

COUNTERACTING SELECTIVE REGIMES AND HOST PREFERENCE EVOLUTION IN ECOTYPES OF TWO SPECIES OF WALKING-STICKS

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Abstract.—The evolution of ecological specialization has been a central topic in ecology because specialized adaptations to divergent environments can result in reproductive isolation and facilitate speciation. However, the order in which different aspects of habitat adaptation and habitat preference evolve is unclear. *Timema* walking-stick insects feed and mate on the host plants on which they rest. Previous studies of *T. cristinae* ecotypes have documented divergent, host-specific selection from visual predators and the evolution of divergent host and mate preferences between populations using different host-plant species (*Ceanothus* or *Adenostoma*). Here we present new data that show that *T. podura*, a nonsister species of *T. cristinae*, has also formed ecotypes on these host genera and that in both species these ecotypes exhibit adaptive divergence in color-pattern and host preference. Color-pattern morphs exhibit survival trade-offs on different hosts due to differential predation. In contrast, fecundity trade-offs on different hosts do not occur in either species. Thus, host preference in both species has evolved before divergent physiological adaptation but in concert with morphological adaptations. Our results shed light onto which traits are involved in the initial stages of ecological specialization and ecologically based reproductive isolation.

Key words.—Ecological speciation, ecological specialization, host preference, phytophagous insects, polymorphism, reproductive isolation, visual predation.

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The divergence of lineages through evolutionary time typically involves changes in several traits. Determining which traits and corresponding selective pressures initiate divergence allows us to better understand the causes of speciation and diversification. For example, scientists have debated the reasons for insects being so diversified as a group and yet specialized at the species or population level (Futuyma and Moreno 1988; for reviews see Jaenike 1990). One hypothesis is that adaptations that increase fitness in one environment may hinder survival or fecundity in another, resulting in performance trade-offs between habitats and thus selection for specialization (Joshi and Thompson 1995; Fry 1996; Via et al. 2000, for review see Schluter 2000). Many examples of specialized adaptations come from herbivorous insects and include adaptations involved in predator and parasitoid defense (such as sequestration of toxic chemicals from the plant) and tolerance to plant chemicals (e.g., Edmunds and Alstad 1978; Price et al. 1980; Rausher 1982; Strong et al. 1984; Futuyma et al. 1984; Bernays and Graham 1988; Thompson 1988; Singer et al. 1992; Via 1999; but see Rausher 1984; Keese 1998).

Multiple traits can be involved in specialization (e.g., resistance to plant chemical defenses, defense from predators, host preference). It is logical to assume that not all traits appeared at once. Most likely, a novel trait is the precursor of specialization, and later other traits that promote a closer relationship with the host plant (such as host preference) are also favored. It is important to determine which traits are involved in the first stages of host specialization because they will eventually affect the evolution of many other traits, the path to specialization, and perhaps speciation by host shifting. Determining which traits evolved first can be difficult because many species often already exhibit suites of specialized traits. However, plant-insect associations that have

evolved recently or that have stable polymorphisms provide a window into the initial stages of specialization. Several studies have measured survival or performance of insects across host plants (references above), but few have studied more than a single trait.

Speciation through the evolution of specialization in parapatric or sympatric conditions can involve two phases. First, there is the formation of a polymorphism (ecotype) that is adaptive to different resources. Second, there is the evolution of reproductive isolation between the ecotypes. Specialized adaptations to divergent environments have been documented to result in reproductive isolation and speciation in some animals (Funk 1998; Via 1999; Schluter 2000; Via et al. 2000; Funk et al. 2002; Coyne and Orr 2004; Rundle and Nosil 2005). Reproductive isolation may evolve as either an indirect result of adaptive divergence (Mayr 1963; Jiggins et al. 2001) or an adaptation to prevent maladaptive habitat switching and the production of ecologically unfit hybrids (Dobzhansky 1951; Servedio and Noor 2003). For example, populations of herbivorous insects using different host-plant species can diverge in host preferences such that individuals from different hosts are unlikely to encounter one another, thereby reducing gene flow between host-associated populations (Via 1999; Funk et al. 2002). Additionally, divergent adaptations can result in reduced survival of immigrants from populations adapted to divergent habitats relative to residents (Mallet and Barton 1989; Funk 1998; Via et al. 2000; Hendry 2004). This process constitutes a form of reproductive isolation (i.e., it reduces gene flow due to a nongeographic barrier) when it reduces encounters, and thus interbreeding, between individuals from divergent populations (“immigrant inviability”; Nosil et al. 2005a).

In recent years, the theory of evolution of specialized traits, particularly within coevolved systems, has incorporated the

variation in ecological conditions and phylogenetic relationships in geographically structured populations to explain the complexity of species interactions in nature (Sandoval 1994a, Thompson 1997). These studies have helped us to understand biodiversity in general.

We studied two species of *Timema* walking-stick insects that exhibit *host ecotypes* (see below) to determine the level of specialization in behavioral, physiological, and morphological traits. We determined the level of specialization by measuring variation in fitness components across different hosts. Our results indicate which traits are involved in the initial stages of ecological specialization and ecologically based reproductive isolation and demonstrate the important effect of natural selection on color pattern.

Study System

Timema species live in patchy environments, feeding and mating on a variety of host-plant species. Nymphs and adults are wingless, rest on the leaves of their host plant during the day, and feed on the leaves at night (Sandoval 1994a,b). While resting on the plants, these insects are vulnerable to predation by birds (scrub jay, *Aphelocoma coerulescens*), and lizards (western fence lizard, *Sceloporus occidentalis*; Sandoval 1994a). Insects are alive from January to June of each year, spending the rest of the year as diapausing eggs. Previous studies of *T. cristinae* have documented divergent selection on color pattern from visual predators (Sandoval 1994a,b; Nosil 2004) and reproductive isolation between populations using different host-plant species caused by divergent host preferences (Nosil et al. 2005b), immigrant inviability (Nosil 2004; Nosil et al. 2005a) and divergent mating preferences (Nosil et al. 2002, 2003).

Here we focus on two species, *T. podura* and *T. cristinae*, whose geographic ranges do not overlap. We define a “population” of insects as all the individuals captured within a homogeneous patch of a single host species (as in Sandoval 1994a,b; Nosil et al. 2002, 2003; Nosil and Crespi 2004; Nosil 2004). Thus, some populations contain one color morph, whereas others contain multiple color morphs (see below). We define individuals from different plant species as different populations, even though the plants are sometimes sympatrically distributed, because previous studies have shown reduced mating frequency between individuals from different hosts (Nosil et al. 2002, 2003; Nosil 2004).

Both species have multiple populations in a large geographical area living mainly on two different host-plant species (*Ceanothus* species and *Adenostoma fasciculatum*). We refer to these populations on different hosts collectively as *host ecotypes*. Ecotypes within each species likely interbreed in the wild in areas of population contact, indicating they belong to one biological species. For example, both morphological and molecular DNA divergence in *T. cristinae* is consistently lower between populations that are in geographic contact than between geographically separated populations (Sandoval 1994a; Nosil et al. 2003; Nosil and Crespi 2004), a pattern that indicates interbreeding and gene flow in areas of contact (Coyne and Orr 2004). Note that this observation is also relevant to host preference evolution because it indicates that movement (i.e., migration) between hosts does

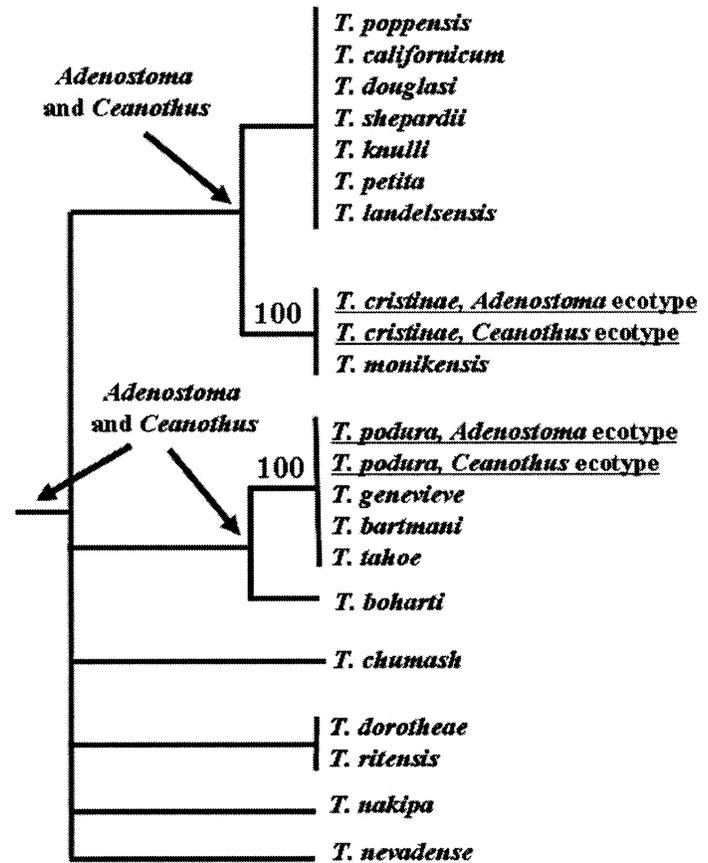


FIG. 1. Phylogeny of the genus *Timema* inferred from mitochondrial DNA (for details see Crespi and Sandoval 2000; Law and Crespi 2002). The ecotypes (underlined) on different host plants within each species examined in the current study (*T. cristinae* and *T. podura*) are more closely related to one another than to populations from the other species that use the same host plant (for details see Law and Crespi 2002). Thus, populations of the same species that use different hosts are united with 100 percent certainty in a bootstrap analysis (denoted by “100” above the relevant branch). It is unknown how much independent evolution occurred between ecotypes within each species because ancestor state reconstructions indicate that the ancestor to both species used both hosts (Crespi and Sandoval 2000). For example, the root of the entire tree is reconstructed as a generalist that used both *Ceanothus* and *Adenostoma* (denoted by arrows).

occur. In laboratory conditions, the ecotypes mate and produce offspring. We consider them ecotypes because they exhibit morphological and behavioral variation on different hosts (Sandoval 1994a; Nosil et al. 2002, 2003; Nosil and Crespi 2004) but do not imply that neutral, molecular-genetic differentiation will be structured along host lines (Dres and Mallet 2002).

The ecotypes on different host plants within each species are more closely related to one another than to populations from the other species that use the same host plant (for details see Law and Crespi 2002). For example, a mitochondrial DNA (mtDNA) phylogeny unites populations of the same species that use different hosts with 100 percent certainty in a bootstrap analysis (Fig. 1). However, it is unknown how much independent evolution occurred between ecotypes within each species because ancestor state reconstructions indicate that the

ancestor to both species used both hosts (Crespi and Sandoval 2000; see also Fig. 1). For example, the root of the species-level phylogenetic tree for the entire genus is reconstructed as a generalist that used both *Ceanothus* and *Adenostoma*. Although the two host plants appear to have been used by both species' ancestors, each species has found a novel way to match *Adenostoma* and perhaps *Ceanothus* as well. For example, *T. cristinae* uses a stripe to match *Adenostoma* whereas *T. podura* uses brown coloration to match that host (and neither species uses both; see Results). Thus, each cryptic color pattern may have arisen independently.

The host plants belong to different families with differing phytochemistry. *Adenostoma fasciculatum* (Rhamnaceae) contains cyanogens, simple phenols, and gallotannins whereas *Ceanothus* species (Rosaceae) contain many triterpenoids and possibly anthraquinones (Hegnauer 1992). Nonetheless, predators have not been observed rejecting *Timema* species.

Timema cristinae is endemic to the Santa Ynez Mountains in southern California and exhibits two main genetically determined color-pattern morphs (Sandoval 1993), with an unstriped green morph more common on *Ceanothus spinosus* and a striped green morph more common on *Adenostoma fasciculatum* (Sandoval 1994a,b). Previous field studies with *T. cristinae* and manipulative field experiments (Sandoval 1994b; Nosil 2004) have shown that each morph is most cryptic (i.e., less preyed upon) on the host on which it is more common.

Timema podura is common in the chaparral of mountainous regions of south-central California. The species is typically gray or dark red throughout its range and matches the trunks and twigs of its usual host plant, *Adenostoma fasciculatum*. An unusual green color morph from San Jacinto Mountain is associated with a different host plant, *Ceanothus leucodermis*. This species of *Ceanothus* has bluish green leaves and twigs and does not appear to have a visually matching background for the gray and red morphs (referred hereafter as gray morph for simplicity).

Here we explicitly examine three aspects of specialization in *Timema*: (1) defense against predators via crypsis, (2) physiological performance on the host plants, and (3) host preferences. Defense against predators via crypsis and host preference has been examined in previous studies for *T. cristinae* only. Physiological performance has not been examined previously in either species.

MATERIAL AND METHODS

Polymorphism in Timema podura

Here we describe the color polymorphism in *T. podura* and its association with different host plants. To quantify the association between color morph and host plant in *T. podura*, we collected walking-sticks from each host plant by shaking branches inside of a large sweep net and recording their color morph. Collections were made from several locations within two main areas in San Jacinto Mountain in 1994, 1995, and 1996, one area was dominated by *A. fasciculatum* and the other area by *Ceanothus* (Table 1).

TABLE 1. Frequency of different color morphs of *Timema podura* on their host plants in two different main habitats (C, *Ceanothus*; A, *Adenostoma*). Sample sizes represent the total number of individuals collected.

Site	Host collected	Date collected	Gray	Red	Green	Total
<i>Ceanothus</i>	A	2 April 1994	15	8	2	25
	C	2 April 1994	5	6	14	25
	C	23 April 1996	1	12	23	36
<i>Adenostoma</i>	A	2 April 1994	36	28	0	64
	A	20 February 1995	13	16	0	29
	C	20 February 1995	9	11	0	20
	C	23 April 1996	20	16	0	36

Predation Experiment with Timema podura

We measured differential visual predation on *T. podura* using wild scrub jays (*Aphelocoma coerulescens*) in an experiment similar to one conducted for *T. cristinae* (Sandoval 1994b). These birds are common in habitats of *T. podura* and are likely to be important visual predators. Three treatments were conducted to test the following hypotheses: (1) on *Ceanothus* the green morph is more cryptic than the gray morph, (2) on *Adenostoma* the gray or red morphs are more cryptic than the green morph, and (3) the gray and red morphs are more cryptic on *Adenostoma* than the green morph on *Ceanothus*. The third hypothesis may help explain why the green morph is uncommon.

The experiments were conducted in May 1996 when different scrub jay pairs were holding distinct territories, so it was possible to distinguish different pairs in the field. Only birds from urban areas, where *Timema* do not occur, were used to avoid effects of previous experience. All jays used were found within a 10-mile radius around the campus of the University of California, Santa Barbara, and were attracted to an experimental setup by offering them nuts, one at a time. The experimental setup consisted of an unpainted wood board measuring 60 cm × 40 cm, covered with 5-cm branches of a particular host plant as the background. Out of sight of the birds, we pithed one walking-stick of the gray morph and one of the green morph (matched by size and sex) and placed them on a randomly selected piece of plant, mimicking their natural position in the field. The pithed insects clung naturally to the plants but did not move. The bird arrived and ate the first insect, after which we recorded the morph eaten.

The three treatments, corresponding to the three hypotheses outlined above, were: (1) one individual of the gray morph and one of the green morph on *Ceanothus*, (2) one individual of the gray morph and one of the green morph on *Adenostoma*, and (3) a gray morph resting on *Adenostoma* and a green morph resting on *Ceanothus* (i.e., the correct hosts). Each bird received each treatment only once to avoid pseudoreplication and to minimize the effects of learning. Thus each of seven birds was subjected to one trial of each of three treatments presented in random order.

Fecundity Experiment with Timema cristinae and T. podura

To measure the effect of food quality or chemistry on lifetime fecundity and survivorship, we raised field-collected newborn nymphs inside of cages on each host in their natural

TABLE 2. Shown is an ANOVA of a split-plot design used to test for the effects of food plant of origin (FROM) and food plant on which insects were reared (TO) on lifetime fecundity (number of eggs) of *Timema cristinae* and *T. podura* (*Adenostoma* site, A site; *Ceanothus* site, C site). The FROM effect is nested within the TO effect. Blocks are groups of plants, each spatially segregated from one another and containing all treatments. FROM, TO, and their interaction are fixed effects and BLOCK is a random effect. The BLOCK \times TO interaction was used as the error term for the TO effect. The probability value for the BLOCK effect is not analyzable because there is no replication within blocks, but the term was included to improve the efficiency of the analysis.

Source	df	Sum of squares	F	P
<i>T. cristinae</i>				
BLOCK	33	137,261.3	1.31	—
TO	1	11,359.2	5.23	0.03
BLOCK \times TO	23	50,000.1	0.69	0.81
FROM	1	5,428.27	2.08	0.16
FROM \times TO	1	119.1	0.04	0.84
<i>T. podura</i> (A site)				
BLOCK	12	112,686.8	2.35	—
TO	1	22,234.9	8.88	0.031
BLOCK \times TO	5	12,519.3	0.63	0.693
FROM	1	13,940.1	3.49	0.135
FROM \times TO	1	4,294.1	1.07	0.358
<i>T. podura</i> (C site)				
BLOCK	14	72,348.1	1.89	—
TO	1	43,858.8	7.55	0.016
BLOCK \times TO	13	75,495.5	2.13	0.118
FROM	1	8,633.6	3.17	0.106
FROM \times TO	1	5,487.6	2.01	0.186

habitat. Each individual was kept inside a fine mesh bag that enclosed a branch of the food plant sufficiently large (40 cm \times 60 cm) to support it until maturity and exclude predators. Upon maturity, a male from the same site and host as the female was added to each bag containing a single female (bags with males were discarded because we were measuring fecundity). A cup of soil was added because these walking-sticks coat their eggs with soil. At the end of their life cycle, the bags were collected and all the eggs within them counted.

For *T. cristinae*, the experiment was conducted from 7 January to 30 May 1992 with individuals from various plants of each host species within 7 km of the transplantation site. Only individuals from the striped morph were collected from *Adenostoma*, and only the unstriped morph was collected from *Ceanothus*. Immediately after collection, insects were transplanted to either *Ceanothus* or *Adenostoma* plants at the experimental site.

A split-block design was used yielding four different treatments in a 2 \times 2 factorial design based on the host species of origin and the host species to which the insects were transplanted. In each of 40 blocks, there was one shrub of each host species separated by less than 2 m. Every block was 3–10 m from its nearest neighbor with the farthest blocks approximately 200 m apart. Since the number of replicates was limited by finding paired plants for the blocks, two walking-sticks per bag were used to increase the chances of obtaining a female. When males had to be added to a bag (i.e., both individuals were female), one female was discarded randomly.

For *T. podura*, the experiment was conducted from 19 February to 30 June 1995. There were two experimental sites.

One site, at approximately 1000 m elevation, was dominated by *Adenostoma* and contained only the gray morph (*Adenostoma* site hereafter). This morph was collected from either *Adenostoma* or *Ceanothus* and transplanted to either of these same hosts. The other site, at approximately 1800 m, had more *Ceanothus* than *Adenostoma* and contained both green and gray morphs (*Ceanothus* site hereafter). Both morphs were collected from either *Adenostoma* or *Ceanothus* and transplanted to either of these same hosts.

The dominance of the host plants was estimated visually based on canopy cover. The different species were distributed in a complex mosaic, with plants of the same species occurring alone, or clustered with other individuals. First instar nymphs were transplanted within their own experimental site. The *Adenostoma* site had 13 blocks and the *Ceanothus* site had 15 blocks.

We investigated lifetime fecundity (number of eggs) using ANOVA. The model followed a split-plot design using the MGL program guideline provided in SAS (Littell et al. 1991; for details see Table 2), with the main effect being the host species reared upon. A significant TO effect (the host on which the insects were raised) indicates that one host has better quality than the other or that the walking-sticks were better adapted physiologically to it. A significant FROM effect suggests that the walking-stick populations differ regarding their ability to use the host plants. A significant FROM \times TO interaction indicates that the effect of host species on fecundity is dependent on the population, indicative of local adaptation and fitness trade-offs (Via 1991).

Host Preference Experiment with *Timema podura*

Host preference in *T. cristinae* is presented elsewhere because it was studied in numerous populations ($n = 33$) in addition to those used in the fecundity experiment here, to investigate the causes of geographic variation in host preference (Nosil et al. 2005b; see Discussion for summary).

Preference tests for *T. podura* were conducted in March 1995 using individuals collected from the same sites as the fecundity experiment. Individual walking-sticks were placed in the bottom of a 500-ml plastic cup (height, 15cm) with one 12-cm host cutting from each host-plant species in the cup (total $n = 114$). The bottom end of each host cutting was inserted in a plastic aqua-pick filled with water which held the cutting upright and kept it fresh. The top of each container was covered with wire mesh, which was secured by elastic bands. These assays were initiated in the evening, and test animals were left in darkness overnight. In the morning, we recorded which host species each individual was resting on. Each individual was used only once and the branches of each host species were paired by collection site within each cup. As noted, the *Adenostoma* site contains only one morph. The *Ceanothus* site does contain both morphs, but the gray morph is rare. Thus, our analyses focus on whether the dominant host in the environment or the immediate host of origin (rather than color morph per se) affects host preference.

RESULTS

Morph Frequencies of Timema podura

We tested whether morphological divergence in color has occurred between populations feeding on different hosts. At the *Adenostoma* site, the green morph was absent and the gray and red morphs occurred in similar frequencies on the two host plants (Table 1). At the *Ceanothus* site, color morph was dependent on host of origin such that gray and red individuals were more common on *Adenostoma* and green individuals were more common on *Ceanothus* ($\chi^2 = 29.2$, $P < 0.001$; samples pooled among dates after being tested for homogeneity, heterogeneity $\chi^2 = 1.6$, $P > 0.05$; Table 1). Thus the two sites differed in morph frequency ($P < 0.01$, chi-squared test).

Predation Trials with Timema podura

We tested for host-specific, differential predation on color morphs of *T. podura* to assess whether the divergence in color pattern documented above represents host adaptation. The results from treatments 1 and 2 are relevant for testing this hypothesis (for each individual trial the background host was the same, but color-morphs differ; in treatment 1 the host is *Ceanothus* for both morphs; in treatment 2 the host is *Adenostoma* for both morphs). The results from these treatments are consistent with color pattern representing host adaptation. Thus scrub-jays preyed differentially on the color-morphs of *T. podura* depending on which host was the background ($\chi^2 = 5.33$, $df = 1$, $P = 0.021$ for chi-squared probability, and $P < 0.05$ for Fisher's exact probability). The gray and red morphs were eaten disproportionately more than the green morph on *Ceanothus* and the inverse was found on *Adenostoma* (Fig. 2).

When both morphs were placed on each of their native host (treatment 3), the green morph resting on *Ceanothus* was eaten significantly more often than the gray or red morphs resting on *Adenostoma* (7 green eaten on *Ceanothus*, and 1 gray eaten on *Adenostoma*, $P < 0.05$).

Fecundity Experiment

We conducted a reciprocal-transplant experiment to test for fecundity trade-offs on different hosts. We found no evidence for such trade-offs. In both species, we found significant fecundity effects of the host on which the insect was reared, but no interaction between host reared upon and host of origin. Thus fecundity was always highest when individuals were reared on *Ceanothus* (Table 2, Fig. 3).

For *T. cristinae*, the rearing host plant affected fecundity but not survival of the experimental insects (number surviving in different treatments, where the first letter indicates host of origin and second indicates host transplanted to; A, *Adenostoma*; C, *Ceanothus*: AA = 48, AC = 49, CC = 49 CA = 50).

For *T. podura*, lifetime fecundity depended on the site of transplantation (*Adenostoma* or *Ceanothus* site) and on host reared upon (with no interaction between these effects; Table 2). Walking-sticks had almost three times higher fecundity when raised at the *Ceanothus* site (mean fecundity = 141.9, ± 16.2 SE, $n = 25$) than when raised at the *Adenostoma* site (mean fecundity = 47.0 ± 12.7 SE, $n = 41$; $F = 21.3$, $P < 0.001$).

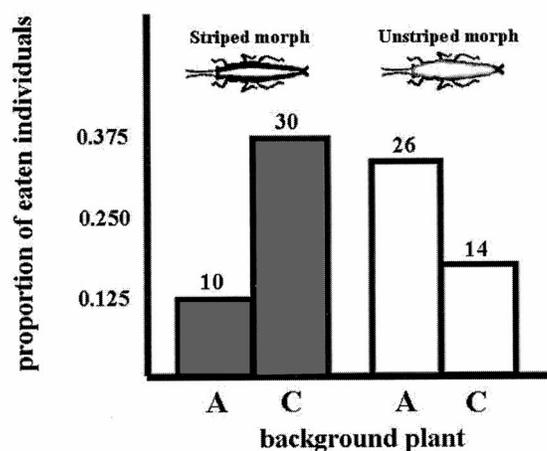
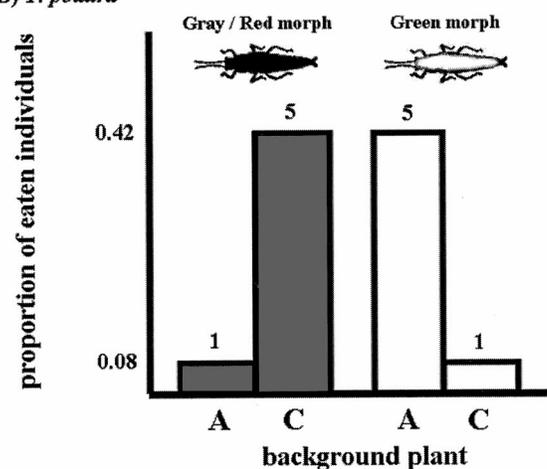
A) *T. cristinae*B) *T. podura*

FIG. 2. Predation experiments showing differential predation by birds. (A) *Timema cristinae*: proportion of eaten individuals that were unstriped versus striped individuals on different backgrounds (from Sandoval 1994a). (B) *Timema podura*: proportion of eaten individuals that were gray/red versus green morphs on different backgrounds. For both species, the results are shown from trials where the color morphs differed, but the background was the same host (i.e., treatments 1 and 2, see Materials and Methods). Proportions refer to the proportion of the total number of eaten insects that were a particular morph on a particular background. Numbers above the bars refer to the number of individuals eaten. (A, *Adenostoma* background; C, *Ceanothus* background).

At both sites, fecundity was higher for individuals reared on *Ceanothus* than for those reared on *Adenostoma* (FROM effect; Table 2, Fig. 3). There was no effect of host species of origin (FROM effect, Table 2) and no interaction between host species reared upon and host of origin (FROM \times TO).

There was a significant effect of site on survivorship of each morph (Table 3). In the *A. fasciculatum* site, gray and red morphs survived in similar proportions (89% and 90% respectively; $n = 18$ and 29 respectively, heterogeneity $\chi^2 = 0.01$, $P > 0.25$). At the *Ceanothus* site, the green and red morphs had higher survivorship than the gray morph (71%, 48%, and 18% respectively, $n = 14$, 21, and 17 respectively, heterogeneity $\chi^2 = 9.2$, $P < 0.005$). There was no significant

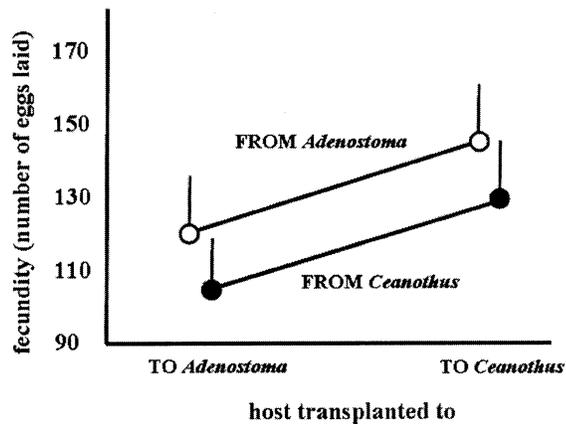
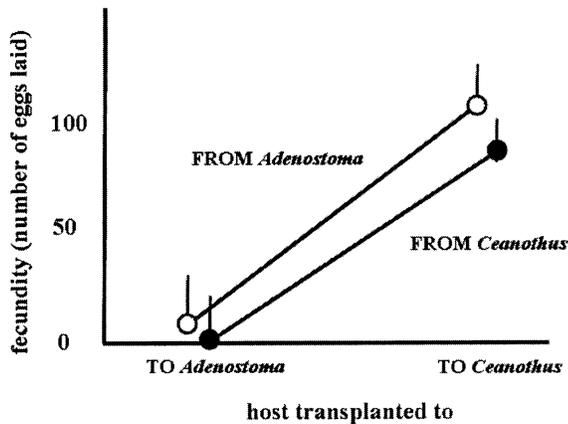
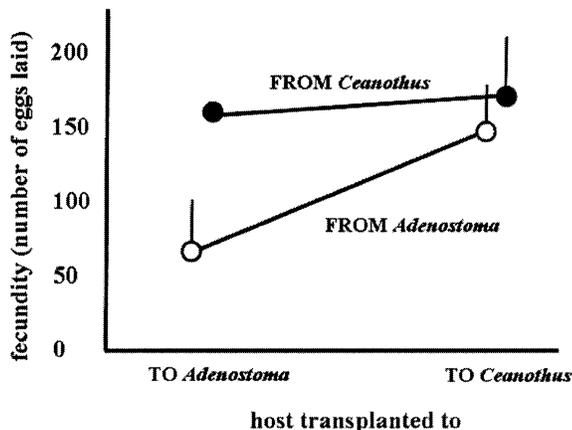
A) *T. cristinae*B) *T. podura*, *Adenostoma* siteC) *T. podura*, *Ceanothus* site

FIG. 3. Norm-of-reaction plots showing means and standard errors of fecundity of walking-sticks from *Ceanothus* or *Adenostoma* populations raised on their native or the alternative host-plant species. Fecundity is always higher when insects are reared on *Ceanothus* (for statistics see Table 2 for statistics). (A) *Timema cristinae*, (B) *T. podura* at the *Adenostoma* site, (C) *T. podura* at the *Ceanothus* site.

effect of host plant on survivorship on either site (*Ceanothus* site: survival on *Adenostoma* = 82%, on *Ceanothus* = 85%; *Adenostoma* site: survival on *Adenostoma* = 32%, on *Ceanothus* = 27%).

Host Preferences of *Timema podura*

We assessed whether individuals of *T. podura* from populations feeding on different hosts had diverged in host preference. At the *Adenostoma* site, the host plant chosen was independent of host of origin ($\chi^2 = 2.37$, $n = 48$, $df = 1$, $P = 0.12$) such that individuals from both hosts preferred *Adenostoma* (source host pooled, $\chi^2 = 8.33$, $df = 1$, $P < 0.005$; Fig. 4a). At the *Ceanothus* site, individuals collected from *Adenostoma* exhibited no preference ($\chi^2 = 0.04$, $n = 27$, $df = 1$, $P = 0.85$) and individuals collected from *Ceanothus* preferred *Ceanothus* ($\chi^2 = 5.23$, $n = 43$, $df = 1$, $P < 0.05$, Fig. 4b).

DISCUSSION

Evolution of Ecological Specialization

Ecological specialization is an important process by which biodiversity is created and maintained. Yet, we are just beginning to understand how it influences speciation. Previous phylogenetic studies have suggested that species of *Timema* walking-sticks have evolved from generalists to relative specialists in host plant use (Crespi and Sandoval 2000). We investigated the selective mechanisms favoring specialization and diversification in *Timema* by studying three traits that typically provide adaptation and specialization to host plants: crypsis, fecundity, and host preference.

The selective agent causing divergence in color pattern was similar in both species of *Timema* examined (*T. podura*, this study; *T. cristinae*, Sandoval 1994a,b; Nosil 2004). Selection from visual predators favored the evolution of a specialized cryptic color pattern. Thus, selection from predators is divergent and favors specialization on a single host, depending on the color morph. The genetic basis of color is not known in *T. podura*. As with *T. cristinae*, the morphs did not change color when fed a different host in the laboratory (C. P. Sandoval, pers. obs.). Future genetic crosses are necessary to determine the inheritance of color pattern in *T. podura*.

In contrast to the results for selection by predators, reciprocal transplant field experiments in the absence of predation show that fecundity is higher on *Ceanothus* independent of morph and host of origin in both species. Thus, fecundity selection is directional and favors specialization on *Ceanothus*, regardless of morph, and in both species.

Host preference depended on various factors including host of immediate origin, dominant host in the environment, and color-pattern morph. We expect that this variation is caused by ecological differences such as community structure, relative abundance of hosts (which affect gene flow), relative abundance of predators (which affect the strength of selection), and proximity of hosts (which affect the opportunity for selection for host preference). We will present the causes of geographic variation in host preference in *T. cristinae* elsewhere. In brief, this other work indicates that relative host abundances and host proximity are both important factors

TABLE 3. Survivorship to maturity of different color morphs of *Timema podura* in the *Adenostoma* and the *Ceanothus* sites. Individuals were raised since first instar inside mesh bags where they were protected from predators. An initial number of 52 individuals and 60 individuals were raised in the *Adenostoma* and the *Ceanothus* sites, respectively. The total numbers in the table is smaller because a few cages were vandalized during the experiment.

<i>Adenostoma</i> site		Gray	Red	Total	
Died		2	3	5	
Survived		16	26	42	
<i>Ceanothus</i> site		Green	Gray	Red	Total
Died		4	14	11	29
Survived		10	3	10	23

such that both selection and gene flow affect host preference evolution (Nosil et al. 2005b).

In this study, we studied host preference in ecological situations where the selective agents are expected to be the strongest; for example, the hosts are common and near each other so host choice can be exercised. Notably, in the population of *T. podura* where there was only the gray morph, individuals preferred the visually matching host (*Adenostoma*), despite the fact that *Ceanothus* is a better food plant with respect to fecundity, and it was available in the environment. Likewise, the striped morph from some populations of *T. cristinae* preferred *Adenostoma*, the matching host, de-

spite having higher fecundity on *Ceanothus* (Nosil et al. 2005c). These results suggest that the balance between protection from predators and food quality favors predator avoidance. Further work could focus in more detail on the causes of geographic variation in host preference in *T. podura*, and on its genetic basis (e.g., with common garden experiments).

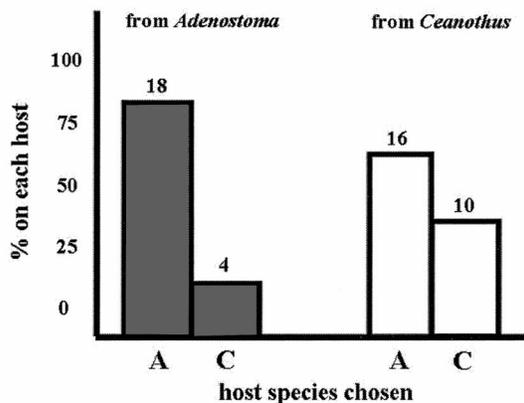
These results suggest that both food quality and predator avoidance select for specialization in the species studied because they favor the use of a single host (see Table 4 for a summary of selective forces at the level of morphs and populations). A number of questions still remain. Why has each morph not evolved to better adapt itself physiologically to the best matching host? Is physiological adaptation slower to evolve than color pattern and host preference in these insects? If so, innovative color patterns that provide good protection on a new host plant could be followed by the evolution of host preference, and host switching could occur even on lower quality plants. When placed on their correct matching hosts, the green morph of *T. podura* had higher predation than the gray morph (Fig. 3). This uncommon morph may be the result of a more recent host shift and not be as fine-tuned in crapsis as the gray and red morphs.

Several authors have suggested that predation or parasitism is an important selective agent in the evolution of food plant specialization (Gilbert and Singer 1975; Lawton and McNeill 1979; Bernays and Graham 1988; Denno et al. 1990; Bernays and Singer 2005). Most of these studies focused on the sequestration of chemicals from plants as a way to gain protection. However, the study presented here offers a different perspective: crapsis is a means of obtaining enemy-free space and may allow for further host adaptation and specialization (especially since it evolved prior to physiological adaptation in the species examined). Crapsis is perhaps the most common mechanism of predator avoidance (relative to physical or chemical defense) in herbivorous insects, and yet it is the least studied in relation to host specialization (but see Gillis 1982; Janzen 1985).

Another aspect of the study of *T. podura* is that the sites themselves differed in quality. Individuals raised at the *Ceanothus* site had a much higher fecundity than individuals raised on the *Adenostoma* site, independent of the host species they were reared upon. Perhaps site quality, independent of host quality, could select for specialized, site-adapted genotypes.

In summary, host-plant quality and predators select for specialization in the two *Timema* species studied, and selection was divergent only for traits associated with predator avoidance. Host preference evolution appears to be complex

A) *T. podura*, *Adenostoma* site



B) *T. podura*, *Ceanothus* site

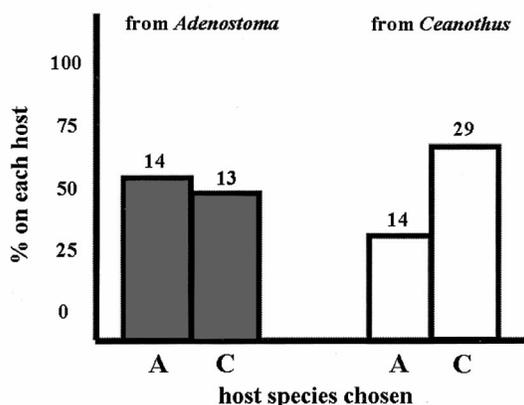


FIG. 4. Host preference of *Timema podura* collected from *Adenostoma* or *Ceanothus* at two different sites. Numbers of individuals are denoted above the bars. (A) *Adenostoma* site. (B) *Ceanothus* site.

TABLE 4. Summary of results of selection types on each morph and on each species.

Morph	Host with higher fecundity	Host with higher crypsis	Direction of selective agents (fecundity vs. crypsis) on color morph	Host preference
<i>T. cristinae</i>				
Unstriped	<i>Ceanothus</i>	<i>Ceanothus</i>	congruent	<i>Ceanothus</i>
Striped	<i>Ceanothus</i>	<i>Adenostoma</i>	counteracting	<i>Adenostoma</i>
<i>T. podura</i>				
Green	<i>Ceanothus</i>	<i>Ceanothus</i>	congruent	<i>Ceanothus</i>
Brown	<i>Ceanothus</i>	<i>Adenostoma</i>	counteracting	<i>Adenostoma</i>
Form of selection on populations	directional	diversifying		diversifying

and reflect various combinations of opposing or convergent selective pressures and gene flow. This study suggests that in mosaic environments, where gene flow and selection vary in space, genetic variation for traits associated with specialization can be highly variable. This provides support to a growing number of studies showing that geographically variable and complex ecological factors can explain variation among populations (Thompson 1994, 1997; Gomulkiewicz et al. 2000; Thompson and Cunningham 2002). Thus, heterogeneity in the types and distribution of plants may affect the diversification of herbivorous insects.

Ecological Speciation

In a recent discussion of speciation Coyne and Orr (2004, p. 57) state that “the central problem of speciation is understanding the origin of those isolating barriers that actually or potentially prevent gene flow in sympatry.” This involves two major tasks: determining which reproductive barriers were involved in the initial reduction in gene flow between populations and then understanding which evolutionary forces produced these barriers.

Our results have potential implications for both of these questions. First, they show that several forms of ecologically based reproductive isolation can evolve during the initial stages of divergence. Within both *T. cristinae* and *T. podura*, divergent host preferences between populations feeding on different hosts could cause partial premating isolation because insects feed and mate exclusively on the hosts upon which they rest. Second, individuals migrating between hosts should have, on average, lower survival relative to residents (because populations have diverged in morph frequency such that the average immigrant is the nonvisually matching morph). This process can reduce encounters, and thus gene flow, between populations (see also Nosil 2004; Nosil et al. 2005a). Reduced viability of hybrids might also occur in *Timema*, as has been observed in host race forms of the *Rhagoletis* fruit flies (Bierbaum and Bush 1990). In *T. cristinae*, sexual isolation has evolved between populations using alternate hosts and has been reinforced in parapatry in response to maladaptive “hybridization” (Nosil et al. 2002, 2003). Whether sexual isolation occurs in *T. podura* is not known.

With respect to evolutionary forces, physiological trade-offs do not occur and thus have not actively favored, nor indirectly driven, the evolution of any premating barrier. Conversely, visual predation may play a role in the evolution of each barrier (and clearly causes the “immigrant inviability”).

Finally, our findings also have implications for sympatric

speciation because the populations examined meet the requirements of many verbal and mathematic models of sympatric speciation: a strong divergent selection and a host- or habitat-based preference (Maynard Smith 1966; Bush 1969; Garcia Dorado 1986; for review see Kawecki 2004). Consistent with these models, partial premating isolation has evolved. An outstanding question then is why complete speciation has apparently not occurred? Future studies addressing this question should focus on how the spatial distribution of host plants affects gene flow, and how the strength of selection varies in space. The *Timema* walking-sticks offer opportunities for examining the complexity of natural systems where heterogeneity in ecological factors maintains genetically based variation among populations. Replicated studies across different groups in this genus could provide insight into the ecological and evolutionary factors driving the transition from a host race or ecotype to a species.

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LITERATURE CITED

- Bernays, E., and M. Graham. 1988. On the evolution of host specificity in phytophagous arthropods. *Ecology* 69:886–892.
- Bernays, E., and M. Singer. 2005. Taste alteration and endoparasites. *Nature* 436:276.
- Bierbaum, T. J., and G. L. Bush. 1990. Genetic differentiation in the viability of sibling species of *Rhagoletis* fruit flies on host plants, and the influence of reduced hybrid viability on reproductive isolation. *Entomol. Exp. Appl.* 55:105–118.
- Bush, G. L. 1969. Sympatric host race formation and speciation in the frugivorous flies of the genus *Rhagoletis* (Diptera: Tephritidae). *Evolution* 23:237–251.
- Coyne, J. A., and H. A. Orr. 2004. *Speciation*. Sinauer, Sunderland, MA.
- Crespi, B. J., and C. P. Sandoval. 2000. Phylogenetic evidence for the evolution of ecological specialization in *Timema* walking-sticks. *J. Evol. Biol.* 13:249–262.
- Denno, R. F., S. Larsson, and K. L. Olmstead. 1990. Role of enemy-free space and plant quality in host-plant selection by willow beetles. *Ecology* 71:124–137.

- Dobzhansky, T. 1951. Genetics and the origin of species. Columbia Univ. Press, New York.
- Dres, M., and J. Mallet. 2002. Host races in plant-feeding insects and their importance in sympatric speciation. *Philos. Trans. R. Soc. Lond. B* 357:471–492.
- Edmunds, G. F., and D. N. Alstad. 1978. Coevolution in insect herbivores and conifers. *Science* 199:941–945.
- Fry, J. D. 1996. The evolution of host specialization: Are trade-offs overrated? *Am. Nat.* 148S:84–107.
- Funk, D. J. 1998. Isolating a role for natural selection in speciation: host adaptation and sexual isolation in *Neochlamisus bebbianae* leaf beetles. *Evolution* 52:1744–1759.
- Funk, D. J., K. E. Filchak, and J. L. Feder. 2002. Herbivorous insects: model systems for the comparative study of speciation ecology. *Genetica* 116:251–267.
- Futuyma, D. J., and G. Moreno. 1988. The evolution of ecological specialization. *Annu. Rev. Ecol. Syst.* 19:207–233.
- Futuyma, D. J., R. P. Cort, and I. van Noordwijk. 1984. Adaptation to host plants in the fall cankerworm (*Alsophila pomataria*) and its bearing on the evolution of host affiliation in phytophagous insects. *Am. Nat.* 123:287–296.
- García Dorado, A. 1986. The effect of niche preference on polymorphism protection in a heterogeneous environment. *Evolution* 40:936–945.
- Gilbert, L. E., and M. C. Singer. 1975. Butterfly ecology. *Annu. Rev. Ecol. Syst.* 6:365–397.
- Gillis, J. E. 1982. Substrate colour-matching cues in the cryptic grasshopper *Circotettix rabula* (Rehn and Hebard). *Anim. Behav.* 30:113–116.
- Gomulkiewicz, R., J. N. Thompson, R. D. Holt, S. L. Nuismer, and M. E. Hochberg. 2000. Hot spots, cold spots, and the geographic mosaic theory of coevolution. *Am. Nat.* 156:156–174.
- Hegnauer, R. 1992. Chemotaxonomie der Pflanzen; eine Übersicht über die Verbreitung und die systematische Bedeutung der Pflanzenstoffe, von R. Hegnauer. Birkhäuser, Basel.
- Hendry, A. P. 2004. Selection against migrants contributes to the rapid evolution of ecologically dependent reproductive isolation. *Evol. Ecol. Res.* 6:1219–1236.
- Jaenike, J. 1990. Host specialization in phytophagous insects. *Annu. Rev. Ecol. Syst.* 21:243–273.
- Janzen, D. E. 1985. A host plant is more than its chemistry. III. *Nat. Hist. Surv. Bull.* 33:141–174.
- Jiggins, C. D., R. E. Naisbit, R. L. Coe, and J. M. Mallet. 2001. Reproductive isolation caused by colour pattern mimicry. *Nature* 411:302–305.
- Joshi, A., and J. N. Thompson. 1995. Trade-offs and the evolution of host specialization. *Evol. Ecol.* 9:82–92.
- Kawecki, T. J. 2004. Genetic theories of sympatric speciation. Pp. 36–53 in U. Dieckmann, M. Doebeli, J. A. J. Metz, and D. Tautz, eds. Adaptive speciation. Cambridge Univ. Press, Cambridge, U.K.
- Keese, M. C. 1998. Performance of two monophagous leaf feeding beetles (Coleoptera: Chrysomelidae) on each other's host plant: Do intrinsic factors determine host plant specialization? *J. Evol. Biol.* 11:403–419.
- Law, J. H., and B. J. Crespi. 2002. The evolution of geographic parthenogenesis in *Timema* walking-sticks. *Mol. Ecol.* 11:1471–1489.
- Lawton, J. H., and S. McNeill. 1979. Between the devil and the deep blue sea: on the problem of being a herbivore. *Symp. Br. Ecol. Soc.* 20:223–244.
- Littell, R. C., R. J. Freund, and P. C. Spector. 1991. SAS system for linear models. 3rd. ed. SAS Institute, Inc., Cary, NC.
- Mallet, J., and N. H. Barton. 1989. Strong natural selection in a warning-color hybrid zone. *Evolution* 43:421–431.
- Maynard Smith, J. 1966. Sympatric speciation. *Am. Nat.* 100:637–650.
- Mayr, E. 1963. Animal species and evolution. Harvard Univ. Press, Cambridge, MA.
- Nosil, P. 2004. Reproductive isolation caused by visual predation against migrants between divergent environments. *Proc. R. Soc. Lond. B* 271:1521–1528.
- Nosil, P., and B. J. Crespi. 2004. Does gene flow constrain adaptive divergence or vice versa? A test using ecomorphology and sexual isolation in *Timema cristinae* walking-sticks. *Evolution* 58:102–112.
- Nosil, P., B. J. Crespi, and C. Sandoval. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. *Nature* 417:441–443.
- . 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. *Proc. R. Soc. Lond. B* 270:1911–1918.
- Nosil, P., T. H. Vines, and D. J. Funk. 2005a. Perspective: Reproductive isolation caused by natural selection against immigrants from divergent habitats. *Evolution* 59:705–719.
- Nosil, P., B. J. Crespi, and C. P. Sandoval. 2005b. The evolution of host preference in allopatric versus parapatric populations of *Timema cristinae* walking-sticks. *J. Geol. Biol.* *In press*.
- Nosil, P., B. J. Crespi, C. P. Sandoval, and M. Kirkpatrick. 2005c. Migration and the genetic covariance between habitat preference and performance. *Am. Nat.* *In press*.
- Price, P. W., C. E. Bouton, P. Gross, B. A. McPheron, J. N. Thompson, and A. W. Weis. 1980. Interaction among three trophic levels: influence of plants on interactions between insect and natural enemies. *Proc. R. Soc. Lond. B* 11:41–65.
- Rausher, M. D. 1982. Population differentiation in *Euphydryas editha* butterflies: larval adaptation to different hosts. *Evolution* 36:581–590.
- . 1984. Trade-offs in performance on different hosts: evidence from within- and between-site variation in the beetle *Deloya guttata*. *Evolution* 38:582–595.
- Rundle, H. D., and P. Nosil. 2005. Ecological speciation. *Ecol. Lett.* 8:336–352.
- Sandoval, C. P. 1993. Geographic, ecological and behavioral factors affecting spatial variation in color morph frequency in the walking-stick *Timema cristinae*. Ph.D. diss. University of California, Santa Barbara.
- . 1994a. Differential visual predation on morphs of the walking-stick *Timema cristinae* (Phasmatoidea: Timemidae) and its consequences for food plant utilization. *Biol. J. Linn. Soc.* 52:341–356.
- . 1994b. The effects of gene flow and selection on morph frequencies in the walking-stick *Timema cristinae*. *Evolution* 48:1866–1879.
- Schluter, D. 2000. The ecology of adaptive radiation. Oxford Univ. Press, Oxford, U.K.
- Servedio, M., and M. Noor. 2003. The role of reinforcement in speciation: theory and data. *Annu. Rev. Ecol. Syst.* 34:339–364.
- Singer, M. C., D. Ng., D. Vasco, and C. D. Thomas. 1992. Rapidly evolving associations among oviposition preferences fail to constrain evolution of insect diet. *Am. Nat.* 139:9–20.
- Strong, D. R., J. H. Lawton, and R. Southwood. 1984. Insects on plants: community patterns and mechanisms. Blackwell Scientific Publications, London.
- Thompson, J. N. 1988. Coevolution and alternative hypotheses on insect/plant interactions. *Ecology* 69:893–895.
- . 1994. The coevolutionary process. Univ. of Chicago Press, Chicago.
- . 1997. Evaluating the dynamics of coevolution among geographically structured populations. *Ecology* 78:1619–1623.
- Thompson, J. N., and B. M. Cunningham. 2002. Geographic structure and dynamics of coevolutionary selection. *Nature* 417:735–738.
- Via, S. 1991. The genetic structure of host plant adaptation in a spatial patchwork: demographic variability among reciprocally transplanted pea aphid clones. *Evolution* 45:827–852.
- . 1999. Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. *Evolution* 53:1446–1457.
- Via, S., A. C. Bouck, and S. Skillman. 2000. Reproductive isolation between divergent races of pea aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. *Evolution* 54:1626–1637.