

***Selaginella* and the satyr: *Euptychia westwoodi* larval performance on an ancient plant lineage**

Members of the plant genus *Selaginella* are often considered living fossils, as extant taxa are virtually indistinguishable from 300 Ma fossils. In contrast, the brush-footed butterflies are a relatively recent radiation, having diversified primarily within the last 60 Ma. Satyrs are among the most derived of these butterflies, likely radiated ~35 Ma and are known primarily for their high diversity and propensity to feed on grasses and sedges. In contrast to its close relatives, the Neotropical satyr genus *Euptychia* also feeds on *Selaginella*, which is thought to be nutrient poor. Using no choice feeding experiments, I compared growth rates Costa Rican *E. westwoodi* that were offered two species of *Selaginella*, to those that were offered *Lasiacis rusifolia*, a grass commonly fed upon by close relatives. I discovered *E. westwoodi* larvae fed on two species of *Selaginella* and there was no difference in mass gained between the two species. However, larvae refused to feed on *L. rusifolia* and lost mass over the course of the trial and expired unless they were transferred to *Selaginella*. To the best of my knowledge, these are the first data to report larval performance of the butterfly genus *Euptychia* or any *Selaginella* feeding insect. . Though far from conclusive, these results support the proposition that *Euptychia* have lost the ability to feed on other host plants and are now specialized on grasses.

***Selaginella* and the satyr: *Euptychia westwoodi* larval performance on an ancient plant lineage**

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1 Introduction

2 Butterflies of the family Nymphalidae underwent a period of rapid diversification during
3 the late Cretaceous period, approximately 90 Ma, resulting in ~6000 extant species in ~540
4 genera (Wahlberg et al. 2009). The timing of the nymphalid radiation is highly correlated with
5 the diversification and spread of the angiosperms (their primary host plants), such that this
6 pattern is considered a classic example of co-evolution (Ehrlich & Raven 1964; Crane, Friis, &
7 Pedersen 1995; Janz & Nylin 2008). The Satyrinae is the most speciose subfamily within the
8 Nymphalidae, containing ~2200 species in ~200 genera (Hamm & Fordyce 2014), and
9 experienced a burst of diversification in the Oligocene (~25 Ma) (Peña 2007). Consistent with the
10 co-evolutionary hypothesis, this expansion is associated with diversification of the satyr's
11 primary host plants, which are graminoids such as Poaceae and Cyperaceae (Strömberg 2005;
12 Spriggs, Chistini & Edwards 2014; Peña 2007). Graminoid feeding is likely an ancestral condition
13 in the Satyrinae as this regime is found in the vast majority of satyrs (Ackery 1988; Peña 2007).

14 The satyrine genus *Euptychia* Hübner 1818 is found in the Neotropics but does not follow
15 the dietary trends of its relatives (i.e. other member of the subfamily or tribe Euptychiini). While
16 there are records of *Euptychia* feeding on Poaceae (Beccaloni et al., 2008; Janzen & Hallwachs
17 2009), species within this genus primarily feed on two plant lineages, the Selaginellaceae
18 (Lycopodiophyta) and Neckeraceae (Bryophyta) (Singer et al., 1971; Singer & Mallet 1985;
19 DeVries 1987 Mound, Martin & Polaszek 1994). These are interesting host plants for *Euptychia*,
20 not only because these are not graminoid plants, but also because they are two of the most
21 ancestral plant lineages known (Finet et al., 2010).

22 Members of the Lycopodiophyta reached peak diversity during the Carboniferous period
23 (approximately 310 Ma) and are among the ancestral taxa to the gymnosperm/angiosperm

radiation (Nickrent et al., 2000; Banks 2009). The genus *Selaginella* is typically considered a “relict” species as it has exhibited very little phenotypic change over the last 300 Ma, yet few insects feed on it (Mound et al., 1994; Banks 2009). Only 16 species from 6 orders of insects have been reported to feed on *Selaginella*, yet there are multiple accounts of *Euptychia* spp. feeding on *Selaginella* (Singer et al., 1971; DeVries 1987; Mound et al., 1994; Janzen and Hallwachs 2009). It is intriguing that *Euptychia* would make the apparent switch to *Selaginella* and mosses considering these plants typically grow in nutrient poor soils and are thought to have less nutrient content relative to the more derived plant graminoids (Scriber & Slansky 1981; Egorov 2007).

Despite this apparent transition to feed on nutrient poor and ancient plants, little is known about the larval ecology of *Euptychia* on *Selaginella*. I set out to address outstanding questions in *Euptychia* ecology, such as: what is the degree of feeding specialization on different *Selaginella* species, and at what rate do larvae add mass under different feeding conditions? To this end I conducted a series of no-choice feeding studies using *E. westwoodi* Butler 1866, a species of *Euptychia* common to the lowland forests of Costa Rica, and has only been reported feeding on one species of *Selaginella*, *S. eurynota* (DeVries 1987).

Materials and Methods

This study was conducted at both the La Selva Biological Research Station and the Tirimbina Biological Reserve in Costa Rica between 18 and 23 May 2010. These sites are located in lowland tropical wet forest of Sarapiquí County, Heredia province. Larvae of *E. westwoodi* (3rd-4th instars) were collected from *S. eurynota*, a locally abundant species. I was restricted to using larvae of this size because the balance at my disposal could not reliably measure smaller

larvae. In total, 54 *E. westwoodi* larvae were collected in the field. Once in the laboratory, each individual larva's mass was measured to the nearest 1/10th of a mg using an SM-50 semi-micro balance (Curtiss-Wright Corp.). Larvae were then haphazardly placed into one of three treatment groups, each with an initial n=18.

Individual larvae were placed into 2 oz. sealable containers with the respective food plant for that treatment. Treatment group S₁ was offered *S. eurynota*, the host plant the larvae were collected on; treatment group S₂ was offered *S. arthritica*, another *Selaginella* species found in proximity to *S. eurynota*; and treatment group G₁ were offered the grass *Lasiacis ruscifolia*. This grass was chosen because it is host plant to other Euptychiini species and its sympatric with *Selaginella* (DeVries 1987; Janzen & Hallwachs 2009). All treatment groups were offered fresh, undamaged plant material *ad libidum* in the morning and again in the evening. The experiment was maintained in an open-air classroom under ambient conditions (~25° C, 80% RH). Larvae were allowed to feed for 48 hrs, after which time all larvae were measured for mass.

Treatment effects were estimated using repeated measures ANOVA (rmANOVA) as implemented in R 3.1.2 (R Core Team 2014). All data and the code necessary to reproduce the results presented here are freely available on the FigShare website ([DOI to be established on ms is finalized](#)). I modeled this in two ways, using an additive model and one with interaction terms. First, I asked if the mass (mg) of an individual larva acted as a function of treatment group plus its starting mass. Second, I asked if the starting mass of a larva was a function of treatment group interacting with starting mass. I then used ANOVA to ask if the two models were significantly different from one another. These, and all other comparisons, were considered statistically significant at $\alpha = 0.05$. Given that rmANOVA is the equivalent of the one-way ANOVA for non-independent groups, I used pairwise t-tests with Holm's corrections as a *post hoc* test to compare

differences among groups. At the conclusion of the no-choice trial, all larvae were maintained through to the adult stage so their identity could be confirmed.

Results and Discussion

After 48 hrs of no-choice trials the final experimental group sizes were: *S. eurynota* n = 18, *S. arthritica* n = 17, and *L. ruscifolia* n = 17. The additive and interactive models could not be distinguished statistically, so results from the additive model are reported for ease of interpretation. The rmANOVA appeared to sufficiently capture the variation I sought to explain, with a well-fit slope ($P_{2,48} = 2 \times 10^{-16}$) and high R^2 (0.78) (Table 1). *E. westwoodi* larvae offered *Selaginella* were observed feeding, and most gained mass over the course of the experiment (Fig 2-S₁, S₂). One individual from the *S. eurynota* group lost mass and expired shortly after the conclusion of the feeding trial. Necropsy revealed what appeared to be fungal mycelia, which filled the body cavity. Additionally, one individual from each of the *Selaginella* groups maintained mass during the experiment. In these cases the individuals molted, which suggests that each would have continued to gain mass had the experiment continued.

Contrary to expectations, larvae offered *L. ruscifolia* were never observed feeding and all individuals in this treatment lost mass during the trial (Fig 2-G₁). This was surprising given that *L. ruscifolia* is a common host plant of close relatives of *Euptychia* (DeVries 1987; Beccaloni et al., 2008; Janzen & Hallwachs 2009), and because grasses are generally considered to have low levels of allelochemicals relative to other angiosperms (Ackery 1988). Behaviorally, larvae placed with *L. ruscifolia* would crawl over leaf plant material but were never observed feeding; in contrast to *Selaginella* groups, which appeared to feed continuously. When fresh food was

exchanged for old, I microscopically examined *L. ruscifolia* leaves for damage and observed none.

Given these observations, it was not surprising that *post hoc* tests detected statistically significant differences between the *S. eurynota* / *S. arthritica* and *L. ruscifolia* groups ($P < 0.001$ for both comparisons). The average mass change (with 95% CIs) over the two days the experiment were as follows: *S. eurynota* = +11.2 mg (6.7 – 15.7), *S. arthritica* = +12.5 mg (7.9 – 16.9), *L. ruscifolia* = -3.1 mg (-7.6 – 1.4; *N. b.*, the upper 95% CI exceeds 0, which is a side effect of the method used predict confidence intervals; no individuals in this group gained mass). I failed to detect a difference in mass gain between the two *Selaginella* groups ($P = 0.64$, t-test with Holm's correction). Once the no-choice trials were concluded, half of the larvae in the *L. ruscifolia* treatment group were offered *S. eurynota*, or maintained on *L. ruscifolia*. All larvae switched to *S. eurynota* immediately resumed feeding and eventually completed metamorphosis, while individuals that remained on *L. ruscifolia* expired within 96 hrs.

While these data suggest that *E. westwoodi* are specialized on *Selaginella*, it is premature to make that claim for a number of reasons. There are many instances of local herbivore populations evolving some degree of host specificity that the species as a whole does not exhibit. This phenomenon has been referred to as the “mosaic pattern of coevolution” (Thompson 1994; Thompson 2005) and is commonly recognized in butterflies. Furthermore, we know from different regions that other *Euptychia* feed on both grasses and *Selaginella* (Janzen and Hallwachs 2009). Additionally, it is possible that *E. westwoodi* is capable of feeding on grasses other than *L. ruscifolia*, and was not offered a suitable alternative.

Another possible explanation for the refusal of *L. ruscifolia* was the use of 3rd – 4th instars. Other researchers have demonstrated *Pieris rapae* (Lepidoptera: Pieridae) larvae will reject host

plants if switched during later instars (Kerowe 1989; Renwick & Lopez 1999). I cannot discount this, but note that transferring *E. westwoodi* larvae from *S. eurynota* to *S. arthritica* had no apparent effect on mass; however the magnitude of difference between *Selaginella* and *Lasiacis* could be much greater than the differences among *Selaginella*. In contrast, other Lepidoptera are capable of switching host plants at later instars and still complete development (Scriber 1979; Scriber 1982). A longer experiment using neonate caterpillars and additional alternative host plants are needed to further understand the degree of host specialization present in *E. westwoodi*.

What is the diet breadth of *Euptychia*? This is not an easy question given the high level of divergence among *Euptychia*'s three host plants. Using a metric such as Faith's Phylogenetic Diversity (PD) index (Faith 1992; Symons & Becalloni 1999) and functions in the "picante" package in R (Kembel et al., 2010), *Euptychia* has an unrooted PD of ~0.578 (branch lengths from Finet et al., [2010]). This value is strongly influenced by the phylogenetic distance between Neckeraceae, Selaginellaceae, and Poaceae. This influence is observed when analyzing the genus *Adelpha*, which has a PD of 0.576, but feeds on 22 host plant families. Other methods that consider host breadth, such as the ordinated diet breadth (ODB) (Hamm and Fordyce, 2014; Fordyce et al. *in prep*), may provide a more intuitive answer to the question of diet breadth. ODB asks, based on insect diets, what is the effective diet breadth of a taxon (Fordyce et al. *in prep*)? Using this metric, *Euptychia* has an ODB of 1.4, while *Adelpha* has an ODB of 12.8, which seems more in line with the number of host families that these genera are known to feed on.

To the best of my knowledge, these are the first data reporting insect performance on *Selaginella*. These data are useful but indicate other experiments should be performed. Future directions for research on *Euptychia* diet breadth include: beginning the experiment with eggs, conducting the experiment for a longer period, and comparing final adult dry mass and size

among experimental groups. In addition, it would be useful to investigate other *Selaginella* feeding insects and butterflies, such as the two Oriental satyr genera, *Acrophtalmia* and *Ragadia*. Both have been reported to feed on *Selaginella*, and would provide an important comparative framework for future work (Igarashi & Fukuda 1996).

Conclusions

Larval (3rd-4th instar) *E. westwoodi* feeding on *Selaginella* gained an average of ≥ 10 mg during a two day no-choice feeding experiment, while larvae offered a common grass refused to feed and lost ~ 3 mg during that same time. Larvae that were initially offered grass, but were later switched to *Selaginella*, resumed feeding and eventually completed metamorphosis. A number of issues warn against a broad interpretation of these results, and calling *E. westwoodi* a specialist based on these results would be over reaching. However, it appears that 3rd-4th instar *E. westwoodi* from the Sarapiquí region of Costa Rica were adapted to *Selaginella* and incapable of feeding on a common grass.

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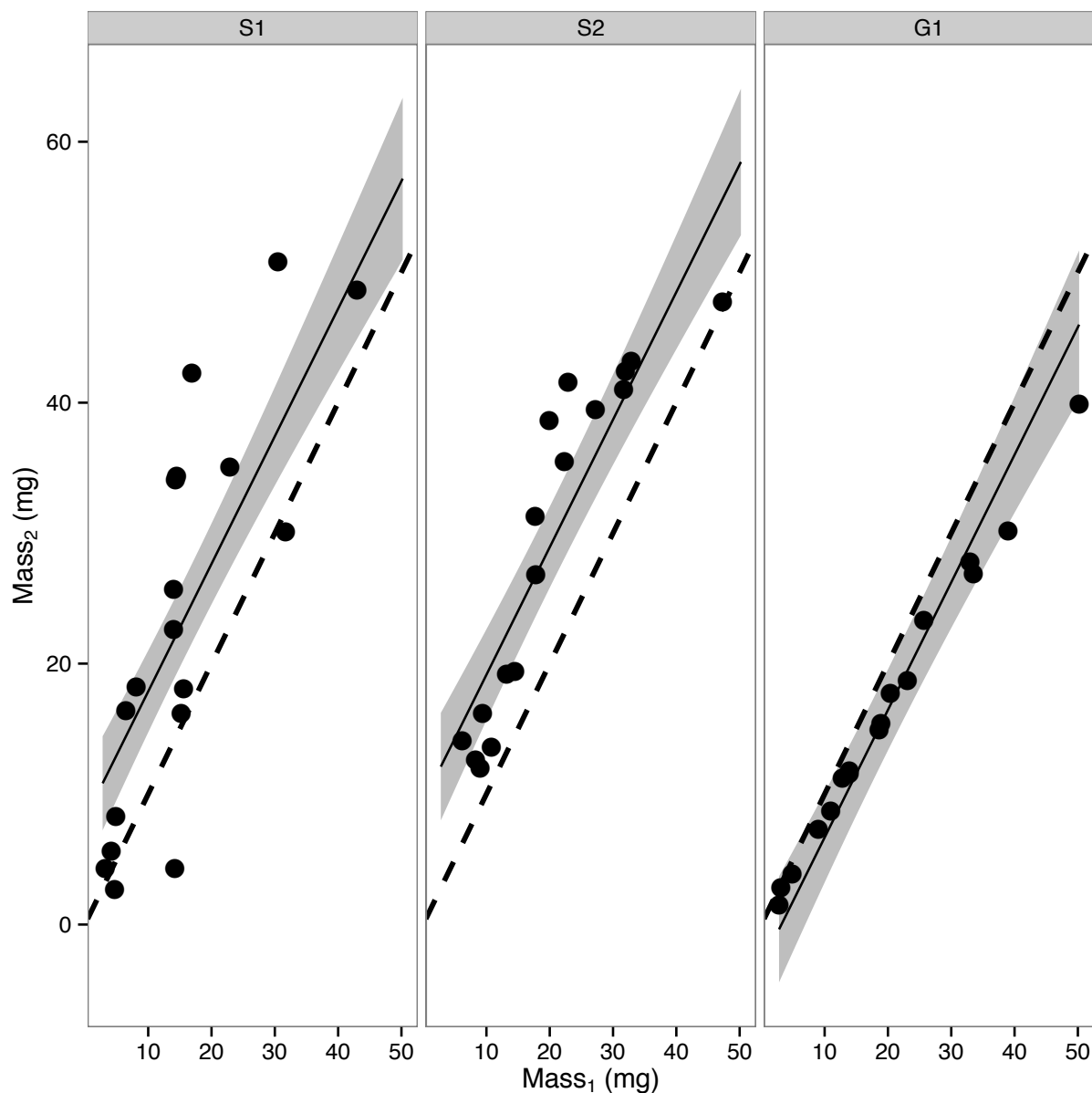


Figure 1. Plot of rmANOVA results for *E. westwoodi* individual mass (mg) at Time1 (X-axis) and Time 2 (Y-axis) for three treatment groups: S1 = *Selaginella eurynota*, S2 = *S. arthritica*, G1 = *Lasiacis ruscifolia*. Solid line indicates the model slope and the grey band indicates the 95% confidence interval. The dashed line is the 1:1 line; individuals above it gained mass and individuals below it lost mass during the course of the experiment.

257 **Table 1.** rmANOVA results from *E. westwoodi* feeding experiment. $S_1 = S. eurynota$, $S_2 = S.$
258 *arthritica*, $G_1 = L. ruscifolia$. “ T_1 slope” indicates the slope of the regression at time 1, “ S_1 mass”
259 indicates that group S_1 was ~11 mg heavier than G_1 at the conclusion of the experiment.

	Estimate (\pm SE)	t value	<i>P</i>
slope	0.98 (0.08)	12.3	$< 2 \text{ e}^{-16}$
S_1 mass	11.2 (2.23)	5.0	7.7 e^{-6}
S_2 mass	12.4 (2.24)	5.5	1.1 e^{-6}

260