Females of the specialist butterfly *Euphydryas aurinia* (Lepidoptera: Nymphalinae: Melitaeini) select the greenest leaves of *Lonicera implexa* (Caprifoliaceae) for oviposition

Constantí STEFANESCU[1], Josep PEÑUELAS[2], Jordi SARDANS[2] and Iolanda FILELLA[2]

[1]Butterfly Monitoring Scheme, Museu de Granollers-Ciències Naturals, Francesc Macià 51, E-08400 Granollers, Spain; e-mail: canliro@teleline.es

[2]Ecophysiology Unit CSIC-CEAB-CREAF, CREAF (Center for Ecological Research and Forestry Applications), Edifici C, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain; e-mails: josep.penuelas@uab.es, j.sardans@creaf.uab.es, i.filella@creaf.uab.es

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Abstract. In Mediterranean habitats, the specialist butterfly *Euphydryas aurinia* oviposits on *Lonicera implexa*. Previous work has shown that ovipositing females select and lay a higher number of egg clusters on certain plants. In this paper the results of a field study aimed at assessing whether females use plant size and/or plant or leaf greenness (i.e., chlorophyll concentrations) as cues for oviposition are described. Size of plants did not appear to be an important factor in determining host plant selection, probably because even small plants provide enough resources for the young larvae to reach the diapausing stage and because last instar larvae, the most likely to face resource depletion, can move great distances in search of food. Measurements of both spectral reflectance and chlorophyll concentration of plants failed to reveal differences between host and non-host plants. On the other hand, reflectance and chlorophyll concentration of leaves were found to be important in oviposition choice as egg clusters were generally located on the greenest leaves with the highest chlorophyll contents. This suggests that females use visual cues to select the leaves that will provide optimal growth opportunities for newly hatched larvae. Although there was some indication that plants receiving a greater number of egg clusters also had more leaves of high chlorophyll content, multiple egg batches on single plants could also be a consequence of females being attracted by the presence of conspecific egg clusters.

INTRODUCTION

Euphydryas butterflies (Lepidoptera: Nymphalinae: Melitaeini) have been the subject of much ecological research (Ehrlich & Hanski, 2004). For instance, a great deal of effort has been devoted to the investigation of oviposition behaviour and the understanding of the evolution of diet preferences (see revisions in Singer, 2003, 2004). One of the central themes underlying these issues is the fact that *Euphydryas* species lay eggs in clusters of several to many dozens, a strategy that has important implications for their population biology and the searching behaviour of females. Thus, it has been suggested that the choice of egg-laying sites may be particularly important for batch-layers since females make just one or only a few decisions, which will have great repercussions on offspring survivorship. In line with this argument, the discrimination phase in searching *Euphydryas* and other Melitaeini females is much longer than in most butterflies that lay eggs singly (Singer, 2004; C. Stefanescu, pers. obs.).

This complex searching behaviour often results in interesting patterns in the spatial distribution of eggs on host plants (Singer & Wee, 2005); some of these patterns are unrelated to plant quality per se and are commonly reported in other butterfly taxa. For example, for an individual plant the risk of attack is highly influenced by its spatial location. In particular, isolated plants tend to receive more eggs of *Euphydryas editha* than clumped plants, a phenomenon that can be explained in terms of the mobility patterns of searching females (e.g., Rausher et al., 1981; Singer & Wee, 2005; see also Mackay & Singer, 1982; Root & Kareiva, 1984). Several other physical factors also influence the acceptability of host plants. Thus, Murphy et al. (1984) showed that egg densities in a population of *Euphydryas chalcedona* declined as distance from nectar sources increased, a pattern that is also reported in other non-related butterflies (e.g., Grossmueller & Lederhouse, 1987). In another study, Williams (1981) showed that females of *Euphydryas gillettii* inhabiting a thermally constrained montane habitat choose warmer microsites for egg-laying, resulting in egg clusters being clumped on host plant leaves orientated towards the sun.

As well as these factors, variation in plant quality is also a major cause of non-random patterns in egg laying. Intrinsic properties of plant quality (i.e., its chemical profile) may vary both at the individual and population levels (Singer & Parmesan, 1993; Singer & Lee, 2000), rendering some hosts more acceptable than others to ovipositing females. As recently shown by Nieminen et al. (2003), complex tritrophic interactions may underlie these preferences. These authors found a positive correlation between the concentration of iridoid glycosides in plants of *Plantago lanceolata* and *Veronica spicata* and their

use by ovipositing *Melitaea cinxia* females, as well as a negative correlation between plant iridoid concentration and the incidence of parasitism in the larvae developing on these plants. However, it was not clear from this study whether ovipositing females selected plants with higher concentrations of iridoids or whether it was the act of oviposition itself that elicited the production of iridoids in the plants. A recent study by Peñuelas et al. (2006) seems to favour the latter possibility.

In practice, several of the above-mentioned factors may interact and result in a few plants being highly accepted by ovipositing females and receiving a disproportionally high number of egg clusters. These plants may present a diversity of cues for searching females: once a potential host has been located, one such possible cue is the colour of the leaves, which at least enables ovipositing females of some pierid and papilionid butterflies to choose the young and soft leaves most favourable for the survival of their offspring (Ilse, 1937; Kolb & Scherer, 1982; Scherer & Kolb, 1987; Kelber, 1999).

Clumped distributions of eggs on host plants is common in Mediterranean populations of *Euphydryas aurinia* feeding on *Lonicera implexa* (e.g., Singer et al., 2002). In fact, plants bearing up to five or even more egg clusters are not unusual in these populations. Given that egg clusters of *E. aurinia* usually consist of 200–300 eggs, this means that more than a thousand larvae may develop on a single plant, a situation that can easily result in larval food shortage and competition leading to mass mortality. Under these circumstances, plant size or host density would seem to be further crucial factors that ovipositing females should take into consideration.

In this paper the results of a field study aimed at investigating the possible cues used by females of *E. aurinia* when egg-laying on *L. implexa* are presented. In particular, cues related to plant size and leaf-colour, vital for future resource availability and quality for the hatching larvae, were studied.

MATERIAL AND METHODS

System studied: butterfly, plants and experimental design

Euphydryas aurinia (Rottemburg, 1775) is a univoltine butterfly occurring throughout Europe, temperate Asia and North Africa (Tolman & Lewington, 1997). In most of northern and central Europe it is monophagous on *Succissa pratensis* (Dipsacaceae), while in the southern parts of its range it uses a diversity of hosts, mainly Dipsacaceae (e.g., *S. pratensis, Knautia arvensis, Scabiosa columbaria* and *Cephalaria leucantha*) and Caprifoliaceae (*Lonicera implexa* and *Lonicera etrusca*) families (Mazel, 1986; Kankare et al., 2005). Despite this diversity of hosts, populations are usually monophagous (Singer et al., 2002). For instance, in Catalonia (north-east Spain) most populations in typical Mediterranean habitats (e.g., the population studied in this paper) are monophagous on *Lonicera implexa*.

During the flight period, from mid-April to early June, females lay large egg-batches of about 200–300 eggs on the underside of *L. implexa* leaves. Larvae hatch synchronously within three weeks and spin a large silken web around the leaves, in which they feed for about three weeks. Immediately after their third moult, larvae enter diapause in a winter web (usually at the base of the plant) and do not resume feeding until early next spring. Pupation takes place at the end of the sixth instar, normally by the end of March or beginning of April. This life cycle is very similar to that of most other Melitaeini butterflies (see Kuussaari et al., 2004 for a comprehensive review).

It was known from previous research that a good population of *E. aurinia* occurs in an area known as Olesa de Bonesvalls, in the Garraf Natural Park (Barcelona province), which is dominated by evergreen oak (*Quercus ilex*) forest and holly oak (*Quercus coccifera*) shrubland. There is also a high density of *L. implexa*, an evergreen climber usually with several shoots that grows both in dense scrub and forest or as an isolated plant in hedgerows or on slopes. During the growing season in June 2004, the *L. implexa* plants in this area were searched for *E. aurinia* egg clusters. In that year, the flight period of *E. aurinia* occurred between the second week of May and the second week of June (assessed by weekly counts following the standard methodology of the Catalan Butterfly Monitoring Scheme; see Stefanescu, 2000). On 12 June, that is, right at the end of the flight period, all plants of *L. implexa* along a 1-km transect were carefully monitored. Previous observations indicated that females very rarely oviposit on plants growing in dense clumps in dense scrub and so searching was restricted to those plants growing more or less in isolation (e.g., on the edge of paths). In all, 18 plants were investigated, half of which bore egg clusters.

Plant size, foliar chlorophyll concentrations and spectral reflectance

Data on host and non-host plants were collected in order to study the factors affecting plant selection by ovipositing *E. aurinia* females: plant size (maximum diameter and height) was measured and the maximum volume (assuming a cylindrical form) was estimated. In addition, the chlorophyll concentrations of the leaves bearing egg clusters (17 leaves from 9 different plants; see Results) and of several leaves (6–8) from each of the 18 plants studied were measured with a SPAD chlorophyll meter (Minolta Model 502, Valencia, Spain) (Monje & Bugbee, 1992). For the plants oviposited on, leaves with no egg clusters were selected at random from among those similar to the leaves with egg clusters (i.e., comparable in size and shape). Then measurements were taken of the spectral reflectance of (1) the plant at the whole plant-canopy level (using a Spectron narrow-bandwidth visible/near-infrared spectroradiometer fitted with 15° field-of-view optics; model SE590 with detector model CE390WB-R, Spectron Engineering, Inc., Denver, CO) and (2) of the previously selected leaves (using a field portable spectroradiometer; Unispec, PP Systems, Haverhill, MA). Spectral reflectance was calculated after standardisation using white standard (Labsphere Inc., North Sutton, NH). The Spectron instrument detected 252 approximately evenly spaced spectral bands between 390 and 1100 nm (Full Width Half Maximum around 15 nm). The whole plant-canopy measurements were made by holding the radiometer in a boom and pointing it vertically downwards (nadir) from approximately 1 m above the top of the plants. The area viewed by the sensor was a circle of 0.26 m diameter on the top of the plant canopy. In each measurement four scans were averaged. White standard measurements were conducted immediately before or after the canopy spectral measurement. The measurements were made around solar midday and in cloudless conditions. The Unispec instrument used at leaf level (measurements were taken by attaching a fibre optic to the leaf) has a nominal spectral range from 350 to 1100 nm with an approximately 3 nm nominal bandwidth (10 nm full width, half maximum). Thus, for each measurement, the spectrometer programme automatically collected 256 data points covering the entire spectral range. A linear interpolation routine
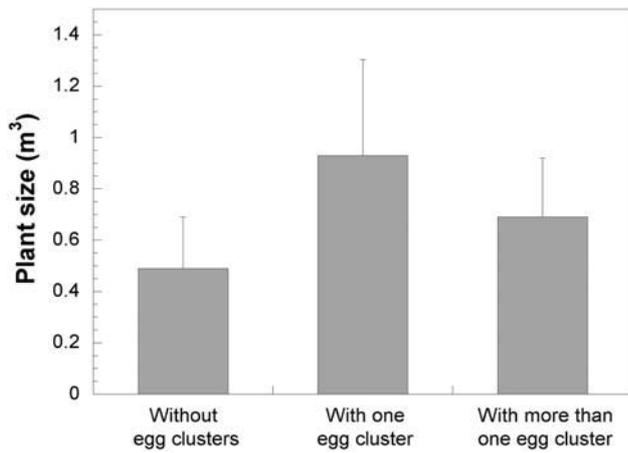
Fig. 1. Size (estimated maximum volume) (mean ± SE) of *Lonicera implexa* plants with no egg clusters (n = 9), one egg cluster (n = 7) and several egg clusters (n = 2). There were no statistically significant differences between the three groups.

was used to estimate values at 1-nm intervals prior to the calculation of indices.

Several reflectance indices, computed from the reflectance data obtained with the spectroradiometer, were taken as indicators of green biomass (NDVI, normalised difference vegetation index), photochemical performance (PRI, photochemical reflectance index), carotenoids/chlorophyll ratio (SRPI, simple ratio reflectance index, and SIPI, structural independent pigment index), water content (WI, water index) and chlorophyll concentrations (ND705, normalised difference chlorophyll index), amongst others (see Peñuelas & Filella, 1998 for a list of all such indices and their significance). Here, only the equation for the chlorophyll index ND705 (Sims & Gamon, 2002), based on the chlorophyll index developed by Gitelson & Merzlyak (1994), is presented owing to the fact that this is the only index that differed significantly for leaves that were oviposited and no-oviposited on (Rx denotes the reflectance at x nm): ND705 (normalised difference chlorophyll index) = (R750 − R705)/ (R750 + R705).

Statistical analyses

We used the non-parametric Mann-Whitney U-test to compare the sizes of groups of plants with different numbers of egg clusters (non-normal distribution of the variable). Independent and paired t-tests, as well as one-way ANOVA and post hoc tests (Statistica, StatSoft Inc., Tulsa, USA), were used to compare the reflectance and chlorophyll concentrations of plants with and without egg clusters, and to compare the reflectance and chlorophyll contents of leaves with egg clusters and those without egg clusters for those plants bearing eggs. For plants with multiple egg clusters the mean of the values for each leaf bearing eggs as a single reflectance or chlorophyll concentration was used.

RESULTS

Of the 18 plants studied, two had multiple egg clusters (one on four leaves, the other on six), while seven plants only had one leaf with an egg cluster. Nine plants had no egg clusters. Given that searching was conducted at the very end of the butterfly's flight period, it is highly unlikely that any further egg clusters would have been laid on the monitored plants.
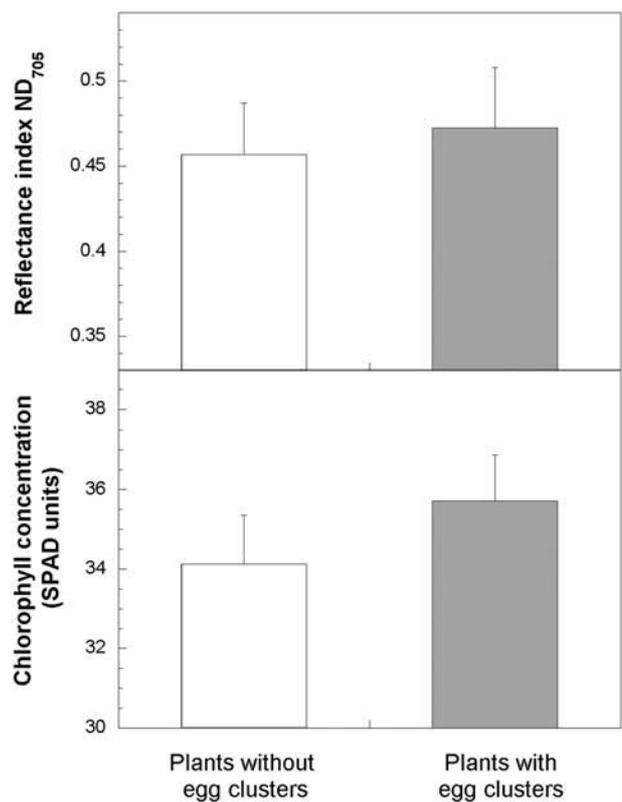

Fig. 2. Whole plant reflectance index ND705 (mean ± SE; n = 9) and plant mean foliar chlorophyll concentrations (SPAD units) (means ± SE; n = 6–8 foliar measurements for each plant) of plants of *Lonicera implexa* with no egg clusters and egg clusters. There were no statistically significant differences in either comparison.

No significant differences in plant size were found between plants receiving and not receiving egg clusters (Mann-Whitney U-test: $Z = 0.44$, $P = 0.66$), or between the two plants with several and those with only one egg cluster (U-Mann Whitney test: $Z = 0.44$, $P = 0.77$) (Fig. 1).

Visible and near infrared reflectance of whole plants and chlorophyll concentrations in the leaves that were selected randomly did not differ between plants bearing and not bearing egg clusters (Fig. 2). No significant differences were found either for these variables between the two plants with several and those with only one egg cluster. However, there was a statistically non-significant trend for greener plants to have more egg clusters (37.98 ± 2.46 SPAD units (means ± SE) for the two plants with more than one egg cluster, 35.08 ± 1.32 for the seven plants with one egg cluster and 34.12 ± 1.23 for the nine plants with no egg-clusters).

Significant differences in reflectance and in chlorophyll concentrations were, however, found at the leaf level. The reflectance index for chlorophyll, ND705, and foliar chlorophyll concentrations were 10% greater (two-tailed paired-sample t test: $t = 2.88$ and $t = 4.67$, respectively, both $P < 0.05$) for leaves with egg clusters than for similar leaves of the same plants without egg clusters (Fig. 3).
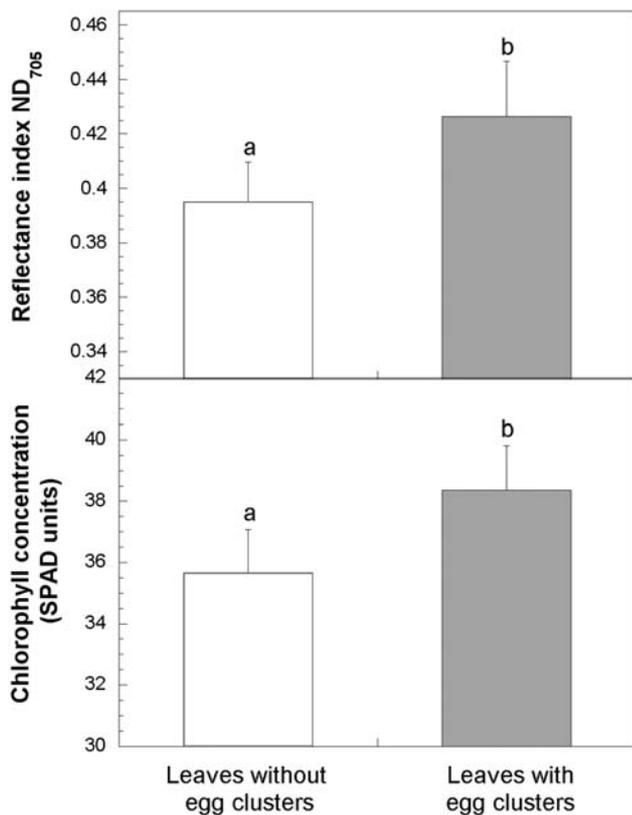
Fig. 3. Leaf reflectance index ND705 and leaf chlorophyll concentrations (SPAD units) of leaves of *Lonicera implexa* plants that had and lacked egg clusters. The values are means ± SE (n = 9 single leaves or means of leaves bearing eggs of the two plants with multiple egg clusters; n = 9 means of 6–8 leaves without egg clusters). The letters *a* and *b* above the bars indicate statistically significant differences (Paired t-test, *P* < 0.05) between leaves of the same plants with and without eggs.

DISCUSSION

That several *E. aurinia* egg clusters can be laid on a single plant of *L. implexa* (e.g. Singer et al., 2002) was confirmed once again by our data. Although the distribution of egg clusters on plants did not differ from random (Poisson distribution: $\chi^2 = 4.14$, $\nu = 2$, $P = 0.25$), it should be noted that the sample size was small and that the two plants bearing a high number of egg clusters were pooled into a single category in the goodness of fit testing (the expected frequency of a plant with six egg clusters was very low: 0.007). In fact, the finding of just two plants hosting more than half the egg clusters seems to confirm the suggestion of Singer et al. (2002) that, in practice, most plants in a *L. implexa* population are probably non-hosts for the co-occurring population of *E. aurinia*.

The characteristics of plants bearing egg clusters as compared to those without hint at some of the cues that may be used by ovipositing females. Although these results must be treated with caution, they suggest that the size of the plants in the range found in the area studied (30–180 cm high and 50–300 cm wide) is not an important factor in determining host plant selection by *E. aurinia*. This finding agrees with previous observations (J.

Planas & C. Stefanescu, pers. obs.), although it does seem to contradict the expectation that egg-clustering species should prefer bigger plants to reduce the risk of resource depletion (e.g. Le Masurier, 1994). However, in the case of *E. aurinia*, most plant consumption occurs 8–10 months after eggs are laid since larvae enter diapause soon after hatching and do not resume feeding until early the following spring. Therefore, even small plants provide enough resources for the young larvae to reach the diapausing stage. Moreover, last instar larvae, that is, the stage most likely to face resource depletion the following season, can move great distances in search of food (maximum distance recorded: 30 m; C. Stefanescu, pers. obs.), thus minimizing the risk of starvation in areas where host plant density is high (cf. Kuussaari et al., 2004).

Neither measurements of spectral reflectance nor of chlorophyll concentration at plant level distinguished between host and non-host plants in the *L. implexa* population. On the other hand, reflectance and chlorophyll concentration at the leaf level appeared to be important factors in determining the presence or absence of egg clusters, which suggests that colour is an important short distance cue for oviposition. For instance, on a particular plant egg clusters were located on the greenest and apparently healthiest leaves (those with highest chlorophyll contents), a result that coincides with those of studies on other butterflies (Ilse, 1937; Kolb & Scherer, 1982; Scherer & Kolb, 1987; Kelber, 1999). As shown in many other studies, chlorophyll content correlates positively with nitrogen availability and nutritional value (Peñuelas & Filella, 1998). Therefore, colour may permit females to select leaves that are optimal for the growth of newly hatched larvae, as indeed was found experimentally by Kelber (1999) in the case of *Papilio aegeus*.

The mechanism by which the greenest leaves are selected by females cannot be deduced from our data. Once a searching female has located and landed on a potential host plant, she assesses the quality of that plant by analysing a combination of visual, chemical and physical stimuli (cf. Renwick & Chew, 1994; Singer, 2004). It is possible, however, that the greenest leaves are not selected on the basis of their colour, but instead are chosen more indirectly in response to other correlated stimuli.

The question as to why some plants receive a disproportionately high number of egg clusters has still to be resolved. A simple explanation is that these plants have more leaves with a high chlorophyll content than plants receiving just one egg cluster. This reasoning seems to fit the finding of a slight trend for greener plants to have more egg clusters. However, there are other possible explanations. For example, ovipositing butterflies may be attracted by conspecific eggs, leading to many egg batches on a single plant. This behaviour was confirmed experimentally, at least in the case of Finnish populations of *Melitaea cinxia* (M.C. Singer, pers. com.), and may have evolved because of the benefits associated with increased larval group size. In many cluster-laying spe-

cies (including several Melitaeini butterflies) group size correlates positively with larval survival and larval growth, especially during the first larval instars (e.g., Clark & Faeth, 1997; Denno & Benrey, 1997; and see Kuussaari et al., 2004 for a recent review).

In conclusion, our study provides further insights into the understanding of oviposition behaviour in Melitaeini butterflies and suggests that the colour of the leaves of their host plant probably acts over short distances as a cue in the selection of suitable oviposition substrates. However, further work is needed not only to ascertain if other correlated traits are actually the main oviposition stimuli, but also to discover what the ultimate reasons are for selecting the greenest leaves for egg laying.

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