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Host Plant Suitability in a Specialist Herbivore, *Euphydryas anicia* (Nymphalidae): Preference, Performance and Sequestration

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Abstract

The checkerspot butterfly, *Euphydryas anicia* (Nymphalidae), specializes on plants containing iridoid glycosides and has the ability to sequester these compounds from its host plants. This study investigated larval preference, performance, and sequestration of iridoid glycosides in a population of *E. anicia* at Crescent Meadows, Colorado, USA. Although previous studies showed that other populations in Colorado use the host plant, *Castilleja integra* (Orobanchaceae), we found no evidence for *E. anicia* ovipositing or feeding on *C. integra* at Crescent Meadows. Though *C. integra* and another host plant, *Penstemon glaber* (Plantaginaceae), occur at Crescent Meadows, the primary host plant used was *P. glaber*. To determine why *C. integra* was not being used at the Crescent Meadows site, we first examined the host plant preference of naïve larvae between *P. glaber* and *C. integra*. Then we assessed the growth and survivorship of larvae reared on each plant species. Finally, we quantified the iridoid glycoside concentrations of the two plant species and diapausing caterpillars reared on each host plant. Our results showed that *E. anicia* larvae prefer *P. glaber*. Also, larvae survive and grow better when reared on *P. glaber* than on *C. integra*. *Castilleja integra* was found to contain two primary iridoid glycosides, macfadienoside and catalpol, and larvae reared on this plant sequestered both compounds; whereas *P. glaber* contained only catalpol and larvae reared on this species sequestered catalpol. Thus, although larvae are able to use *C. integra* in the laboratory, the drivers behind the lack of use at the Crescent Meadows site remain unclear.

Keywords *Castilleja integra* · *Penstemon glaber* · Iridoid glycosides · Plant defense · Host plant selection · Population variation

Introduction

Host plant selection is typically a complex process: environments are dynamic and, while specialist herbivores might have multiple acceptable host plants in an area, variation in preference for particular host plant species is often evident at a local scale (e.g., Carrasco et al. 2015; Gardner and Stermitz 1988; Meiners 2015). For many herbivorous insects, secondary compounds in host plants are the basis for host plant selection and specialization (Bernays 2001; Dethier 1954); however, other factors such as plant phenology (Singer et al. 1988), nutrient content (Prudic et al. 2005), size (Talsma et al. 2008), physical defenses (Eaton and Karban 2014) and interactions with higher trophic levels such as predators and parasitoids (e.g., Ali and Agrawal 2017; Murphy 2004) may also be important. In addition, ovipositing females may vary in their preference for particular plant species, even though the same sets of species are available. For example, the specialist butterfly, *Euphydryas editha* (Nymphalidae), varied in patterns of host plant utilization across populations, even though similar host plant species were available in those populations (Thomas and Singer 1998).

Euphydryas anicia (Lepidoptera: Nymphalidae), the focus of the present study, is a checkerspot butterfly species that specializes on host plants containing a particular group of plant secondary compounds, the iridoid glycosides (Gardner and Stermitz 1988). They have been observed using host plants from several genera including *Besseya, Plantago* and *Penstemon* (Plantaginaceae), and *Castilleja,* and *Orthocarpus* (Orobanchaceae) (Damtoft 1993; Gardner and Stermitz 1988; Mead et al. 1993; Scott 1986). *Euphydryas anicia* sequesters iridoid glycosides (henceforth IGs) from its host plants (Gardner and Stermitz 1988; Stermitz et al. 1986) and is

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unpalatable to predators (Bowers and Farley 1990). Previous studies on *E. anicia* indicated that a primary host plant at several sites in Colorado is *Castilleja integra* (Gardner and Stermitz 1988; Stermitz et al. 1986). More recently, we have been studying a population of *E. anicia* at a different site: Crescent Meadows, Colorado (details in methods). Although *C. integra* occurs at this location, there has been no evidence of *E. anicia* feeding or ovipositing on *C. integra* in over 15 years of observation (Bowers and Kelly, pers. obs.). Instead, the primary host plants at the Crescent Meadows site are two species of *Penstemon*: *P. glaber* and *P. virgatus* (Kelly and Bowers 2016). *Penstemon glaber* is preferred for oviposition over *P. virgatus,* although both species are used in this population (Kelly and Bowers 2016). We wanted to determine whether *E. anicia* larvae in this Crescent Meadows population could use *C. integra* as a host plant, and if so, how its use affects larval performance and chemical defense. Prior studies have not compared *E. anicia* response to *Penstemon* and *Castilleja.*

To examine host plant suitability and larval preference and performance in this population of *E. anicia,* we conducted a series of experiments addressing three questions: 1) Can *E. anicia* larvae from this Crescent Meadows population successfully develop on *Castilleja integra* (henceforth *Castilleja*) and how do their growth and survivorship on this plant compare to those on their preferred host plant *Penstemon glaber* (henceforth *Penstemon*)? 2) Do unfed, naïve larvae of *E. anicia* from this site show a preference for one of these plant species over the other? 3) Can *E. anicia* larvae sequester IGs when fed *Castilleja* from this site and, if so, how does this compare to sequestration by larvae reared on *Penstemon*?

Methods and Materials

Study Organisms and Field Site The anicia checkerspot butterfly, *Euphydryas anicia*, is native to the western half of the United States (Scott 1986; Stermitz et al. 1986). Adult butterflies have a wingspan of approximately two and a half centimeters, with black bands and spots of red, yellow, and white giving them a 'checkered' appearance (Scott 1986). Adult females lay eggs on the underside of host plant leaves. Newly hatched larvae are gregarious, living in webs formed on the host plant on which they hatched (Scott 1986; Singer et al. 2004; White 1979). They enter diapause during their fourth instar and overwinter until post-diapause larvae emerge in the spring and complete development, pupating in early June. Adults fly during June and July at this field site. Egg masses for this study were collected at the Crescent Meadows field site, near Coal Creek, Colorado (Eldorado State Park, Boulder County, Colorado, USA; 39° 55′ 51.60" N 105° 20' 16.80" W, elevation 2258 m) during July and caterpillars were reared in growth chambers (Percival model LLVL, 25 °C day: 20 °C night, 14 h day length) at the University of Colorado at Boulder.

Host plant material used in the experiments was also collected in July at the time that caterpillars are feeding at this site. *Castilleja integra* and *Penstemon glaber* are both herbaceous perennials native to Colorado and the southwestern United States. *Castilleja* is hemiparasitic on roots of grasses and forbs (Schädler et al. 2005). It produces several iridoid glycosides, three of which are sequestered by *E. anicia*; macfadienoside, catalpol, and aucubin (Gardner and Stermitz 1988); *Penstemon glaber* has also been shown to contain multiple IG's of which only one, catalpol, is sequestered (Fig. 1; Kelly and Bowers 2016; Stermitz et al. 1994).

Growth Tests To determine whether larvae could successfully feed and develop on these two host plant species, we compared the relative growth rates of *E. anicia* caterpillars reared on *Penstemon* and *Castilleja*. Newly hatched groups of larvae from 20 different families (different females for each egg mass) were split between the two host plant treatments ($N =$ 20 larvae from each family on each host plant species). Groups of larvae were weighed, then placed with a small paintbrush onto leaves of each host plant and allowed to feed for 11 days. The experiment was conducted in a growth chamber with a temperature regime of 25 °C day: 20 °C night and a day length of 14 h light and 10 h dark. This experiment was stopped after 11 days because some individuals began entering diapause and were no longer feeding. In six families, egg masses contained fewer than 40 individuals, and for these families, the mass was split and half the larvae were used on each host plant. After 11 days, larvae in each of the groups were counted to determine survivorship and surviving larvae re-weighed as a group to determine the relative growth rate (mg/mg/day). Larvae were fed ad libitum and checked every day to maintain adequate food supply and clean containers.

Choice Tests To determine food preference of naïve caterpillars when offered these two plant species, 10 newly hatched, unfed *E. anicia* larvae from 14 different families were presented with two pieces of leaf (4 × 10 mm), one of *Castilleja* and one of *Penstemon*. Trials were conducted in a 5 × 3 cm plastic container with a 5 × 3 cm sheet of moist filter paper on the bottom, placed in a growth chamber under the same conditions as above for rearing. In each container, 10 larvae were placed at one end and both leaf disks were placed equidistant from the larvae at the other. The species of plant on the left and right sides was varied randomly. Prior to starting the experiment, leaf disks were weighed, and then weighed again after a 24-h feeding period to quantify consumption. As leaf material dries out over the course of a trial, four trials were conducted for each plant species to estimate water loss during the experiment. To correct for water loss during the feeding trials, average percent weight loss from these trials was applied to initial weights of plant samples at the start of the 24-h period.

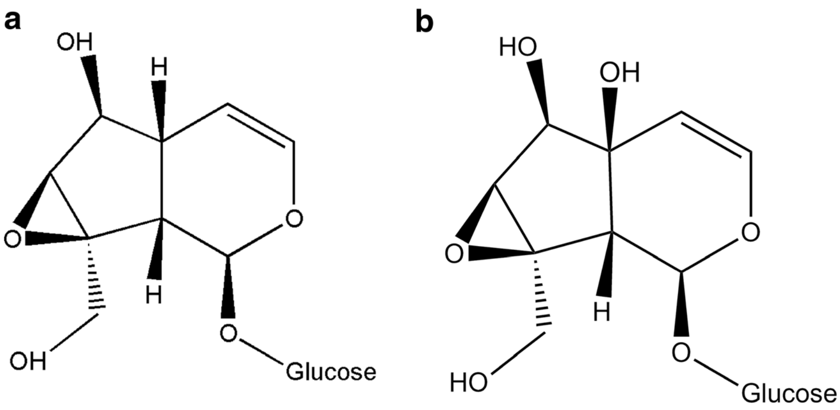


Fig. 1 Chemical structures of (**a**) catalpol and (**b**) macfadienoside

Chemical Analyses We analyzed plant and caterpillar iridoid glycoside content via gas chromatography (see details below). Single ramets from individual plants were collected in the field at the Crescent Meadows site ($n = 18$ for *Castilleja*, $n = 20$ for *Penstemon*), leaves were removed from stems, combined for a single ramet, oven dried at 50 °C, and then ground to a fine powder using a mortar and pestle. For each sample, 25 mg (± 1 mg) was weighed to the nearest 0.01 mg and placed in a test tube. Each sample was extracted overnight in methanol before filtering out solid materials and evaporating the residue to dryness.

For caterpillar samples, we used diapausing caterpillars, which have emptied their gut prior to entering diapause. Five diapausing caterpillars from each family (20 families on *Castilleja* and 17 on *Penstemon*) were weighed, frozen, and placed in test tubes with a small amount of sand. Five ml methanol was added to each tube and samples were ground with a glass rod until individual caterpillars were indistinguishable and their exoskeletons broken into fine pieces. Samples were then allowed to extract overnight before being filtered and the residue dried. Caterpillar wet weights were converted to dry weights to allow direct comparison with dried plant material. Concentrations of IGs are reported as percent dry weight for both plants and caterpillars. A set of five additional groups of five diapausing caterpillars were weighed wet, dried at 50 °C to a constant weight (72 h) and weighed again. Based on these measurements, the percent dry weight was calculated as 30.5% of the wet weight.

From this point forward, treatment of the samples was identical for caterpillar and plant samples. An internal standard phenyl-β D-glucopyranoside (PBG), at a concentration of 0.500 mg/ml was added to each sample. Then, samples were partitioned between water and ether. The ether portion containing waste materials was discarded and the water fraction containing iridoid glycosides was evaporated again. We then added 1 ml of methanol to each sample and a 100 μl aliquot was removed, evaporated, and derivatized using Tri-Sil Z (Sigma-Aldrich Corporation), before being run on an Agilent 7890A gas chromatograph (GC; Agilent Technologies) equipped with a flame ionization detector (FID) and Agilent DB-1 column. The GC was calibrated prior to running these samples with a standard containing purified catalpol, aucubin and macfadienoside (for details see Bowers and Collinge 1992; Bowers et al. 1992; Gardner and Stermitz 1988). Data were processed with Agilent ChemStation software (version A.03.34).

Statistical Analyses All statistical analyses were conducted in R version 3.2.2. Choice tests were conducted as paired t-tests and all others were conducted as Welch's two sample t-tests.

Results

Growth and Survivorship Larvae reared on *Penstemon* grew more than twice as fast as those reared on *Castilleja* ($t = 4.217$, $df = 25.968$, $p < 0.01$, Fig. 2). Only data from replicates where living individuals remained at the end of the experiment were included in this analysis. As a result, the sample size for *Castilleja* was decreased from 20 families to 10, and for *Penstemon* from 20 to 18. Survivorship of larvae reared on *Penstemon* was also nearly twice that of those reared on *Castillea* ($t = 3.088$, $df = 36.406$, $p < 0.01$, Fig. 2).

Choice Tests In choice tests, caterpillars ate significantly less of *Castilleja* (mean = 31.54 mg ± 3.15 SE) than of *Penstemon* (mean = 51.59 mg ± 2.19 SE) indicating a significant preference for *Penstemon* ($t = 5.228$, $df = 23.168$, $p < 0.01$, Fig. 3).

Chemical Analyses of Plants and Caterpillars *Penstemon* contained eighteen times as much catalpol as *Castilleja* which only contained an average of 0.30% dry weight ($t = 8.543$, $df = 19.252$, $p < 0.01$; Fig. 4). Macfadienoside occurred only in *Castilleja* and was not found in *Penstemon* (Fig. 4). Due to the high concentrations of macfadienoside, *Castilleja* had twice the concentration of total sequesterable IGs as compared to *Penstemon* ($t = -4.8267$, $df = 27.172$, $p < 0.01$; Fig. 4).

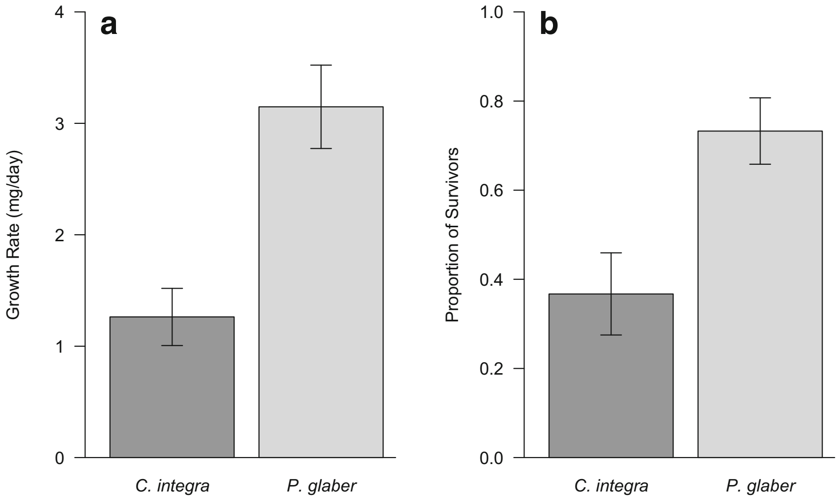


Fig. 2 Growth rate in mg/day and Survivorship of *Euphydryas anicia* when reared on either *Castilleja* or *Penstemon*. Means ± s.e are shown

Comparison of total IG concentrations in diapausing caterpillars showed that larvae fed *Castilleja* had significantly higher levels than those reared on *Penstemon* ($t = -3.2007$, $df = 47.555$, $p < 0.01$, Fig. 4). Larvae reared on *Penstemon* had 10 times more catalpol than those reared on *Castilleja* ($t = 10.86$, $df = 17.588$, $p < 0.01$; Fig. 4), however only those reared on *Castilleja* contained macfadienoside.

Discussion

These results show, first, that *Euphydryas anicia* caterpillars of the Crescent Meadows population are able to use *Castilleja integra* as a host plant. However, their performance, in terms of both growth rate and survivorship, was much higher on *Penstemon glaber.* Indeed, over half the replicates reared on *Castilleja* did not survive. Second, naïve larvae showed a strong preference for *Penstemon* over *Castilleja*. Together, these data indicate that *C. integra* is a less suitable host plant than *P. glaber* for *E. anicia* larvae at the Crescent Meadows population. Finally, the specific IGs sequestered by the caterpillars reflected those in the host plants (macfadienoside and catalpol for caterpillars reared on *Castilleja* and catalpol for those reared on *Penstemon*) and total IG concentration in both caterpillar populations was largely a function of total IG concentration in the plant species eaten. Poor larval performance on *Castilleja,* along with the preference for *Penstemon,* indicates that *Castilleja* is a poor host plant at the Crescent Meadows population. In contrast, other *Euphydryas* populations in Colorado showed evidence of selection for the use of *Castilleja* (Gardner and Stermitz 1988; Stermitz et al. 1986). Although our data suggest that *Penstemon* is the more suitable host plant at the Crescent Meadows site, the drivers behind this apparent host ranking remain unclear.

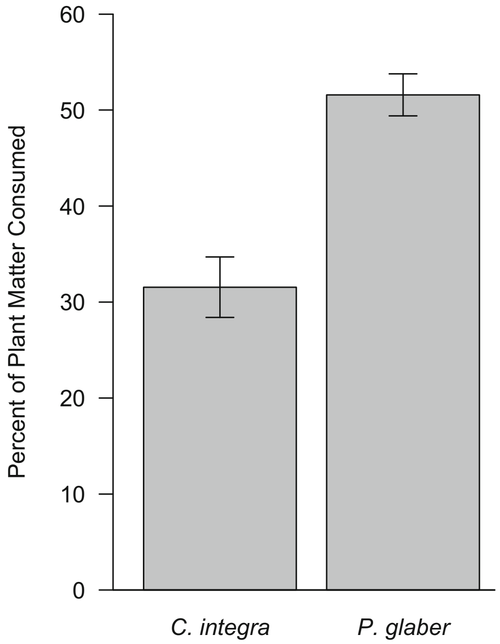
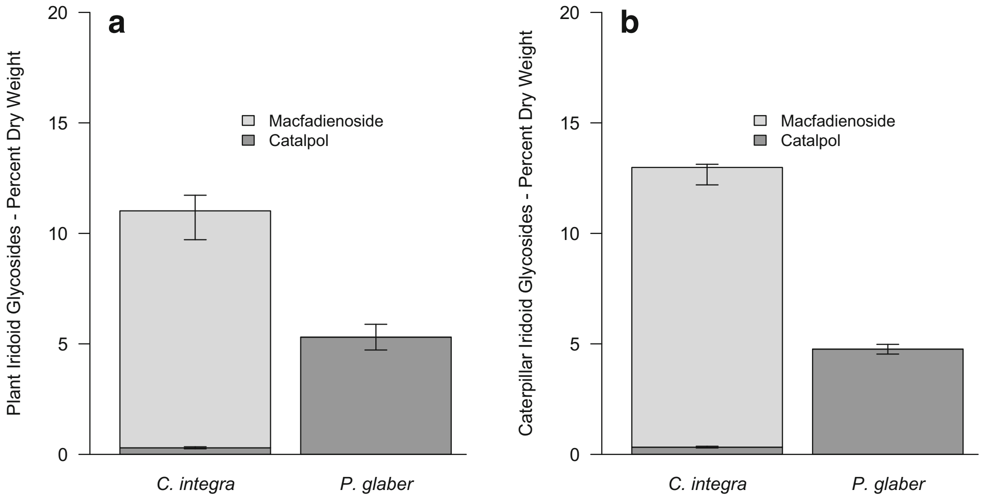


Fig. 3 Leaf consumption of *Castilleja* and *Penstemon* by *Euphydryas anicia* larvae during naïve choice tests. Means ± s.e are shown

Previous research has found similar evidence for variation in the host plant use of specialist herbivores. One experiment compared the host plant preferences of *Euphydryas editha* in two California populations (about 150 km apart) that each contained the same two host plant species (Singer and Parmesan 1993). Individuals from one population preferred *Penstemon rydbergii* while those from the other population preferred *Collinsia parviflora* (Plantaginaceae; Singer and Parmesan 1993). In these populations, the variation was driven by an interaction between plant traits and genetic variation among ovipositing butterflies rather than simply one of those factors alone. Another study examining *E. editha* populations from Colorado found that ecological differences between sites, such as host plant abundance, phenology, and plant size, largely explained the avoidance of certain potential host plant

Fig. 4 Iridoid glycoside (IG) composition of *Castilleja* and *Penstemon* leaves and of *Euphydryas anicia* larvae reared on either *Castilleja* or *Penstemon*. Means ± s.e are shown



species; however, nutritional and biochemical differences did not appear to significantly contribute to host plant choice (Holdren and Ehrlich 1982). Similar ecological factors could explain the avoidance of *Castilleja* exhibited by *E. anicia* at our study site. At Crescent Meadows, *Castilleja* plants were less abundant than *Penstemon* in locations where adult females were commonly found (pers. obs.). Also, *Castilleja* has narrower leaves with higher levels of trichomes, which may negatively affect oviposition preference and the ability of newly hatched larvae to feed (Eaton and Karban 2014; Fordyce and Agrawal 2001; Gripenberg et al. 2010; Westoby et al. 2002). It may also be the case that feeding on *Penstemon* provides enemy free space (Murphy 2004).

Ecological factors can vary substantially between sites and through time, potentially resulting in variation in female ranking of host plant suitability. For example, plant size may be an important determinant of female oviposition choice (e.g., Cohen and Brower 1982; Nieminen et al. 2003) and female preference may change over the course of a growing season due to changes in host plant abundance or quality (e.g., Rausher 1980). Furthermore, the presence or absence of natural enemies may also influence female oviposition choice (e.g., Kessler and Baldwin 2002). The factors that lead to one plant species being the most suitable host plant for a particular insect population often result in it also being the most preferred for feeding and oviposition. Oviposition preference is a heritable trait (e.g., Thompson 1988), and while various ecological may also influence oviposition (see above), selection can relatively quickly drive oviposition preference in populations that are genetically isolated (Singer 2004). These initial host shifts may then lead to rapid evolution of preference and performance, resulting in substantial differences in host plant use among populations (Schoonhoven et al. 2005; Scriber et al. 2008; Singer and Parmesan 1993; Singer et al. 1988; Singer 2004). Such differences in host plant use among populations have previously been studied in *E. editha*; our study provides possible evidence that other members of the genus *Euphydryas* show similar patterns of population variation in host plant use and suitability.

Iridoid glycoside variation between plant species could also be important for the relative suitability of host plant species and for the interaction of the insect herbivore with its natural enemies. Previous research examined populations of *E. anicia* elsewhere in Colorado that used different host plant species, specifically *Castilleja integra, Besseya alpina, and B. plantaginea* (Gardner and Stermitz 1988; Stermitz et al. 1986). At those sites, *E. anicia* sequestered macfadienoside, catalpol and aucubin (Gardner and Stermitz 1988; Stermitz et al. 1986). Those studies show that other populations in Colorado successfully use *Castilleja* and that adults from those populations contained sequestered macfadienoside. Our laboratory experiments confirmed that *E. anicia* larvae from our site have the ability to sequester macfadienoside from *Castilleja*. However, in 15 years of analyzing iridoid glycosides in adult *E. anicia* from our site, we have never found macfadienoside in any individuals (Bowers, unpublished data). The relative effectiveness of catalpol and macfadienoside as predator deterrents remains unclear. Thus, while experiments with vertebrate predators showed that *E. anicia* is unpalatable (Bowers and Farley 1990), the importance of sequestering differing amounts of catalpol and macfadienoside for higher trophic levels is unknown. It is possible that one compound provides a more effective chemical defense than the other (Bowers and Farley 1990; Harvey et al. 2004), but further studies are needed to confirm this.

Localized specialization among populations has been observed in a number of different herbivorous insect taxa (Fox and Morrow 1981; Thompson 2008) and the factors driving localized specialization may be complex and differ among taxa. Our results suggest that *Euphydryas anicia* might be another species exhibiting such characteristics. While we are unable to pinpoint specific ecological drivers behind the differences in host choice that we observed in our population compared to others, it is clear that strong population differences occur. Future studies, involving multiple populations and including more consideration of higher trophic levels (e.g., predators, parasitoids and pathogens), might provide insight into the mechanisms driving these varying patterns of host plant preference and performance.

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