EXPERIMENTAL DESIGN AND THE OUTCOME OF PREFERENCE-PERFORMANCE ASSAYS, WITH EXAMPLES FROM MITOURA BUTTERFLIES (LYCAENIDAE)

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ABSTRACT. Investigations into adult host preference and the performance of larvae on different host plants have played a central role in ecological and evolutionary plant-insect research. Here I present two sets of experiments that address aspects of the experimental design of preference-performance assays, using a well-studied system of lycaenid butterflies. First, I compare results from sequential, no-choice oviposition assays to previous results reported from simultaneous choice tests with Mitoura nelsoni. Second, I describe an experiment in which the larvae of two closely related species (M. nelsoni and Mitoura muiri) were reared in parallel on plants in the laboratory and in the field to assess the potential influence of environmental conditions on performance. Results from the no-choice preference assays are consistent with previous results, suggesting that, at least in this system, the two types of experimental design lead to similar conclusions. The experiment rearing larvae in the field and in the laboratory revealed a significant effect of environment on pupal weights, but did not detect a species by environment interaction. Thus for pupal weights, a laboratory-based study is sufficient to compare performance between M. nelsoni and M. muiri. However, a species by environment interaction was observed for development time, which has implications for host-associated speciation in this group that would not have been detected in a solely laboratory-based study.

Additional key words: Callophrys, specialization, choice test, no-choice test.

Preference-performance assays are used to address a range of questions in the ecology and evolutionary biology of herbivorous insects (Dethier 1954; Thompson 1985; Jaenike 1990; Wackers 2007; Craig & Itami 2008). Preference refers to the choices made by ovipositing females or feeding individuals for different host plant species (Singer 2000), and performance refers to the development of juvenile stages on specific hosts. The questions addressed by preference-performance experiments may be as simple as: will a species of insect accept a particular species of plant as a host, and is the same plant a suitable host for larval development? Questions may also involve genetic variation and correlations among genetic elements; in particular, is preference for a particular plant species genetically correlated with the ability of larvae to utilize the same host species (Via 1986; Thompson 1985; Mayhew 1997). Experiments involving preference and performance are also central to the practice of biocontrol, in which behavioral and physiological host range must be determined before the release of a control agent (Marohasy 1995). These and related topics have been reviewed by many authors, including Jaenike (1990), Thompson & Pellmyr (1991), Craig & Itami (2008), and Berenbaum & Feeny (2008). The goal of this paper is to address two methodological and experimental issues involved in preference-performance assays: choice versus no-choice preference tests, and the influence of laboratory versus field conditions on performance experiments.

Two of the more common ways in which preference assays can be constructed include choice and no-choice tests (for a review of other experimental designs and related issues not discussed here, see Courtney et al. (1989), Singer & Lee (2000), Barton Browne & Withers (2002), Singer et al. (2002) Van Driesche & Murray (2004), and Mercader & Scriber (2007)). In choice tests, host plants are presented to an adult female or group of females in an array and the response is typically the number of eggs laid on the different plants in a set amount of time. In a no-choice assay, the behavioral response (oviposition) is scored with plants in isolation, often sequentially, with plants being presented one after the other to adults. Simultaneous choice tests have been criticized as being unrealistic, as different host plant species may not be in immediate physical proximity in the wild (Singer et al. 1992). On the other hand, an argument can be made that simultaneous choice tests are conservative: the juxtaposition of plants in an experimental arena could make it more difficult for an ovipositing female to make a choice (since information gathered from volatile plant cues may be overlapping or mixed).

In any event, the two types of test, choice and no-choice, potentially provide different and complementary information (Withers & Mansfield 2005). Consider a simple, hypothetical scenario: two host plants (A and B) are used by a particular insect herbivore. In choice tests, plant A is overwhelmingly preferred to the exclusion of plant B, but in no-choice tests both plants receive a comparable number of eggs from ovipositing females. It might be the case that the volatile and tactile cues that characterize plant A are sufficiently more stimulating to ovipositing females such that B is ignored in the presence of A. While in the absence of A, B is recognized as a suitable host and will
be utilized. The choice test tells us not only what could happen in the wild when plants are interdigitated or in very close proximity, but it tells us something about the inherent ranking of host cues by the herbivore (e.g. Thompson 1993). The no-choice test on the other hand might give a clearer picture of what could happen in the wild as a female moves from one isolated patch of plants to another. Choice tests are more common in the literature, perhaps because they are logistically more efficient. What is not often tested (and which I address here with one butterfly species) is how often the results from choice and no-choice tests provide different lines of information (as in the hypothetical example above), or how often results are congruent or redundant.

Preference experiments are often rather contrived in that females are typically presented plants under artificial conditions (cages or preference arenas), and in arrays or sequences that they might never encounter in the field (though more realistic preference tests have been conducted, e.g. Singer & Thomas 1985). In contrast to this, performance experiments need not be quite so highly abstracted from natural conditions: it is possible to rear larvae in the field by confining them to small cages or bags. Despite this, the majority of performance experiments have addressed the performance of larvae in laboratory conditions, often with larvae reared singly in petri dishes (Zalniki et al. 2002). Whatever measure of performance is taken (pupal weight, development time, etc.), it seems intuitively obvious that results may be biased by laboratory conditions. For example, the architecture of a given species of plant might provide a microclimate that allows larvae to feed throughout the heat of the day, resulting in faster development than on a host that does not have the same architecture (Alonso 1997). This effect would only be apparent if larvae were reared in the field. Other environment-dependent effects could include interactions with predators and parasitoids.

I used two species of lycaenid butterflies, *Mitoura nelsoni* Boisdruval and *Mitoura muiri* H. Edwards, to address these issues in the design of preference and performance experiments. The oviposition behavior of *M. nelsoni* females in choice tests has been previously described: they have consistent preferences for their host incense cedar (*Calocedrus decurrens* Torrey), laying the most eggs on that host in both four-way and two-way choice tests involving other hosts of *Mitoura* in Northern California (Forister 2004, 2005a). Here I ask if the preferences of *M. nelsoni* females for incense cedar are expressed in no-choice tests as a willingness to lay eggs on incense cedar and a reticence to lay eggs on an alternate host when encountered in isolation. The larval performance of *M. nelsoni* and *M. muiri* on multiple hosts, as expressed in pupal weight and survival, has been previously described (Forister 2004, 2005a). Here I focus on one host, a host of *M. muiri*, and ask if differences between the two butterfly species in performance on that host are consistent between laboratory and field environments.

**Materials and Methods**

**Butterflies and plants.** *M. nelsoni* and *M. muiri* are part of a complex of host-specific lycaenid butterflies in North America associated with plants in the family Cupressaceae which have been the focus of recent investigations into the ecology of speciation (Nice & Shapiro 2001; Forister 2004, 2005a, 2005b). *M. nelsoni* is found in association with incense cedar at low to middle elevations in mesic forests from southern British Columbia to Baja California. *M. muiri* is an edaphic-endemic associated with cypress hosts (primarily MacNab cypress, *Cupressus macnabiana* A. Murray, and Sargent cypress, *Cupressus sargentii* Jepson) on low elevation, ultramafic soils such as serpentine in California (Gervais & Shapiro 1999).

The experiments described here used *M. nelsoni* adults in preference experiments, and caterpillars of both species in performance experiments. The *M. nelsoni* adults consisted of wild-caught and laboratory-reared individuals. Wild-caught individuals were taken from the following locations in 2004 on the west slope of the Sierra Nevada Mountains near interstate 80: Drum Powerhouse Road and Lang Crossing (see Forister 2004 for more details on these locations). Laboratory-reared adults were part of a colony that was being maintained for other experiments at the University of California, Davis. These individuals were the offspring of females collected from a number of populations in the Sierra Nevada and North Coast Ranges in the previous season.

Larvae used in performance experiments were generated from individuals reared and mated in the laboratory. For both *M. nelsoni* and *M. muiri*, larvae were pooled from multiple lines without regard to genetic background within species. In other words, *M. nelsoni* larvae were the product of matings between *M. nelsoni* adults from a number of locations throughout California (and the same for *M. muiri*). These matings are described in detail in Forister (2005a).

Three host plant species were involved in these experiments: incense cedar (the host of *M. nelsoni*), Sargent cypress and MacNab cypress (hosts of *M. muiri*). For preference experiments, incense cedar and Sargent cypress were collected from Goat Mountain in the North Coast Range of California, where the two hosts grow sympatrically. For the performance experiments, MacNab cypress was used both in the field
and through collection from one location, Knoxville Public Lands, also in the North Coast Range.

Preference assays. In order to assess the oviposition behavior of *M. nelsoni* in no-choice assays, females were confined individually with sprigs of host plants in oviposition arenas (cylinders of wire mesh, 3600 cm$^3$). They were exposed to one host for 24 hours, and then switched to the other host for 24 hours (the two hosts, as mentioned above, were incense cedar and Sargent cypress). The switch from one host to the other was done in the early morning of the second day, before butterflies were active. Experiments were only conducted for 48-hour periods because previous experience with Mitoura butterflies had shown that females become considerably less vigorous and egg-laying begins to drop off after 48 hours when they are kept in a greenhouse in full sun (Forister, pers. obs.). At the start of the experiment, each female was haphazardly assigned to one of two groups, with one group being confined first with incense cedar, and the second group being confined first to Sargent cypress. Sugar water was applied to the cages as an artificial nectar source that was readily consumed by butterflies throughout the experiment. The number of eggs on plants was counted at the end of each interval as a measure of host preference (*Mitoura* butterflies very rarely oviposit on any surface in preference arenas other than the host plants; and if eggs were found on the side of the cage they were not counted).

Results from preference assays were analyzed in two ways. First, the number of eggs laid by each female on the two hosts was treated as a pair in a nonparametric Wilcoxon matched-pairs test. This analysis addressed the question: which host received more eggs without reference to the order of the hosts? Second, a Wilcoxon rank-sum test was used to ask: does the first host encountered affect the number of eggs laid on incense cedar? In this case, each female is represented by one data point (the number of eggs laid on cedar), and females are identified as belonging to either the treatment that received incense cedar first or Sargent cypress first.

Performance assays. The goal of performance assays was to ask if differences in performance between the two butterfly species observed in the laboratory (Forister 2004, 2005a) are also observed in the field. To address this question, ten trees of MacNab cypress, the host of *M. mutiri*, were selected at a field site that has been studied previously (Knoxville, see Forister 2004). Trees were selected haphazardly within a small area (approximately 100 square meters), and caterpillars of both *M. mutiri* and *M. nelsoni* were reared to pupation simultaneously on these trees in the field and on cuttings from these trees brought back to the laboratory. Caterpillars in the laboratory were reared in groups of five in large drinking cups nested within smaller cups so that the cut ends of branches could be pushed through holes in the larger cup and into water held in the smaller cup. Upon pupation, pupae were weighed on a Mettler Toledo microbalance to the nearest hundredth of a milligram. Caterpillars that became part of the field component were reared initially in the laboratory through the first instar. They were then transferred to the field, where they were reared to pupation in groups of five in spun mesh bags enclosing tree branches. Each of the ten trees in the field had two bags (one *M. mutiri* bag and one *M. nelsoni* bag). Caterpillars in bags were checked weekly and moved to new branches on the same trees when foliage had been depleted. Upon pupation, pupae were removed from bags, brought back to the laboratory and weighed. In addition to pupal weight, survival and days to pupation were recorded for both the laboratory and field-reared individuals.

Analyses of variance (ANOVA) using restricted maximum likelihood (REML) mixed models were used to analyze results from performance experiments (Littell et al. 1996). Fixed factors in models included species, location (field or laboratory), and an interaction between species and location. Random factors were tree, and interactions between tree and species, and between tree and location. Barring group is not included in models because values within groups (for pupal weight, development time and survival) were simply averaged prior to analysis (individuals within groups are not statistically independent).

No transformations were found to be necessary to meet the assumptions of ANOVA for pupal weight or development time. Residual error from analysis of survival (the fraction of individuals surviving to pupation within each rearing group) was highly non-normal (even following arcsine transformation) due to the large number of groups in which survival was 100%. Therefore, two separate nonparametric Wilcoxon rank-sum tests were performed to compare survival between the two species in the laboratory and in the field. JMP-IN software, version 7.0 (SAS Institute, Cary, NC, U.S.A.), and Kaleidagraph, version 3.6 (Synergy Software, Reading, PA, U.S.A.), were used for nonparametric analyses (both for survival data and preference results, described above), and PROC MIXED in SAS, version 9.1 (SAS Institute, Cary, NC, U.S.A.), was used for REML analyses of variance.

Results

Preference assays. A total of 45 *M. nelsoni* females were tested in no-choice assays using incense cedar, the
host of *M. nelsoni*, and Sargent cypress, the host of *M. muiri*. As can be seen in Fig. 1a, females laid a majority of their eggs on incense cedar in these no-choice assays (*T* = 4.63, *P* < 0.0001). The behavior of females was not influenced by the order in which plants were presented to them: a comparable number of eggs was laid on incense cedar regardless of whether that host was presented first or second in sequence (Fig 1b; *T*₂ = -1.11, *P* = 0.26).

**Performance assays.** A total of 185 larvae were reared to pupation in 30 rearing groups (20 in the laboratory and 19 in the field; larvae from one *M. muiri* group in the field escaped). As has been observed in previous work (Forister 2004), *M. nelsoni* individuals develop to pupal weights that are greater than *M. muiri* (on average 10% greater), even though the host in question is the natal host of *M. muiri*. The results reported here demonstrate that this difference (between the two species) is not affected by rearing environment (Fig 2a, and note the insignificant species by location interaction in Table 1). In contrast, rearing environment did have a differential effect on the development time of the two species: in the field, *M. muiri* individuals reach pupation 4.62 days earlier than *M. nelsoni* individuals (Fig. 2b, Table 2). In general, larvae of both species developed more slowly in the field, and this might be because they did not feed at night: when checking the bags in the early morning, I found larvae to be inactive, while larvae in the laboratory are capable of feeding throughout the night. There were no significant differences between the

![Graph](image)

**Fig. 1.** Results from preference assays illustrated as box plots. The same data is shown in two different ways in (a) and (b); data shown in (a) is the fraction of eggs laid on the two hosts, while (b) shows the influence of experimental sequence in sequential no-choice assays on oviposition behavior. In other words, in (b), the data shown is the fraction of eggs laid on incense cedar for females which were exposed to that plant first (the left box), and for females which were exposed to cypress first (the right box).

![Graph](image)

**Fig. 2.** Means and standard errors from assays of performance in the laboratory and in the field. Statistical results for pupal weight (a) and days to pupation (b) are shown in Tables 1 and 2 respectively. See text for more details related to survival (c).
survival of *M. nelsoni* and *M. muiri* caterpillars in the laboratory ($T_1 = 0, P = 1.0$) or in the field ($T_1 = 0.51$, and $P = 0.61$) (Fig. 2c).

Differences among individual trees had a significant effect on pupal weight (Table 1), but not on development time (Table 2). Although tree had an effect on pupal weight, this was not influenced by rearing environment, nor was there a significant species by tree interaction. In other words, larvae of both species did better on certain trees, and this was true whether larvae were reared in the field or on cuttings from the same trees in the laboratory. In order to better visualize the influence of individual trees on pupal weight, Fig. 3 shows the correlation between weights of larvae reared in the field and in the laboratory. One outlier has been excluded from the relationship shown in Fig. 3: one *M. nelsoni* rearing group had high mean pupal weight in the field (50.03 mg), but unusually low weight in the laboratory (66.9 mg). With the outlier excluded, the correlation is significant: Pearson product-moment correlation of 0.73, $P = 0.0006$; with the outlier included the correlation is 0.57, $P = 0.12$.

**DISCUSSION**

*M. nelsoni* females express a clear preference for their natal host, incense cedar, in both choice tests (Forister 2004, 2005a), and no-choice tests, as reported here (Fig. 1). Choice tests are more efficient from the point of view of experimenter effort: there is less manipulation in choice tests, as plants do not need to be changed part way through the test (as compared to a no-choice design with sequential replacement of hosts). The results reported here suggest that, at least in the *Mitoura* system, choice tests provide equivalent information to no-choice tests. There are two important caveats to this conclusion. First, these results should not be used to infer that the two types of choice test are equivalent in other systems. Rather, the results reported here highlight the utility of exploring both types of assay, and the possibility that in some systems choice tests may be sufficient. Second, while it is true that choice and no-choice assays with *M. nelsoni* lead to similar conclusions about the relative ranking of the two hosts by ovipositing females, there may be situations in which no-choice tests would still be uniquely useful. For example, no-choice tests could be used to survey for variation among

### Table 1. Results from analysis of pupal weights. Degrees of freedom and F ratios are reported for fixed effects, covariance estimates and standard errors for random effects. Significant $P$ values are shown in bold text.

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<th>$P$</th>
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**Covariance**

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<td>Tree x location</td>
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### Table 2. Results from analysis of development time (days to pupation). Degrees of freedom and F ratios are reported for fixed effects, covariance estimates and standard errors for random effects. Significant $P$ values are shown in bold text.

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**Covariance**

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females in preference for a less preferred host, while such variation could potentially be harder to detect in choice tests where females always spend a majority of their time ovipositing on the preferred host. No-choice tests could also be used to study factors (such as egg load) which may influence “motivation” and lead to the acceptance of an otherwise less-preferred host (Singer et al. 1992).

With the performance results reported here, it is apparent that a comparison between the two species for at least one element of larval performance (pupal weight) is not greatly influenced by rearing environment. *M. nelsoni* pupae are bigger than *M. muiri* pupae, and individuals reared in the laboratory are bigger than individuals reared in the field (Fig. 2a), but being reared in the laboratory or the field does not change the relative sizes of *M. nelsoni* and *M. muiri* pupae. The foliage quality of individual trees was also consistent across rearing environments (Fig. 3). The vast majority of performance experiments are done in the laboratory (Zalucki et al. 2002), thus the results reported here are heartening; not only may laboratory performance (as measured by pupal weight) be an accurate reflection of performance in the field (at least in the absence of natural enemies), but intraspecific variation in plant quality may in some cases also be reasonably studied under laboratory conditions. Osier et al. (2000) reported a similar consistency between performance in the laboratory and in the field on particular plant genotypes using gypsy moth larvae and quaking aspen clones.

The performance results reported here are also interesting in the light of a scenario of host-associated speciation that has been described in *Mitoura*. Differences in host preference are believed to be a key mechanism in the diversification of this group (Nice & Shapiro 2001; Forister 2004, 2005a), as has been suggested for a number of other phytophagous insect systems in which adults mate and oviposit on their host plants (Berlocher & Feder 2002; Drés & Mallet 2002). Divergent host preferences are expected to evolve in association with host-specific larval adaptations, particularly when divergence is in sympathy or parapatry (Fry 2003) (which appears to be the case for *Mitoura*). *M. nelsoni* fits this model nicely; females have strong preferences and larvae attain considerably larger pupal weights on incense cedar (larger than *M. nelsoni* larvae reared on other hosts of *Mitoura* in northern California, and larger than other *Mitoura* larvae reared on incense cedar). In contrast, *M. muiri* females have strong host preferences but *M. muiri* larvae do not attain greater pupal weights or have higher survival on their natal cypresses relative to *M. nelsoni* larvae on the same hosts. The present study suggests a previously undetected component of local adaptation in *M. muiri*: faster development than *M. nelsoni* on MacNab cypress in the field. Why this difference would only be manifest in the field is not known, though one possibility is that *M. muiri* larvae may be able to feed over a slightly wider range of temperatures than *M. nelsoni* larvae. Faster growth may reduce exposure to natural enemies (Williams 1999), or extreme climatic events (Fordyce & Shapiro 2003). In particular, faster development at low elevations in the dry, inner North Coast Range of California might allow larvae to pupate before temperatures become unfavorably high (three days before the end of the experiment, a maximum daily temperature of 40 degrees Celsius was recorded at the field site). Although the adaptive significance of faster development in the field is unknown, this is a difference between *M. nelsoni* and *M. muiri* that would not have been observed in a solely laboratory-based study.

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**Literature Cited**


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