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Restoration of an endangered plant, *Hygrophila pogonocalyx*, leads to an adaptive host shift of the chocolate pansy (*Junonia iphita iphita*)

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ABSTRACT

Anthropogenic introduction of a plant species may cause novel encounters between the plant and local herbivores, and initiate evolutionary changes in host plant usage by herbivores. Until recently the endemic aquatic plant *Hygrophila pogonocalyx* was endangered and had a restricted distribution in Taiwan. Massive restoration efforts since 1997 have led to an expansion of the plant's distribution and a novel encounter between it and an Asian butterfly, the chocolate pansy, *Junonia iphita* (Nymphalidae). This butterfly appears to have colonized *H. pogonocalyx*, switching from its original host, *Strobilanthes penstemonoides* var. *formosana*. In the present study, we aimed to investigate whether the utilization of *H. pogonocalyx* as a host plant has initiated a differentiation between butterflies using the novel and the original hosts. To this purpose we collected butterflies from patches of the two host plants which grow sympatrically. We tested oviposition preference for the two hosts and larval performance on them. Female adults exhibited distinct oviposition preference toward the host plant their mothers preferred. Offspring showed greater survivorship and pupal weight when fed on the host plant their mothers preferred. Male adults displayed territorial behaviors on the host plant that their mothers had preferred. Finally, the survival rate of offspring produced from cross-mating between individuals with different host plant preference was lower than that of non-hybrids. Taken together, we suggest that genetic differentiation has occurred between individuals preferring *H. pogonocalyx* versus *S. penstemonoides* as host plants via host shifting. This process was likely induced by the mass restoration of the formerly rare and endangered plant species.

1. Introduction

Herbivorous insects encounter novel plant species when they colonize new habitats, or when formerly absent plant species enter the insects' distribution ranges (Keeler and Chew, 2008). The novel encounter may initiate ecological changes that produce novel inter- and intraspecific interactions and community dynamics (Tylianakis et al., 2008; Forister and Wilson, 2013) as well as evolutionary changes in host plant usage patterns by insect herbivores that may lead to speciation (Thomas and Singer, 1987; Graves and Shapiro, 2003; Mercader and Scriber, 2007; Janz and Nylin, 2008). Host shifts of herbivorous insects onto exotic host plants may be followed by genetically based changes in insect life history, morphology, physiology, behavior and phenology (Strauss et al., 2006). The study of these processes should provide good examples of the evolutionary adaptation of insects to novel hosts and of the ecological consequences of colonization by non-native species in local communities (Carroll et al., 2005; Carroll and Fox, 2007; Sax et al., 2007; Cogni, 2010; Forister and Wilson, 2013).

The chocolate pansy, *Junonia iphita iphita* (Lepidoptera: Nymphalidae), is a common nymphalid butterfly in Taiwan. Larvae of this species are originally found associated with *Strobilanthes* spp., including *S. penstemonoides* var. *formosana* (*S.f.*, hereafter), *S. rankanensis*, *S. flexicaulis* and *S. longispicatus* (Acanthaceae) (Hsu, 2013). In northern Taiwan the chocolate pansy is often found in relatively shady and humid woodlands with substantial overhead coverage, where its major host plant *S.f.*, grows (Hsu, 1999). However, in recent years many butterfly enthusiasts observed that *Hygrophila pogonocalyx* (Acanthaceae; *H.p.*, hereafter) had become a new host plant for larvae of the chocolate pansy. *H.p.* is an endemic herbaceous, semi-aquatic plant in Taiwan. Due to wetland destruction and degradation it had once been critically endangered, with only small wild populations left in wetlands along the coasts of Taichung County in western Taiwan (Wang, 2002; Huang, 2006).

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Since 1997, *H.p.* has been widely cultivated all over Taiwan for restoration purposes (Huang, 2006). In contrast to *S.f.*, *H.p.* grows in a relatively bright and open environment.

Host range expansions may exert strong selective pressures that require fundamental changes in both behavioral and physiological traits of phytophagous insects (Futuyma and Peterson, 1985; Singer, 1986; Via, 1986; Singer and Thomas, 1996; Cogni, 2010). Insect populations using the original versus new host plants may diverge genetically (Drès and Mallet, 2002; Carroll and Fox, 2007), as has been shown for the apple maggot, *Rhagoletis pomonella* (Feder, 1998). In the present study, we thus aimed to investigate whether the novel utilization of *H.p.* as a host plant has led to a differentiation between chocolate pansy butterflies associated with the novel and the original host. Specifically, we wanted to know whether individuals sampled from the two hosts differ in oviposition preference or larval performance, and whether hybrids produced by individuals using different host plants are less fit than parental forms. We carried out several experiments to answer these questions. First of all, we conducted oviposition preference tests on insects captured in association with each host and followed subsequent larval performance (survivorship and pupal weight) in the laboratory to examine the effects of choosing either *S.f.* or *H.p.* as host plants. We analyzed the relationship between oviposition preference and larval performance. A positive association between the two is generally considered indicative of host-associated adaptation and may generate reproductive isolation in phytophagous insects (Thompson, 1988a; Forister, 2004). In addition, we performed oviposition preference tests for another generation (F1) of the chocolate pansy to investigate whether the preference would remain, i.e., whether the two populations of butterflies showed host fidelity.

Oviposition preference is a female characteristic, and host preferences of males are not as easily assayed in many systems (Forister and Wilson, 2013). However, several studies indicate that some male nymphalid butterflies display territorial behavior related to the presence of host plants (Baker, 1972; Rutowski and Gilchrist, 1987; Rutowski et al., 1991, 1997; Lederhouse et al., 1992). Territorial males would perch on or near host plant patches and defend against other males while waiting for females to appear. Therefore, males' territorial behavior displayed over a particular plant patch could be an indirect indication of male host preference that increases males' chance of meeting females. McDonald and Nijhout (2000) demonstrated that the buckeye butterfly *Junonia coenia*, a close relative to the chocolate pansy, is territorial, and our preliminary observations revealed that male chocolate pansy adults are territorial. Thus, in order to investigate male host preference, we observed territorial behaviors in the field of marked male chocolate pansies of known parentage.

Finally, if chocolate pansy adults using *S.f.* vs. *H.p.* as host plants had diverged genetically, we should expect hybrids between them (preference-hybrids, hereafter) to suffer an environment-dependent reduction in fitness (Forister, 2005; McBride and Singer, 2010). If fitness of preference-hybrid individuals were reduced, divergence between populations adapted to different host plants might be facilitated through reinforcement. For example, Nosil et al. (2003) demonstrated that joint effects of divergent host adaptation and reinforcement had driven strong reproductive isolation between populations of walking-stick insects (*Timema cristinae*) using two different host plants. Thus, we conducted cross-mating experiments between individuals produced by parents preferring different host plants in order to estimate the fitness of preference-hybrid individuals. Overall, we used four experiments: female oviposition, larval growth, male territory defense, and cross-mating, to examine the hypothesis that the novel utilization of *H.p.* as a host plant has led to differentiation of the chocolate pansy. We made several predictions. First, the host plant species at the collection site predicts the preferences of wild-caught females. That is, a positive association exists between host plants at collection site and oviposition preference. Second, there is a positive association between oviposition preference and larval performance. That is, offspring that grow up feeding on the host plants their mothers prefer will show greater survivorship and pupal weight than those that do not. Third, female offspring will show oviposition preference for the host plants their mothers preferred regardless of what they grow up feeding on. Fourth, male offspring will show territory preference for the host plants their mothers preferred regardless of what they grow up feeding on. Finally, compared to non-hybrid ones, preference-hybrid individuals will suffer an environment-dependent reduction in fitness.

2. Materials and methods

2.1. Study site

The study site was located at the An-Kang Butterfly Ecological Education Park in Sindian City (GPS location data (WGS84): 24.961024, 121.502904) of Taipei County, Taiwan. *S.f.* has been growing naturally in the park. *H.p.* was introduced to the park around 1994, which predated the 1997 mass restoration project. Both species grow lavishly in the park sympatrically. In order to compare the growing environments of *H.p.* vs. *S.f.*, we measured the overhead coverage above the host plants where chocolate pansy larvae were found from March 2007 to February 2008 using a spherical densiometer held at elbow height.

2.2. Female oviposition preferences

We performed oviposition preference trials in the laboratory between July 2007 and October 2008 to investigate the oviposition preferences of wild-caught female chocolate pansies and their offspring (F1). We collected 58 female chocolate pansies from the study site at both *H.p.* and *S.f.* growing patches. We recorded the species of host plant at the collection site of each female by associating a focal female with a host plant (*H.p.* or *S.f.*) when the plant patch occurred within a 5 m radius of the female. Thus, the females were classified as *H.p.*-associated, *S.f.*-associated, and non-associated.

We used plastic containers measuring 36 cm × 26 cm × 18 cm (L × W × H) in all trials. Upon the start of a trial, we randomly placed a branch of similar-sized *H.p.* or *S.f.* at opposite ends of a container. Thus, the end of a container that had, say, *H.p.* was random regarding its spatial relationship with, say, lab windows. The cut end of each branch was wrapped in a cotton ball saturated with water. A cotton ball (5 cm in diameter) soaked with sugar water and placed at the center provided a food source. Two full spectrum sunlight tubes (each 18 W with 75 lm/W) hung 20 cm above the containers provided sunlight which illuminated both sides equally. A female chocolate pansy was then placed in each container for 12 h from 8 am to 8 pm. At the end of 12 h, the female was removed, and the total number of eggs laid was counted. We alternated the sides of the two plants in different trials conducted in a day, and the containers were cleaned thoroughly afterward to avoid side bias.

We observed that females actively flew or walked around inside the container, visiting both sides before laying large amounts of eggs. An adult chocolate pansy female could generally produce >60 eggs (mean = 68, $n = 52$) in a day in the laboratory. Accordingly, if a test female laid <30 eggs, it would be placed into a new container the following day for another day of trial. Data from females that produced <30 eggs over two days (8 individuals) were excluded from further analyses. At the end of each trial, we counted the total number of