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LETTER

When random sampling does not work: standard design falsely indicates maladaptive host preferences in a butterfly

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Abstract

In experiments that investigate species' interactions, individuals are often chosen at random to represent their populations. However, this practice can generate misleading results when individuals of different species do not interact at random. We illustrate this effect by examining oviposition preferences of *Euphydryas aurinia* butterflies from three populations using three different plant genera. We first offered each insect a randomly chosen member of its own host population and a foreign host (*Succisa pratensis*) not present in the insect's habitat. The butterflies uniformly preferred the foreign *Succisa* over their own hosts. Preferences were apparently maladaptive because insects wasted time searching for a nonexistent plant. We repeated the experiment using individual hosts that had naturally received eggs in the field. The overall preference for *Succisa* and the appearance of maladaptation both disappeared. In the original experiments, our random choice of experimental host individuals had combined with strong within-species discrimination by the butterflies and with overlap of acceptability between host species to obscure the true nature of host preference.

Keywords

Parasite–host interactions, insect–plant interactions, acceptability, preference, *Euphydryas*, random sampling.

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INTRODUCTION

Host preferences of herbivorous insects influence life history (Courtney *et al.* 1989), gene flow and speciation (Feder *et al.* 1998; Via 1999; Wood *et al.* 1999), as well as insect-host relationships (Wiklund 1975; Jaenike 1986; Thompson 1994, 1998; Menken 1996; Janz & Nylin 1997; Leebens-Mack *et al.* 1998). Therefore, understanding how preference evolves is important (Singer 1983). In this sense, it is interesting that apparently maladaptive oviposition preferences have been frequently reported. These usually involve adult insects preferring to lay eggs on host species other than those that confer the highest offspring fitness. These cases have been attributed to gene flow from sites where different preferences are favoured by natural selection (Singer & Thomas 1996), to evolutionary lag (Chew 1977; Karowe 1990; Feldman & Haber 1998), or to constraints on the evolution of the preference hierarchy (Thompson 1993; Wehling & Thompson 1997).

We wished to study a case of maladaptive oviposition preference and chose to expand on the work of Mazel (1986), who studied *Euphydryas aurinia* butterflies (Nymphalidae) from populations using several different hosts. He exposed butterflies to an array of hosts and found that eggs accumulated on *Succisa pratensis*, suggesting that this host was generally preferred whether it was locally present or not. In the genus *Euphydryas*, preference for a host that cannot be found is maladaptive because it delays oviposition and reduces fecundity (Singer *et al.* 1992). Between 1998 and 2000, we undertook a series of field and greenhouse experiments in order to investigate preferences in *E. aurinia*. Our present thesis is an unexpected outcome of this study.

MATERIALS AND METHODS

Hosts of *Euphydryas aurinia* across its range are: *Succisa, Knautia*, *Scabiosa*, *Cephalaria* (Dipsacaceae), *Lonicera* (Caprifoliaceae) and *Gentiana* (Gentianaceae) (Mazel 1986; Warren 1994; Warren *et al.* 1994; Tolman & Lewington 1997). All these hosts are perennial. *Lonicera* are shrubs, the others are herbaceous. Butterflies fly in spring, and eggs are laid in large clusters in April–May at sea level and June–July at high elevation. Most populations are monophagous and all are univoltine. Larvae feed gregariously in silk webs during the summer and diapause half-grown within a strong winter nest. We selected four populations of *E. aurinia*, located in an area 200 × 200 km in southern France and north-eastern Spain (Catalonia). Three of these populations were monophagous; the fourth, Pic St. Loup, used two hosts (*Cephalaria* and *Scabiosa*). The names of the sites and the principal hosts used there are given in Table 1, which also shows the approximate distance of each site from the nearest known population of *E. aurinia* using another host.

Oviposition preferences in this set of populations were investigated in three different experiments.

Experiment 1: Comparison of Succisa *with two locally used host species using randomly chosen hosts*

In spring 1998, we gathered butterflies and their hosts at Mas Calç (host: *Lonicera*) and Pic St. Loup (principal host: *Cephalaria*). We tested the preference of each butterfly for two plants—a member of its own host population and a *Succisa* gathered from the population used by the butterflies at Coustouges. We used 10–12 individual plants chosen haphazardly from each host population and from the *Succisa* population. By 'haphazard' we mean that we strove to choose plants at random but did not do so in the strictest sense, which would involve numbering every plant in the population and using a table of random numbers to select test plants. Each insect was tested with just one plant pair comprising an individual plant of each species. Different butterflies were tested with different plant pairs.

The preference-testing technique takes advantage of the phenomenon that each insect accepts a wider range of plants as time passes and that the rate at which this expansion of host range occurs can be measured, provided that butterflies are not allowed to oviposit. During each preference trial, the butterfly was manipulated to encounter each host in alternation, starting when neither host was accepted and continuing till both hosts were consistently accepted. Each encounter lasted a maximum of 3 min. Acceptance of a host was recorded if the insect attempted to oviposit by curling its abdomen, extruding its ovipositor, and pressing the ovipositor against the underside of a leaf for 3 sec. Rejection was simply lack of definitive acceptance. For each insect we estimated the strength of preference as the minimum length of the discrimination phase, that is, the time period during which the most-preferred host was consistently accepted and the second-ranked host consistently rejected. This estimate is the length of time between the first recorded acceptance of the preferred host and the last recorded rejection of the second-ranked host. This technique of preference-testing involves a series of assumptions. For example, we assume that the insects do not learn during the tests, and that the response to a plant is unaffected by prior encounters with the same or with different plants. We also assume here that the results of these tests represent the behaviour that the insects would show if they were at liberty. We have tested this in the past in a related butterfly, *Euphydryas editha*. We followed *E. editha* females until they accepted hosts. We captured them and replaced them on individuals of their host population that they had naturally accepted and rejected. We also captured insects in the act of ovipositing on different hosts and tested their preferences. Results of these and other tests of assumptions involved in manipulative preference-testing are described and referenced by Singer *et al.* (1992). They support the hypotheses that the tests have meaning in the field and that the preferences are strongly heritable in *E. editha*.

Experiment 2: Comparison of Succisa *with locally used host species using randomly chosen hosts grown in a common greenhouse environment*

The second experiment was performed in 1998–99 in response to the results of the first, in which insects from Pic St. Loup and Mas Calç had strongly preferred *Succisa* over their own hosts (see Results). When gathering the plants in the field we had been able to transplant *Succisa* plants with their roots intact but we had been forced to use freshly gathered cuttings of *Cephalaria* and *Lonicera*. The *Succisa* may therefore have been presented to the insects in better condition than the *Lonicera* or *Cephalaria*. Accordingly we grew from seed, in Austin, Texas, a set of plants from each of the four host populations to put them on a more equal footing. We did not use plants that were sibs, so that our greenhouse populations would represent the natural diversity of samples from the field. In this second set of trials we

Table 1 Studied populations of *Euphydryas aurinia*, host associations and approximate distances to the nearest population using another host (in parentheses). Mas Calç is in Spain; the other sites are in France.

Population	Host plant	Distance	Latitude	Longitude
Pic St Loup	*Cephalaria leucantha* (Dipsacaceae)	150 km (*Succisa*)	43°46′	3°45′
Mas Calç	*Lonicera implexa* (Caprifoliaceae)	35 km (*Succisa*)	41°56′	3°04′
Coustouges	*Succisa pratensis* (Dipsacaceae)	25 km (*Lonicera*)	42°23′	2°34′
Etang de Dougnes	*Gentiana alpina* (Gentianaceae)	4 km (*Succisa*)	42°42′	2°23′

included plants and insects from all four study sites listed in Table 1: the two sites used in experiment 1 plus the *Gentiana*-feeding and *Succisa*-feeding sites. Insects were raised from egg clusters obtained in the field. We considered each cluster to be an independent sample from its population. Therefore we combined the data from each set of sibs to a single data point which was the arithmetic mean of the separate discrimination phases.

Experiment 3: Comparison of Succisa *with host individuals actually chosen in the field*

This was performed as a check on the results of the first two experiments, in both of which strong preference for *Succisa* had been detected. We carried to Mas Calç and Pic St. Loup the two most acceptable *Succisa* plants grown in Texas. At each site we identified an acceptable local host from the presence of eggs. In each case we found a plant that bore three natural egg clusters. We had difficulty in deciding whether to remove the eggs from the plants. A close relative of *E. aurinia, Melitaea cinxia,* is known to be attracted to conspecific eggs (M.C. Singer & L. Ramakrishnan, unpublished data), so it was possible that the presence of eggs would increase the acceptability of the host. Host acceptability could also be either increased or reduced by the damage to the plant involved in egg removal. We decided that the likelihood of strong effects was least if we removed the eggs with minimal damage to the plant: we sliced through the petioles of the egg-bearing leaves with a sharp knife. This removed less than 1% of the plant material and left a very small wound. We then covered the naturally accepted host individual with a net cage into which butterflies could be introduced. Freshly captured butterflies were offered staged encounters with the acceptable local host and the imported *Succisa*, in alternation. Preferences were recorded as before.

RESULTS

Experiment 1: Comparison of Succisa *with locally used host species using randomly chosen hosts in the field*

Butterflies from Mas Calç and Pic St. Loup strongly preferred *Succisa* from Coustouges over their own hosts, *Lonicera* and *Cephalaria* (Fig. 1, top).

Experiment 2: Comparison of Succisa *with locally used host species using randomly chosen hosts grown in a common greenhouse environment*

General preference for *Succisa* by insects from Pic St. Loup and Mas Calç was repeated, although one family from Mas Calç showed no preference (Fig. 1, centre). The figure does not show results from the other two populations. Four families from the *Gentiana*-feeding population at Etang de Dougnes all showed mean preferences for *Succisa* over *Gentiana* of > 1 day. Butterflies from the *Succisa*-feeding population at Coustouges (four families) preferred *Succisa* over all other hosts, with discrimination phases > 2 days in all cases.

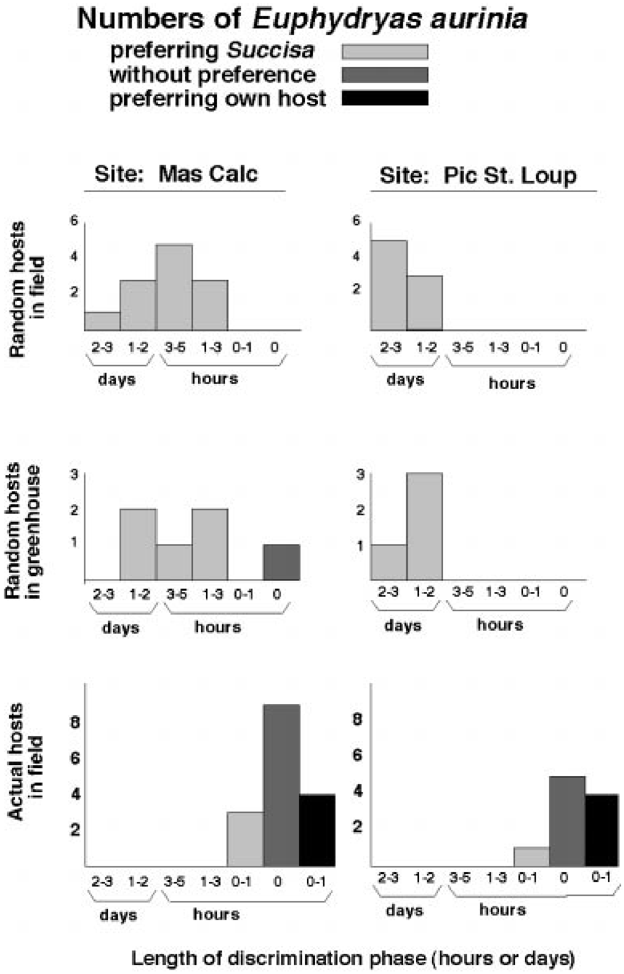

Figure 1 Preferences of *Euphydryas aurinia* for own host vs. *Succisa pratensis*. Only results from two sites, Pic St. Loup and Mas Calç, are shown here. Graphs in the same column represent the same study site. Graphs in the same row represent the same experimental design. The top row is experiment 1, with each insect tested on different plants haphazardly (quasi-randomly) sampled from the plant populations. The second row is experiment 2, done in Texas, with each data point representing the mean preference of a set of sibling butterflies. The third row is experiment 3, done in the field, with each data point the preference of a single butterfly independently sampled from its population, all butterflies tested on the same plants and the hosts chosen from the presence of natural eggs.

Experiment 3: Comparison of Succisa *with host individuals actually chosen in the field*

Strong preferences for *Succisa* were absent. Many insects failed to discriminate between the test plants; those that did

Table 2 Comparison between experiments 1–3 of preferences at Mas Calç and Pic St. Loup.

	Statistic	d.f.	Significance
Mas Calç			
Overall significance	$H = 23.55$	2	***
Expt 1 vs. expt 2	$U = 48.5$	12, 6	NS
Expt 3 vs. expt 1	$U = 270$	12, 16	***
Expt 3 vs. expt 2	$U = 109.5$	6, 16	**
Pic St. Loup			
Overall significance	$H = 16.93$	2	***
Expt 1 vs. expt 2	$U = 20$	8, 4	NS
Expt 3 vs. expt 1	$U = 116$	8, 10	***
Expt 3 vs. expt 2	$U = 50$	4, 10	**

H: Kruskal–Wallis test; U: Mann–Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

discriminate showed weak preferences, and were no more likely to prefer *Succisa* than their own host species (Fig. 1, bottom).

At each site preferences were significantly heterogeneous among experiments (Kruskal–Wallis test, Table 2). Therefore it is legitimate to perform statistical comparisons between the separate experiments. Preferences from the same site did not differ significantly between experiments 1 and 2 but did differ significantly between experiment 3 and both the earlier experiments, considered separately (Table 2). Degrees of freedom are derived from the numbers of butterflies in experiments 1 and 3 and from the number of families in experiment 2.

DISCUSSION

The spatial patterns of association between butterflies and their host plants result principally from an interplay between oviposition preference (a trait of the insects) and acceptability (a trait of the plants). These traits vary among individuals and among populations. They can be difficult to separate both conceptually and experimentally (Singer & Parmesan 1993; Singer 2000; Hanski & Singer 2001). Here, we provide an example of an experimental design that allows us to investigate aspects of the plant–insect interaction while allowing for the possibility that both partners are variable.

Acceptability for oviposition and suitability for insect development vary among conspecific plants (Courtney 1981; Karban 1989; Singer & Parmesan 1993; Janz & Nylin 1997; Mopper & Strauss 1998; Strauss & Karban 1998). The present work illustrates the importance of this variation to experimental design. In our first two experiments, *E. aurinia* butterflies using *Cephalaria*, *Lonicera* and *Gentiana* all showed strong preference for *Succisa* over their own hosts. A *Succisa*-preferring insect with a 5-h discrimination phase would spend 5 h searching for a nonexistent host before it would accept one that it can actually find. This implies maladaptation, assuming that there are costs to prolonged host search. It is expected that locally maladaptive preferences will occur in nature, for all of the reasons suggested in the literature. However, in the present case the appearance of maladaptation is misleading, stemming from the random manner in which we selected test plants. Local host populations are diverse in acceptability to the butterflies and *Succisa* is not generally preferred over the most acceptable hosts, those that actually receive eggs in the field.

It is conceivable that testing of many *Succisa* individuals would reveal some so highly acceptable that the original result of general preference for *Succisa* would be restored; we did not test this possibility. However, the two *Succisa* plants used in experiment 3 were the most acceptable in a set of 15 that we had grown and tested in Texas with insects from several populations.

The acceptability of our test *Succisa* plants could have been reduced by their travel by air from Texas to Britain and subsequently by road to the study sites. To investigate this possibility we tested these same *Succisa* at Pic St. Loup against 30 undisturbed *Cephalaria* plants that did not bear naturally laid *E. aurinia* eggs. We also included in our tests two of the Pic St. Loup *Cephalaria* that we had grown from seed in Texas. The two *Succisa* brought from Texas were both strongly preferred over the two *Cephalaria* brought from Texas, with a discrimination phase of more than a day. This resembles the results obtained in Texas before the plants had been moved. The 30 wild *Cephalaria* were split into groups of 10, and each group was offered to a different butterfly. One butterfly that had accepted both the test *Succisa* and the test (acceptable) *Cephalaria* rejected all 10 wild *Cephalaria*. We tested two other butterflies that were very highly motivated, having accepted both test plants more than a day earlier. These two insects rejected eight and nine out of the 10 *Cephalaria* that they were each offered. So, our test plants, the two *Succisa* brought from Texas and the acceptable *Cephalaria*, were clearly preferred over 27 out of 30 wild *Cephalaria* plants. In 17 out of the 20 cases where we used highly motivated butterflies the discrimination phases were more than a day.

The naturally accepted *Cephalaria*, tested while still growing undisturbed in the field, was strongly preferred over 90% of undisturbed *Cephalaria* in a haphazard sample at the same site, Pic St. Loup. This suggests that the majority of this *Cephalaria* population is extremely unlikely to receive eggs of *E. aurinia*. Although variation of acceptability among individual *Cephalaria* is continuous rather than discrete, it still seems clear that most plants are, from the butterflies' perspective, not part of their host population. So, a truly maladaptive preference for a locally nonexistent host such as *Succisa* would comprise preference for that host over those *Cephalaria* that are likely to be used by the insects. We consider that experiment 3, in which the host populations are represented by naturally accepted individual plants, is the best test of the hypothesis that insects prefer plants that they never encounter. We therefore reject the apparent conclusion from experiments 1 and 2, that *E. aurinia* that do not feed on *Succisa* show maladaptive preferences for this plant.

The results of this study demonstrate that there are serious consequences in assuming that attractiveness to herbivores is a species-specific trait for potential plant hosts, when this and other studies have demonstrated that host acceptability to herbivores is a phenotype-specific trait. The choice of individuals to represent their species or population can have unexpected effects on the results of experiments that investigate species' interactions. In this case, the high variability of resistance to attack among individual plants in a host population rendered most plants effectively non-hosts for the insect. As a result, a random sample from the 'host' population would in practice consist mostly of non-hosts. In comparisons using such a random sample and other host species or populations, the actual host population would appear very resistant and the insects could appear to have maladaptive preferences. One of these conclusions would be correct, the populations of *Cephalaria* and *Lonicera* were in some sense very resistant to attack. The second conclusion, of maladaptive preference, would be wrong. In the study of species' interactions, the method of choosing individuals to test is crucial to the specific question being asked, and random choice is not always best.

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