars? (2) Does the date of oviposition and the size of the flower bud compromises the development of the caterpillars?

Materials and methods

Study area

The study area is located near Lamas de Olo (41°22′N, 7°48′; 974 m) in Alvão National Park, Portugal, and is within a Site of Community Importance (SCI) for the Mediterranean biogeographical region. The vegetation consists mainly of grasses, sedges and heather (*Erica tetralix),* with *Genista falcata* and *Ulex minor* scattered throughout the meadows. This site contains the largest known population of *M. alcon* in Portugal (Rodrigues et al. 2010; Arnaldo et al. 2011). The study area, of about two hectares, is managed by cattle grazing, irregular mowing, and with burning patches by the owners of the pasture.

Field study

The field work was conducted between 5 July and 29 August of 2011, which coincides with the beginning of the flight period of *P. alcon* and the end of the phytophagous phase of the caterpillars in the host plants. A plot of 24 m^2 was created on a representative part of the meadow and protected with a fence to exclude grazing cattle. For 3 days from 5 July, all gentians within the plot were marked with a 30 cm, labelled, stick placed nearby.

Three oviposition periods of 11 days each were identified during the butterfly flight period: period 1 from 8 July to 19 July, period 2 from 20 July to 31 July, and period 3 from 1 August to 11 August, the end of the flight period. Daily, all shoots of the marked gentian plants were checked for eggs. Shoots without eggs were excluded from the subsequent parts of the study. When eggs were detected, the shoot was individually marked with a labelled stick and we recorded: the date of egg deposition; height of the shoot from ground to the tip of the apical bud; total number of flower buds; total number of eggs on the entire shoot; length of the apical flower bud; total number of eggs on the apical bud distinguishing between top, middle and base of the flower bud, calyx, leaf and stem; and phenological stage of the apical flower bud as follows: (1) green/closed flower bud; (2) swollen flower bud and (3) open flower. Immediately after the measurements and record-taking, a small net of fine mesh gauze, sized 15 × 7 cm, was placed over the apical bud. Only the apical flower buds were used to study caterpillar survival (see Fürst and Nash 2010; Van Dyck and Regniers 2010). The net was held tight at the bottom of the stem by a plasticised metal wire, so as to prevent caterpillars from leaving the host plant. The gentian shoots with nets were checked daily for caterpillar emergence, either in the morning or late in the afternoon. When the caterpillars emerged, the data was recorded and their size measured by placing them on a paper with a millimetre scale. Although quite coarse, this way of measuring is adequate for the objectives of the study. The caterpillars were classified into 3 classes: (1) normal fourth instar (>2 mm in length, >1 mm in width), (2) small fourth instar (<2 mm in length, <1 in width) and (3) earlier instar. After measurement, all caterpillars were released under the gentian they were found on. The number of days each caterpillar needed to reach the fourth instar was calculated.

Statistical analyses

Results of oviposition choice were analyzed using the Sign Test (for binomial experiments), considering as null hypothesis the equal choice of apical or not apical flower buds. In cases where shoots had more than one flower bud, the Wilcoxon Signed Rank Test was used to confirm a preference for the apical flower buds considering as null hypothesis, the ratio of oviposition (apical/others) = 1.

For the analysis of the host plant phenology (i.e. number of shoots per plant, height of the shoot, total number of

Table 1 Total number of shoots and mean (±SD) values of characteristics of *Gentiana pneumonanthe* and of *Phengaris alcon* eggs for the studied oviposition periods

Oviposition period	Number shoots	Shoot height (cm)	Number buds/shoot	Apical bud length (cm)	Number eggs/shoot	Number eggs/apical bud
1	39	24.27 ± 6.58	5.41 ± 2.81	1.59 ± 0.81	8.87 ± 8.14	5.00 ± 3.05
2	41	23.74 ± 5.37	6.71 ± 4.05	1.86 ± 0.88	6.00 ± 6.36	3.32 ± 2.86
3	47	23.98 ± 5.88	4.53 ± 2.64	2.60 ± 1.04	5.21 ± 5.30	3.85 ± 2.65
Total	127	23.99 ± 5.90	5.50 ± 3.31	2.05 ± 1.01	6.59 ± 6.75	4.03 ± 2.91

flower buds, length of the apical flower bud, and the developmental stage of the apical flower bud) and oviposition period responses in: (I) oviposition patterns, (II) offspring survival and (III) caterpillar development time, generalized linear models (GLM) were employed (McCullagh and Nelder 1989). Oviposition patterns were analyzed through GLM with a negative binomial distribution of errors and log link function. Offspring survival, calculated as the ratio between the number of caterpillars that were able to reach the final fourth instar and leave the bud, to the number of eggs deposited on the apical flower bud, was also analyzed through GLM but with a binomial distribution of errors and logit link function.

Caterpillar development time was measured as the number of days between the oviposition date and the date the caterpillar left the bud. This way it is possible to link an individual caterpillar to an egg, since all eggs were deposited at the same day. These data were analysed also by GLM with a Gaussian error distribution and the identity link function. Residual analysis and Akaike Information Criterion (AIC) were also calculated as an additional measure of goodness of fit. Because the oviposition period was split in three parts, the comparison of the successive oviposition periods was made against period 1. Finally, the significance of the parameters estimated was tested by a Wald-z statistic. GLM models were run in R 2.14.1 statistical software's package mgcv by gam function. All other statistical analyses were performed in SPSS 20.0.

Results

Oviposition patterns

Within the study plot and during the entire flight period, the alcon blue females deposited 837 eggs on a total of 127 labelled gentian shoots. The shoots chosen for oviposition were on average 23.99 ± 5.90 SD cm above the ground with 5.5 ± 3.31 SD flower buds per shoot. The length of the apical flower buds was on average 2.05 ± 0.09 SD cm. Eggs were mainly laid on the calyx (93 %) or the base of the flower (5.3 %), with the remainder being found on the leaves and the stem. The number of eggs deposited per shoot was on average 6.59 ± 6.75 SD eggs with 1 and 41 eggs of minimum and maximum respectively. From the total of deposited eggs, 512 eggs (mean 4.03 ± 2.91 SD, min 1, max 16 or 61.1 %) were on the apical flower buds. A significant preference for the apical buds as oviposition site ($Z = -6.075$, $p = 0.0001$) was shown within the group of selected plants. On 53 shoots, only the apical bud was used for egg deposition. On the other 74 shoots with eggs, the ratio of eggs on the apical flower buds to mean number of eggs on the other buds was >1, signifying an apical bud preference ($Z = 7.055$, $p < 0.001$).

Concerning the timing of egg deposition, 346 eggs were laid on 39 gentian shoots during the first oviposition period, 246 on 41 shoots during the second period and 245 on 47 shoots in the third oviposition period (Table 1).

The number of deposited eggs significantly increased with the number of flower buds present (Wald-Z = 6.062; $p < 0.001$). Also buds at lower height from the ground were selected (Wald-z = −2.260; $p = 0.024$) and larger flowers were preferentially chosen for oviposition (Wald-z = 2.917; $p = 0.003$). The developmental stage of the bud also explained oviposition differences with significantly more eggs deposited on green/closed flower buds than on other phenological stages (Wald-z = −4.063, $p < 0.001$). A significantly higher number of eggs was deposited by the early flyers (period 1) as compared with period 2 (Wald-z = −4.313, $p < 0.001$) and late flyers (period 3) (Wald-z = −2.128; $p = 0.033$) (Fig. 1; Table 2).

Offspring survival

From the eggs deposited on the apical flower buds, a total of 283 larvae (55.3 %) reached their fourth instar and left the host plant successfully: 195 in the early oviposition period, 136 in the second, and 181 in the last period. No living caterpillar was observed from 12 flower buds with eggs, and the number of caterpillars varied greatly on the flower buds. Ten buds gave rise to >6 caterpillar and two buds had 15 caterpillars each. The average number of larvae per flower bud was 3.12 ± 2.55.

Offspring survival was significantly higher from eggs that were oviposited on bigger flower buds (Wald-

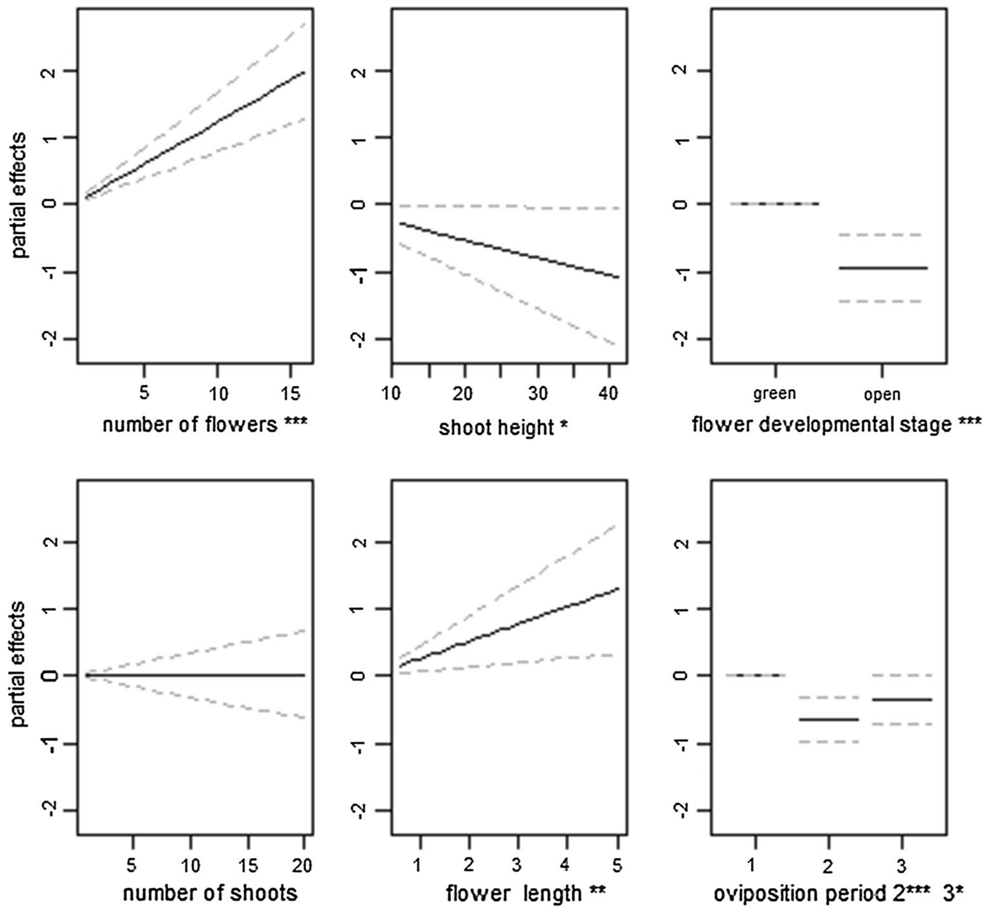

Fig. 1 The number of eggs oviposited in response to predictor variables in the GLM. y-axis = partial effect of each variable on the oviposition pattern (given the effect of other variables). *Solid black lines* response; *dashed lines* 95 % confidence intervals for the mean response. For "oviposition period" the significance levels for period 2 and 3 are for comparison with period 1. The *asterisks* indicate the significance level: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Table 2 Results of the generalized linear models (GLM) testing the effect of plant characteristics and oviposition date on the number of deposited eggs, offspring survival and caterpillar development

Dependent variables	(I) Number for eggs AIC = 682.127			(II) Offspring survival AIC = 465.252			(III) Caterpillar development AIC = 1660.621		
Explanatory variables	Z-value	*p* value	Estimate	Z-value	*p* value	Estimate	Z-value	*p* value	Estimate
Number shoots	0.101	0.919ns	0.001	–	–	–	–	–	–
Shoot height (cm)	−2.260	0.024*	−0.027	−0.938	0.348ns	−0.017	0.477	0.633	0.065
Number flower buds	6.062	0.000***	0.124	–	–	–	–	–	–
Flower bud length	2.917	0.003**	0.261	2.384	0.017*	0.350	−2.718	0.007**	−1.001
Flower developmental stage	−4.063	0.000***	−0.938	−2.824	0.005**	−1.088	−0.651	0.516ns	−0.630
Number of eggs oviposited	–	–	–	0.925	0.355ns	0.025	0.001	0.999ns	0.000
Oviposition period 2	−4.313	0.000***	−0.656	−3.484	0.000***	−0.881	−6.929	0.000***	−4.829
Oviposition period 3	−2.128	0.033*	−0.348	−2.132	0.033*	−0.536	−9.934	0.000***	−7.086

The Z-value as the test statistic, the *p* value and the estimate of the coefficient are given

Z-value is the Wald statistic test; asterisks indicate significant values: $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$, n.s. indicates non-significant values ($p > 0.05$)

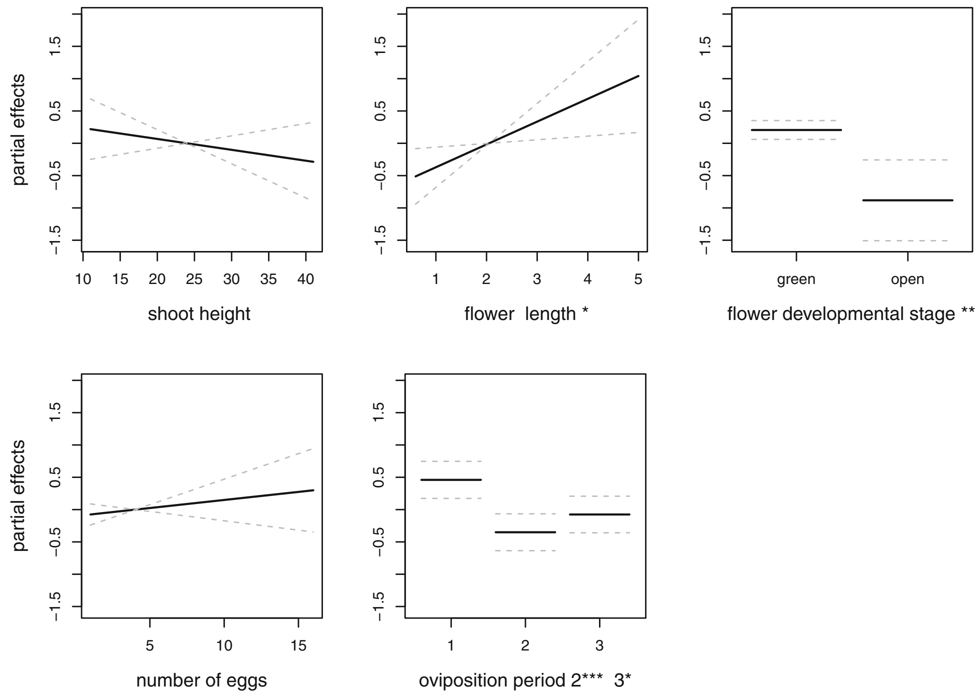

Fig. 2 The response of offspring survival to predictor variables in the GLM. y-axis = partial effect of each variable on offspring survival (given the effect of other variables). *Solid black lines* response; *dashed lines* 95 % confidence intervals for the mean response. The *asterisks* indicate the significance level: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (see further Fig. 1)

z = 2.384, $p = 0.017$) and on green/closed buds (Wald-z = −2.824, $p = 0.005$). The oviposition period used by the females also had an effect on offspring survival, with significantly higher survival of eggs that were deposited by the first flyers (period 1) when compared with period 2 (Wald-z = −3.484, $p < 0.001$) and period 3 (Wald-z = −2.132, $p = 0.033$). No significant effect on offspring survival was found for different shoot heights, numbers of flower buds per shoot or the number of deposited eggs (Fig. 2; Table 2).

Caterpillar development

From the total of the 283 caterpillars that reached the fourth instar and left the host plant, 274 had a normal size for the fourth instars while 9 were smaller. We found no significant differences in caterpillar development across the study period. The average caterpillar size was 3.45 ± 0.03 mm. We found that the size of the flower bud had a highly significant effect on caterpillar development, with many more days needed to reach the final phytophagous stage in smaller flower buds (Wald-z = −2.718; $p = 0.007$). The date of oviposition had also an effect, with a significantly larger decrease in caterpillar development time across the successive oviposition periods (Fig. 3; Table 2).

Discussion

We studied the oviposition site choice of ‘early flyers’ of alcon blue butterflies, *P. alcon*, and the survival of their offspring. The early flyers might have to accept flower buds that are undeveloped at the time they deposit eggs. We tested whether the date of oviposition, host plant phenology and number of eggs had an influence on the survival of caterpillars, and whether the date of oviposition and the size of the flower bud compromised the development of the caterpillars. We found that a significantly higher number of eggs were deposited by butterflies flying early in the season compared with those flying later. Indeed, more eggs were deposited on green/closed flower buds of the host plant than on other phenological stages. The caterpillars from the early flyers had a higher survival and did not show a delay in their development compared to the caterpillars in a later period. Moreover, our results indicate that gentian shoots with more flower buds receive more eggs, which is also found in other studies (Dolek et al. 1998; Nowicki et al. 2005; Van Dyck and Regniers 2010). Also, the apical flower bud was preferentially selected by the females for oviposition. Typically, females did not randomly distribute their eggs but selected an appropriate oviposition site affording protection to the eggs and ensuring appropriate food resources for the offspring (Van Dyck and Regniers 2010). The egg deposition

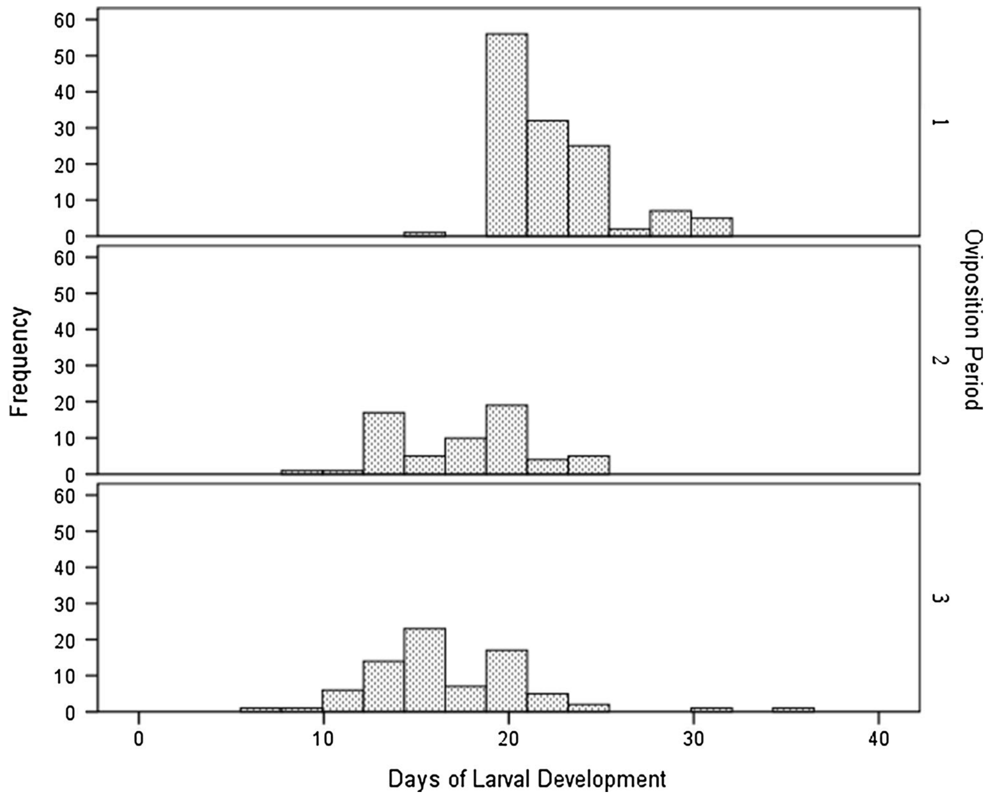

Fig. 3 Larval development time (number of caterpillars emerging from gentian flower buds/day) for each of the three oviposition periods (period 1 from 8 to 19 July, period 2 from 20 to 31 July and period 3 from 1 to 11 August)

pattern we found has also been observed by other authors (Küer and Fatmann 2005; Van Dyck and Regniers 2010). Also, Nowicki et al. (2005) showed that in *G. pneumonanthe*, the apical buds were most attractive to the butterflies.

Visual attraction is an important factor for alcon blues when searching for a suitable host plant (Dolek et al. 1998) and prominent, taller, shoots can be easily found by the butterfly (Küer and Fatmann 2005). At the beginning of the flight period there is no such prominence because the surrounding vegetation is at the same height as the gentian plants. The present study shows differences of egg deposition through time, with significantly more eggs being deposited early in the season. This also happened with other species and according to Boggs (1986) could be explained by the effect of different fecundity of females over time. Under 'good' weather conditions the peak of female butterfly emergence occurs in the beginning of the flight period just after the peak emergence of the male butterflies. It is known that freshly emerged females immediately mate and start laying eggs 1 h after copulation (Van Dyck and Regniers 2010). This strategy has probably developed because of their short life expectancy (Nowicki et al. 2005; Kőrösi et al. 2008). Egg deposition continues throughout the flight period, although the rate (eggs/day) decreases, while the flower buds are developing synchronically.

We found that the phenological stage of the flower bud influences the egg load preference with significantly more eggs being deposited on early stages of the flower bud. At the beginning of the flight period, the majority of the flower buds were at their early phenological stage (i.e. "green buds") leaving the butterflies no choice but to lay their eggs on them. However, when flower buds of all possible phenological stages were available, the butterflies still selected, preferentially, the youngest ones. This may reflect differences in the quality of seeds inside the gentian bud. Probably, the caterpillars are better able to digest the fresh and young developing seeds inside the bud. When flowering starts, the developing seeds inside will mature and dry up, thus becoming unsuitable for caterpillars to feed on. Our results are consistent with the findings of several authors whose results confirm that the youngest available stage of flower bud was selected for oviposition (Figurny and Woyciechowski 1998; Thomas and Elmes 2001; Bonelli et al. 2005; Patricelli et al. 2011).

About half of the eggs (55 %) that were deposited throughout the entire flight period, gave rise to caterpillars that left the gentian bud. The number of caterpillars that leave the flower bud is supposed to be limited by competition from conspecifics and it is suggested that only up to a maximum of 6 caterpillars per bud will survive the phytophagous stage (Elmes and Thomas 1987; Ebert and Rennwald 1991). In the present study, the mean number of eggs per apical bud was 4.03 ± 2.91, which means that for most caterpillars there might have been enough food resources, although the survival rate on the flower buds was only 55 %. Surprisingly, our results showed that in 10 of the flower buds more than 6 caterpillars were produced with a maximum of 15 caterpillars from two buds (a survival rate of 40 %). An explanation for the absence of any effect of the number of eggs on the caterpillar development and survival might be that no further eggs could be deposited on the bud due to our procedure of placing a net around the flower bud after the first day of oviposition. This might explain the relatively low mean number of eggs per bud.

Our results confirm the hypotheses that larger buds should offer more food to caterpillars and so survival should be higher. In addition, survival was higher from eggs deposited on green buds, probably due to the higher quality of the available food. With regard to caterpillar development, and as expected, our results indicate that caterpillars inside the buds needed more time to complete the phytophagous phase in smaller flower buds (fewer days were required for larval development in period 3 when larger flower buds were available).

Concluding remarks

The present research has shown that *P. alcon* oviposition peaks at the beginning of the flight period and the females preferentially oviposit on apical flower buds at the youngest phenological stage. Thus, the initial idea that the oviposition site choice of 'early flyers' and the survival of their offspring could be affected by insufficient food resources was rejected. The nutritional value of the flower buds may be important and explain the greater number of caterpillars surviving in some flower buds in the present study. Also, the size of the host plants and the number of flowers had a decisive impact on the oviposition preferences of *M. alcon*. Larger flower buds offer more food and survival was higher, but we were not able to explain competition inside the flower because we found no effect on the number of laid eggs on survival of the offspring. Finally, the significant differences found across the entire study period, illustrates that the understanding of oviposition over time is important to the conservation of this rare European species. Future global warming might advance the flowering period of *G. pneumonanthe* and might lead to a mismatch between the flight period of the butterflies and one of the caterpillar resources.

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