

Experimental evidence of host race formation in *Mitoura* butterflies (Lepidoptera: Lycaenidae)

Michelle H. Downey and Chris C. Nice

M. H. Downey (mhdowney@txstate.edu) and C. C. Nice, Dept of Biology, Population and Conservation Biology Program, Texas State Univ.-San Marcos, San Marcos, TX 78666, USA.

A population of herbivorous insects that shifts to a novel host can experience selection pressures that result in adaptation to the new resource. Host race formation, considered an early stage of the speciation process, may result. The current study investigates host shifts and variation in traits potentially involved in the evolution of reproductive isolation among populations of the juniper hairstreak butterfly, *Mitoura gryneus*. *Mitoura* are closely associated with their host trees (Cupressaceae) and exhibit host plant fidelity: in addition to larval development and oviposition, host trees support male leks and mating. Female oviposition preference for the natal host, and differential fitness of larvae when reared on natal versus alternate hosts, are indications that specialization and local adaptation to the natal host plant are occurring. Populations with single host plant associations (*Juniperus ashei*, *J. pinchotii* and *J. virginiana*) as well as populations with multiple hosts (both *J. ashei* and *J. pinchotii*) were examined. Concordance between female preference and larval performance was found for *J. ashei*-associated populations. Population-level variation in the patterns of female preference and larval performance, both within and among host associations, may reflect differences in the timing and direction of colonization of hosts. For a single nominal species that otherwise exhibits no morphological or phenological differences, the experimental assessment of specialization and host fidelity in *M. gryneus* provides strong support for the hypothesis of ongoing host race formation in these butterflies.

The recent advent of ecological speciation theory has placed a renewed emphasis on natural selection in promoting reproductive isolation and population divergence (Schluter 2001, Rundle and Nosil 2005, Funk and Nosil 2008). Phytophagous insects, among the most species-rich groups on the planet, often exhibit specialization and host-associated life history adaptations in traits related to feeding, development, oviposition, and mating (Thompson 1988a, Jaenike 1990, Funk et al. 2002). For specialized phytophagous insects, selection experienced during the switch to a novel host can lead to the initial innovations that drive an adaptive radiation (Schluter 2000). Thus, plant–insect systems are ideal for assessing the role and relative importance of ecological factors in the process of speciation (Funk et al. 2002).

The process of speciation is complex and it is unlikely that divergence leading to speciation occurs strictly in allopatric or sympatric conditions (Feder et al. 2003, Michel et al. 2007). Ecological speciation restructures the allopatric and sympatric models of speciation in terms of factors such as life history traits and resource use, rather than biogeography (Schluter 2001, Rundle and Nosil 2005, Nosil 2008). Thus ecological speciation can occur in any spatial arrangement of populations, and understanding the mechanisms of speciation (both those that initiate and also maintain reproductive isolation) are emphasized. Many studies have taken a macroevolutionary approach in examining speciation in

phytophagous insects, comparing phylogenies in relation to host plant use (Janz and Nylin 1998, Moreau et al. 2006). Taking a population-level approach and shifting the focus to ongoing speciation events at early stages is useful in gaining further insight into the initial mechanisms of the process, as well as how divergence may be maintained or inhibited (Fitzpatrick et al. 2009, Via 2009).

Plant–insect systems can be used to evaluate the role of ecology in population divergence, as many insect herbivores are specialized on their hosts and this process appears to be ongoing in several well studied examples (Feder et al. 1998, Funk 1998, Via 1999, Nosil et al. 2002). The switch by herbivorous insects to novel hosts, and the subsequent evolution of specialization and host fidelity, can lead to reproductive isolation between populations resulting in host race formation. Host plant fidelity describes the close association of phytophagous insects with their host plants, with adults reproducing on the same host species that is used in earlier life history stages (Feder et al. 1994). If both sexes evolve responses to visual and chemical cues from the host, and/or aggregate on the host plant, this can facilitate finding mates and lead to assortative mating on the host. Positive assortative mating based on host plant use is important in initiating and/or maintaining reproductive isolation between different host-associated entities, or ‘host races’. Host races are considered to be intermediary stages towards speciation, where

partial gene flow among diverging populations is possible (Diehl and Bush 1984, Drès and Mallet 2002).

The juniper hairstreak butterflies in the genus *Mitoura* represent a species complex of several nominal species (alternatively considered subspecies to *M. gryneus*) that are distributed throughout North America, using different species of trees in the cypress family (Cupressaceae) as host plants (Johnson 1981, Miller and Brown 1981, Scott 1992). *Mitoura* are closely associated with their particular hosts and exhibit behaviors associated with host fidelity and specialization: males lek and mating occurs on host trees, and females oviposit and larvae develop on the host (Forister 2004). Therefore, host race formation is a useful model to explain the divergence of *Mitoura* into different evolutionary units. Species boundaries within the *Mitoura* complex are not agreed upon in the literature (Johnson 1981, Miller and Brown 1981, Scott 1992), which could indicate that speciation is ongoing or incomplete for these butterflies. While the objective of the current study is not to delineate taxonomic boundaries within this group, the concept of a host race is useful in distinguishing different lineages and in understanding the patterns and processes of divergence.

Mitoura butterflies appear to exhibit a high degree of specialization on alternate host plants (Forister 2004, 2005) and thus are a useful system to examine the evolutionary consequences of host fidelity. Forister (2004, 2005) examined three nominal species of *Mitoura* in northern California associated with four different host plants occurring in both sympatry and parapatry. Evidence of host-associated adaptation in the form of female oviposition preference for the natal host was found. Larval performance, in terms of pupal weight, was higher on the natal host for some, but not all, populations. Variation in preference and performance persist despite close proximity of hosts, and may thus reflect different levels of adaptation to hosts. The studies in California included butterflies that differ in morphology and phenology and that use host plants across three different genera of Cupressaceae. The current study examines one nominal species of *Mitoura* (*M. gryneus*) in Texas. These butterflies have minimal phenological and no apparent morphological differences and are associated with three species of juniper trees (*Juniperus*) that occur both allopatrically and sympatrically in the southern United States.

To assess whether host race formation is taking place within *Mitoura*, specialization on the natal host (i.e. the host plant with which butterflies are associated in nature) must first be evaluated. Specialization has frequently been examined in terms of the 'preference-performance' hypothesis (Gripenberg et al. 2010; also the 'naïve adaptationist' hypothesis, Courtney and Kibota 1990). For insects that oviposit directly on the host plant on which larvae will develop, natural selection is predicted to drive female preference for the host on which larval performance is highest (Levins and MacArthur 1969). A concordance between female preference for, and higher larval performance on, the natal host is used to assess the degree of specialization, with two main questions addressed in this study: 1) do females exhibit significant oviposition preference for their natal host versus alternate, potential hosts? and 2) is larval performance, as measured by fitness correlates including percent survival and developmental efficiency, higher when larvae are reared on the natal versus alternate hosts? The

presence of host specificity in oviposition behavior suggests a role for this trait in potentially influencing host-race formation and incipient speciation. Similarly, differential fitness of larvae on natal versus alternate hosts indicates a role for natural selection in driving the process of divergence in this system.

Butterflies from populations that are associated with both single and multiple host plants were examined. While the predictions for populations with single natal host associations can be considered straightforward as mentioned above, a priori predictions regarding populations with sympatric hosts are more complicated. The presence of two natal hosts could result in several possible evolutionary outcomes, including 1) two host races with distinct preference–performance correlations on alternative hosts; 2) one essentially panmictic population using (and thus preferring) two hosts equally; or 3) some intermediate outcome, which could potentially result from gene flow following secondary contact between two host races. While preference–performance analyses may not be sufficient to distinguish among these alternative outcomes, these experiments are a necessary first step in understanding if the conditions for host race formation are present within this system.

Methods

Butterfly biology

Butterflies in the genus *Mitoura* (Lycaenidae) are found throughout North America, with species differing in phenology of flights during the year. The juniper hairstreak, *Mitoura gryneus*, represents a species complex that includes *M. muiri*, *M. nelsoni*, *M. siva*, *M. sweadneri*, *M. thornei* and others, all of which are considered by some taxonomists to be either separate species or subspecies of *M. gryneus* (Johnson 1981, Miller and Brown 1981, Scott 1992). Host plant association is important in many of these taxonomic designations. Throughout much of the eastern United States, *M. gryneus* uses *Juniperus virginiana* as the sole host plant (with exceptions such as *M. sweadneri* with *J. silicicola* in Florida). In the western and northwestern regions of North America, *Mitoura* are more taxonomically diverse and are associated with a greater number of Cupressaceous hosts in several genera including *Juniperus*, *Cupressus* and *Calocedrus* (Johnson 1981).

The ecological landscape for *M. gryneus* in Texas is unique because several species of potential host plants (*Juniperus* spp.) are found in both allopatry and sympatry. Three host associations of *M. gryneus* on juniper trees (*J. virginiana*, *J. ashei* and *J. pinchotii*) are examined in this study. While eastern red cedar (*J. virginiana*) occurs throughout most of eastern North America, the southwestern-most extent of its range lies in eastern Texas. Butterflies were sampled from areas in which *J. virginiana* is allopatric with respect to the range of the other hosts. Ashe juniper (*J. ashei*) occurs throughout central and northern Texas. Redberry juniper (*J. pinchotii*) occurs primarily in western/northwestern Texas. Butterflies were sampled from areas in which *J. pinchotii* and *J. ashei* occur both allopatrically and sympatrically (Fig. 1). For the purposes of this study, a 'population' is considered a discrete area where butterflies were sampled

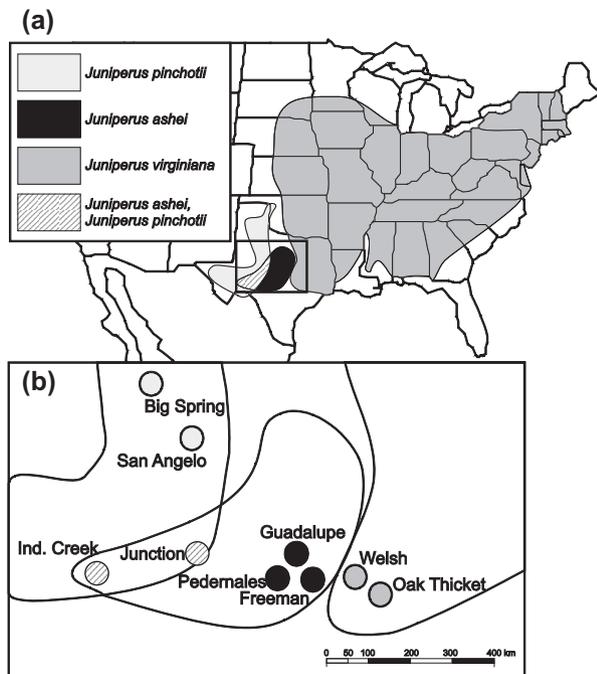


Figure 1. (a) Range of *Juniperus* hosts examined in this study and (b) locations of study populations.

that is approximately ≥ 30 km away from other sampling areas (since these are small butterflies and generally not found far from host plants, this distance was considered sufficiently outside of the normal 'cruising range' for this species) (Fig. 1).

Female oviposition preference

Butterflies were collected during the spring and summer of 2008 and 2009 from nine different populations, with either a single host association (*J. ashei*, *J. pinchotii* or *J. virginiana*) or with sympatric hosts (*J. ashei*–*J. pinchotii*) (Fig. 1). To test the prediction that *Mitoura* butterflies are specialized on their associated hosts, oviposition preference trials were conducted. Wild-caught females were placed individually in cages (~ 30 cm³) with approximately equal amounts of branch clippings from each of the three species of *Juniperus* (e.g. one from the natal host, plus the two alternate hosts). Butterflies were fed periodically with Gatorade and misted daily with water. After 72 h, the number of eggs deposited per host plant per individual female was recorded. In order to minimize any effects from intraspecific variation of host trees, branches were collected haphazardly from trees at all study sites, with no more than one branch taken from an individual tree at a time. Branches were stored in refrigeration at 10°C for up to four weeks (Forister 2005).

The decision to use choice (simultaneous presentation of hosts) instead of no-choice (sequential presentation of hosts, Singer et al. 1992) in the design of oviposition preference trials was informed by previous work by Forister (2008) who found that (for *Mitoura*) choice tests can provide similar outcomes as no-choice tests. Choice tests were also more efficient in terms of sample size (given the small number of females from some populations).

Larval performance

To test the prediction that larval performance will be greater on natal hosts, larvae were reared in a split brood design on the three different host plants (Forister 2004). Eggs from female oviposition trials were removed from branches and placed in petri dishes. Once eggs hatched, larvae were placed (up to five per rearing group) on branch clippings of the different host plant treatments, with roughly equal numbers of progeny from each female reared on all three hosts. Larvae were reared in incubation chambers at constant temperature (27°C) and equal (12 L:12 D) light-dark cycles. Cups were monitored daily, and plant material was replaced as needed. Pupal weights were taken within 24 h of pupation using a scale, and weighed to the nearest milligram. Three fitness correlates were directly measured for each larva: survival to pupation, weight (mg) at pupation, and time (d) to pupation. An index of 'developmental efficiency' (DE) was calculated as the ratio of pupal weight to development time (i.e. days to pupation), with the assumption that a faster development translates to greater efficiency of resource use. In addition, a shorter development time means less time spent in a vulnerable larval stage (i.e. 'slow growth, high mortality hypothesis' Feeny 1976, Clancy and Price 1987; but see Benrey and Denno 1997, Nylin and Gotthard 1998, Fordyce and Shapiro 2003).

Diapause strategy may be a potential source of bias in regard to evaluating DE, given that there can be intraspecific variation in butterflies that will undergo direct development versus diapause. Individuals that are 'set' to diapause potentially have a greater weight at pupation (Hunter and McNeil 1997). *Mitoura gryneus* is multivoltine in the study area and has been observed in the field as late as October (Nice unpubl.). Although diapause has not been specifically examined in *Mitoura*, during laboratory rearing, the majority of individuals (>90%) eclosed after approximately 14 days (Downey unpubl.). To minimize the chance that individuals collected might undergo a facultative winter diapause, sampling was conducted early in the year (no later than August).

Statistical analyses

Female preference was assessed at the level of natal host association, with populations grouped together within each single natal host association (sympatric host populations were analyzed separately). The number of eggs laid per plant for each female was analyzed in a nonparametric Quade test (analogous to a randomized, blocked ANOVA, Conover 1999). Each preference arena for a female was considered a block, and the number of eggs laid on each host determined the relative ranking of hosts within a block. Blocks were then weighted by the range in number of eggs laid (e.g. the greater the range, the more weight assigned to that female). Preference was also assessed at the population level; if significant differences in preference were detected, then a post hoc analysis determining relative ranking of host plants by females was conducted. Finally, if post hoc comparisons revealed that two hosts were equally preferred over the third host, then a heterogeneity G-test (Sokal and Rohlf 1995) was conducted to address the question of whether females were laying eggs

in roughly equal proportions on both of the more-preferred hosts, or if there were distinct groups of females laying a greater proportion of eggs on one host versus the other.

Larval performance was evaluated using analysis of variance (ANOVA). Due to space limitations, larvae were reared in small groups; since each individual larva within a cup could not be treated as independent, the 'rearing cup' was considered the replicate for statistical analyses, and data were collected for individual pupae and averaged per rearing cup. Populations with a single host association were analyzed separately from the sympatric-host populations. Percent survival was evaluated as the proportion of larvae within each rearing cup surviving to pupation. The data were not normally distributed, and most cups had a proportion of survival near 0 or 1. Therefore the data were transformed using the empirical logistic transformation (Cox and Snell 1989). The response variable was z (transformed average per cup survival) weighted by w (that takes into account the number of larvae initially in each cup). Other performance response variables examined included weight at pupation, time to pupation, and the ratio of weight: time to pupation (DE). These measurements were recorded for each pupa, and then an average rearing cup value was calculated. Percent survival, weight at pupation, time to pupation, and DE were examined in separate ANOVAs, with natal host association, population (nested within natal host association), and treatment as fixed factors. While female parent information ('family') was recorded for each rearing cup, uneven sample sizes and missing data (due to variation in number of eggs laid per female) for all treatment/population combinations within each family precluded the use of 'family' as a factor in performance analyses. Interactions examined included population crossed with treatment, and natal host association crossed with treatment. ANOVAs were conducted using JMP-IN software, ver. 8.0 (SAS Inst. 2008).

Results

Female oviposition preference

A total of 138 preference trials were conducted for female *Mitoura gryneus* from seven populations associated with a single host, and two populations associated with both *Juniperus ashei* and *J. pinchotii*. Female preference varied among the different host associations. Females from *J. ashei*-associated populations showed a clear host plant preference hierarchy, and significantly preferred their natal host with *J. pinchotii* ranked second and *J. virginiana* last (Fig. 2, Supplementary material Appendix 1). Females from *J. pinchotii*-associated populations showed equal preference for their natal host and *J. ashei*, with both of these plants preferred over *J. virginiana*. In contrast, butterflies sampled from populations associated with *J. virginiana* did not exhibit significant oviposition preference for any hosts (Fig. 2, Supplementary material Appendix 1).

Female oviposition preference differed between the two populations in which *J. ashei* and *J. pinchotii* host plants are sympatric. Female preference in the Junction population was similar to that found with *J. ashei*-only associated populations, with a preference hierarchy of *J. ashei*, followed by *J. pinchotii*, and finally *J. virginiana*. Females at Independence Creek, however, preferred both *J. ashei* and *J. pinchotii* equally over *J. virginiana*, similar to the *J. pinchotii*-only associated populations (Fig. 2, Supplementary material Appendix 1).

The overall pattern of preference observed for butterflies from Independence Creek was further examined in a heterogeneity G-test to assess whether individual females laid eggs in roughly equal proportions among the two preferred hosts, or whether individual females laid more eggs on one host

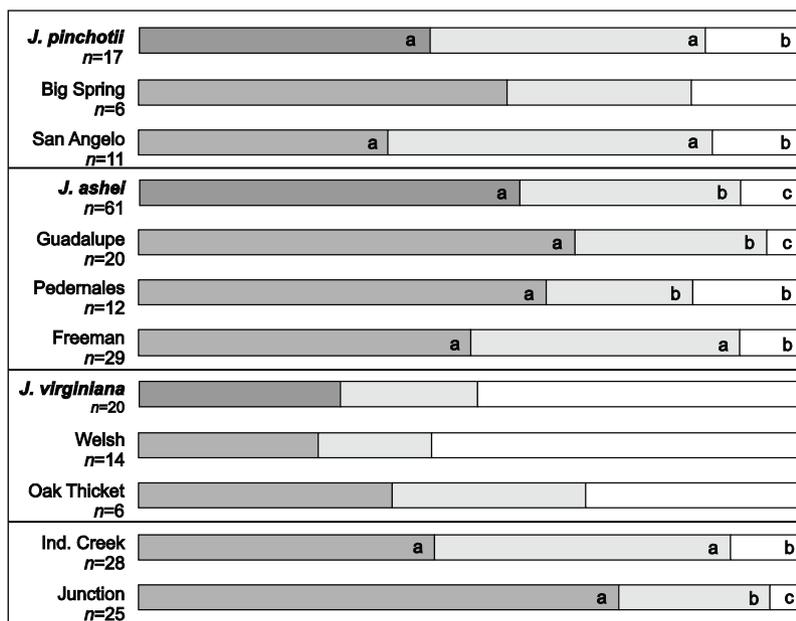


Figure 2. Female oviposition preference results by natal host association (in bold) and for individual populations (Ind. Creek and Junction populations are where the hosts *J. ashei* and *J. pinchotii* are sympatric). Bars indicate the average proportion of total eggs laid on each plant; dark grey is *Juniperus ashei*, light grey is *J. pinchotii*, and white is *J. virginiana*. Lowercase letters on graph indicate results of post hoc analysis if significant differences were found.

versus the other. Significant heterogeneity was found among females for the proportion of eggs laid on *J. ashei* versus *J. pinchotii* (heterogeneity $G = 1496.92$, $DF = 27$, $p < 0.001$), with a post hoc test revealing some females laying a greater proportion on *J. ashei*, and others laying a greater proportion on *J. pinchotii*. There were also females from Independence Creek that were intermediate in their preference, including a few individuals that laid equal proportions on both hosts (which is the pattern predicted if individual females equally preferred two hosts). Significant heterogeneity of preference among individual females was also found within the *J. ashei*-associated Freeman Ranch and the *J. pinchotii*-associated San Angelo populations, suggesting that not all individual females were expressing preference for *J. ashei* and *J. pinchotii* in the same way, and that the finding of 'equal preference' represents the composite preference of individual females.

An ANOVA conducted for each performance response variable (percent survival; weight at pupation; time to pupation; and developmental efficiency) revealed significant interactions between natal host association and treatment plant on which larvae were reared for all performance measures (Supplementary material Appendix 5). A significant interaction was also detected between population (nested within natal host association) and treatment for the response variables of time to pupation ($DF = 8$, $p = 0.045$; Supplementary material Appendix 5) and developmental efficiency ($DF = 8$, $p = 0.0038$; Supplementary material Appendix 5) indicating populations within a host association differ in their response to treatment. However, these differences were not in the form of opposing trends; that is, all *J. ashei*-associated populations had the general trend of having higher DE on their natal host over the alternate hosts. Therefore, the following results will focus on groupings by natal host association and population-level treatment differences.

Larval performance: percent survival

A total of 3640 larvae housed in 1238 rearing cups were established across all host plant treatments. No larvae survived to pupation in approximately 25% of rearing cups; in general, larvae that did not survive to pupation died at an early instar, without establishing a feeding site on the plant. For *J. ashei*- and *J. pinchotii*-associated populations, mean percent survival did not differ between the *J. ashei* and *J. pinchotii* treatments, although each of these treatments resulted in greater survival when compared with *J. virginiana*. For *J. virginiana*-associated populations mean percent survival did not differ when larvae were reared on natal versus non-natal hosts (Fig. 3, Supplementary material Appendix 2, 5).

For host-sympatric populations, the patterns observed in larval survival to pupation mirrored female preference: larvae at Junction had highest survivorship on *J. ashei*, followed by *J. pinchotii* and *J. virginiana*, while at Independence Creek larvae survived equally well on *J. ashei* or *J. pinchotii*, and survivorship on both hosts was greater than on *J. virginiana* (Fig. 4, Supplementary material Appendix 2).

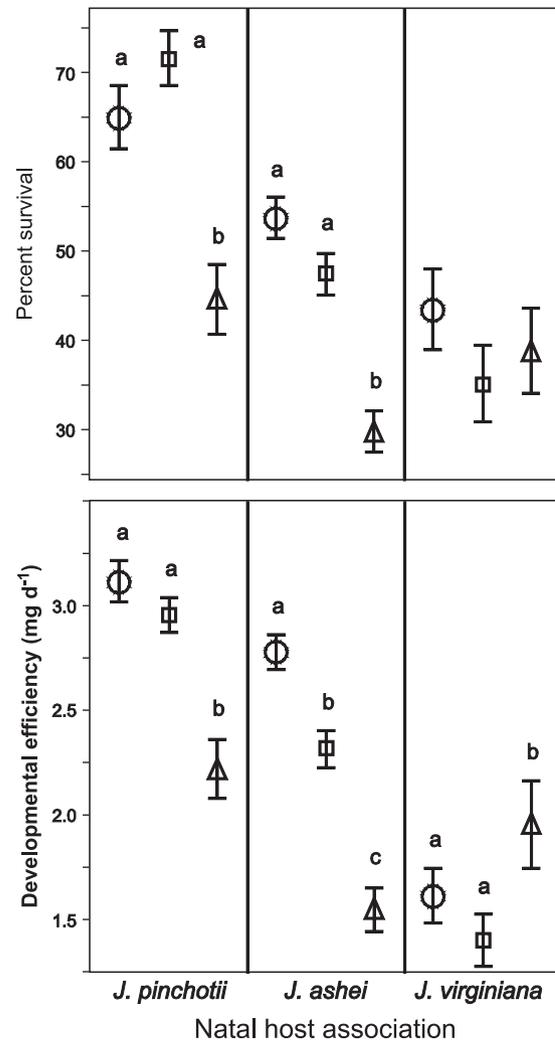


Figure 3. Larval performance results by natal host association (vertical panels of graphs). Symbols are experimental plant treatments: circles = *Juniperus ashei*; squares = *J. pinchotii*; triangles = *J. virginiana*. Lowercase letters represent results of post-hoc analysis within each host association if significant differences were found. Populations with sympatric hosts (Ind. Creek and Junction) were excluded.

Larval performance: weight at pupation

Rearing cups for which ≥ 1 larva survived to pupation were used in subsequent analyses of larval performance, including weight at pupation, time to pupation, and DE (the ratio of weight : time to pupation). For insects, weight at pupation can be used as a fitness correlate related to fecundity, since body size at adulthood has been found to be strongly positively correlated with egg load (reviewed by Honek 1993). *Juniperus ashei*-associated larvae had higher pupal weights when reared on their natal host and *J. pinchotii*; weights were significantly lower when reared on *J. virginiana* (Table 1). Progeny of *J. virginiana*-associated butterflies had significantly higher pupal weights when reared on the natal host in comparison to both *J. ashei* and *J. pinchotii*. Larvae from *J. pinchotii*-associated populations attained the highest weights at pupation on *J. ashei*, and pupal weights did not significantly differ when reared on

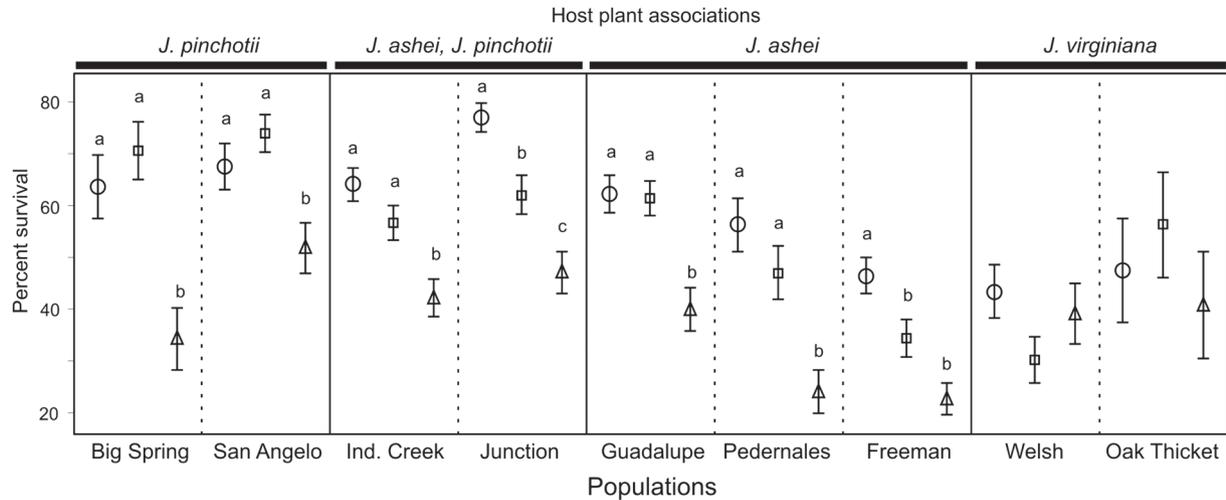


Figure 4. Larval survival for all populations. Symbols are experimental plant treatments: circles = *Juniperus ashei*; squares = *J. pinchotii*; triangles = *J. virginiana*. Lowercase letters represent results of post hoc analysis within each population if significant differences were found.

their natal host and *J. virginiana* (Table 1, Supplementary material Appendix 5).

The patterns of weight at pupation differed between the Junction and Independence Creek populations, where *J. ashei* and *J. pinchotii* are sympatric. Butterflies from Junction produced progeny that weighed significantly more at pupation when reared on *J. ashei* than when reared on *J. pinchotii* or *J. virginiana*. Independence Creek larvae had highest weights at pupation when reared on either *J. ashei* or *J. pinchotii*, with larvae reared on *J. virginiana* having lower weights (Table 1).

Larval performance: time to pupation

Time to pupation gauges how efficiently nutrients are acquired and metabolized in insects that undergo complete metamorphosis. *J. ashei*-associated larvae reached pupation fastest when reared on their natal host (Supplementary material Appendix 3). *Juniperus pinchotii*-associated larvae pupated on *J. ashei* and *J. pinchotii* after a similar length of

time, and this was faster than when reared *J. virginiana*. Butterflies from populations associated with *J. virginiana* had the shortest development time when reared on their natal host, although this did not differ significantly from those reared on *J. ashei* (Supplementary material Appendix 3, 5).

Butterflies from areas of host sympatry again differed in their patterns of time to pupation according to the source population. Larvae from the Junction population pupated fastest on *J. ashei*; whereas for larvae from Independence Creek, time to pupation did not differ significantly between larvae reared on *J. ashei* or *J. pinchotii*, but was faster on either over *J. virginiana* (Supplementary material Appendix 3).

Larval performance: developmental efficiency

While both weight at, and time to, pupation can be considered individually as fitness correlates, the composite metric (pupal weight : development time) provides an estimate of relative DE for larvae. For both *J. ashei*- and *J. virginiana*-associated populations, DE was significantly higher on the

Table 1. Mean weight at pupation (mg) of larvae reared on different hosts. Populations pooled within host plant association at top of table, followed by individual population results. Superscript letters indicate results of post hoc test if significant differences were found.

Host association	Population	n	DF _(N,D)	F	p-value	Mean weight (mg) ± SE when reared on:		
						<i>J. ashei</i>	<i>J. pinchotii</i>	<i>J. virginiana</i>
<i>J. ashei</i>	all populations	338	2,335	9.57	<0.001	95.56 (1.39) ^a	89.79 (1.51) ^b	79.33 (1.98) ^c
<i>J. pinchotii</i>	all populations	160	2,157	6.10	<0.001	97.96 (1.91) ^a	88.31 (1.89) ^b	87.89 (2.14) ^b
<i>J. virginiana</i>	all populations	91	2,88	18.82	<0.001	69.45 (2.21) ^a	67.92 (2.08) ^a	92.00 (2.31) ^b
<i>J. pinchotii</i>	Big Spring	54	2,51	17.13	<0.001	94.59 (3.72) ^a	81.45 (3.71) ^{ab}	85.17 (2.90) ^b
<i>J. pinchotii</i>	San Angelo	106	2,103	7.08	0.0013	103.83 (2.25) ^a	90.45 (2.53) ^b	89.01 (2.20) ^b
<i>J. ashei</i> - <i>J. pinchotii</i>	Ind. Creek	197	2,194	26.16	<0.001	89.55 (2.02) ^a	89.67 (2.14) ^a	78.63 (2.26) ^b
<i>J. ashei</i> - <i>J. pinchotii</i>	Junction	64	2,61	46.85	<0.001	107.98 (2.06) ^a	96.77 (1.84) ^b	94.49 (2.06) ^b
<i>J. ashei</i>	Guadalupe	156	2,153	39.04	<0.001	101.78 (1.98) ^a	93.44 (1.80) ^b	80.32 (2.33) ^c
<i>J. ashei</i>	Pedernales Falls	64	2,61	4.54	0.0145	99.46 (3.65)	91.04 (3.79)	86.79 (4.81)
<i>J. ashei</i>	Freeman Ranch	118	2,115	6.68	0.0018	89.43 (2.18) ^a	83.78 (2.55) ^{ab}	75.85 (2.94) ^b
<i>J. virginiana</i>	Welsh	68	2,65	33.09	<0.001	70.07 (2.41) ^b	66.55 (2.33) ^b	88.66 (2.96) ^a
<i>J. virginiana</i>	Oak Thicket	23	2,20	22.30	<0.001	64.92 (3.54) ^b	68.50 (2.85) ^b	95.26 (3.15) ^a

natal host. For *J. pinchotii*-associated populations, DE did not differ between the natal host and *J. ashei*, but these DE values were significantly higher than when larvae were reared on *J. virginiana* (Fig. 3, Supplementary material Appendix 4, 5).

For those populations that are associated with both *J. ashei* and *J. pinchotii* host plants, DE differed between the two populations, but mirrored the patterns of female preference. Junction larvae had the highest DE on *J. ashei*, followed by *J. pinchotii*, with the lowest DE on *J. virginiana*. For Independence Creek butterflies, DE on *J. ashei* and *J. pinchotii* did not significantly differ, and was higher than when larvae are reared on *J. virginiana* (Fig. 5, Supplementary material Appendix 4).

Discussion

Host plant specialization

Phytophagous insects that both mate and oviposit on their host plant have in place the conditions that can lead to specialization and host race formation, considered an intermediate stage in the evolution of new species (Drès and Mallet 2002). This study tested for evidence of specialization by examining patterns of preference and performance for multiple populations of a single nominal species of hairstreak butterfly, *Mitoura gryneus*, distributed across multiple hosts of *Juniperus* that occur both allopatrically and sympatrically. Butterflies varied in host preference, with *J. ashei*-associated females preferring their natal host, *J. pinchotii*-associated females equally preferring both the natal host and *J. ashei*, and *J. virginiana*-associated females not expressing significant preference for their natal host over the alternate hosts. Larvae exhibited differential fitness, as measured by survival and developmental efficiency (DE), according to host plant treatment. *Juniperus ashei*-associated populations had highest larval survival and DE on the natal host. *Juniperus pinchotii*-associated populations

had higher survival and shorter time to pupation on the natal host as well as on *J. ashei*. For *J. virginiana* populations, larval survivorship did not differ among the different host treatments, although time to pupation was shortest on the natal host, and mean pupal weight was reduced by a third when larvae were reared on alternate hosts. Observations of both female oviposition preference and increased larval performance on natal versus alternate hosts provide evidence for specialization and indicates that the conditions for host race formation are present.

The results presented herein suggest that these putative host races are at different stages of adaptation to their natal host, possibly a reflection of the amount of time accumulated in association with a particular host (Thompson 1988b, Keeler and Chew 2008), differences among populations in the strength of selection leading to local adaptation, and/or varying levels of gene flow between different host-adapted populations. All of these processes in turn may be the result of past biogeographical patterns, both of host plant range and the direction and timing of colonization of these hosts by *Mitoura*. In addition, past host plant use in relatively older evolutionary time may help to explain the plasticity in these butterflies' ability to use different hosts (Nylin and Wahlberg 2008; see also concepts of 'ecological fitting', Jansen 1985). Much work has been done to elucidate the role of oscillations in host plant range and diversification of butterfly lineages on a macroevolutionary scale (e.g. 'oscillation hypothesis' Janz et al. 2006, Janz and Nylin 2008). However the population-level processes described here represent an important transitional stage between host range expansion and a potential host shift, which in turn will shape evolutionary trajectories and the direction of large-scale processes such as lineage splitting.

When considered in combination with previous experimental and population genetic work with *Mitoura* (Forister 2004, 2005, Nice and Shapiro 2001), the results of this study contribute to an emerging body of evidence that suggests that specialization and host race formation are occurring within *Mitoura* on Cupressaceous hosts in North America,

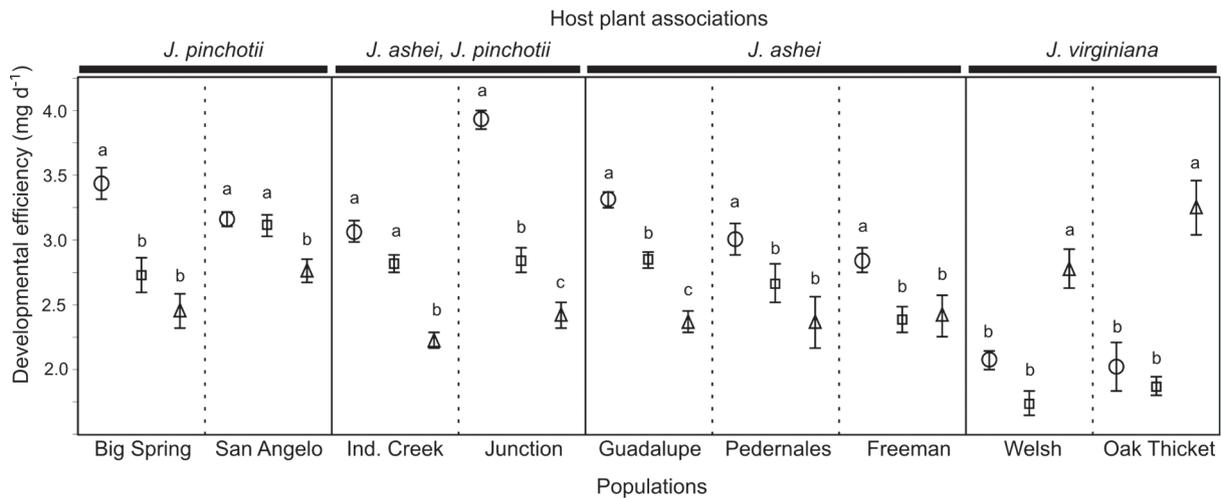


Figure 5. Developmental efficiency (DE) for all populations. Symbols are experimental plant treatments: circles = *Juniperus ashei*; squares = *J. pinchotii*; triangles = *J. virginiana*. Lowercase letters represent results of post hoc analysis within each population if significant differences were found.

with conditions in place for parallel ecological speciation events occurring at different stages in different areas of host association. Forister (2004) examined three nominal species of *Mitoura* (*M. muiri*, *M. nelsoni* and *M. siva*) associated with four different hosts in northern California (two species of *Cupressus*, one *Juniperus* and one *Calocedrus*). Female oviposition preference was correlated with larval performance for some but not all host associations; as in this study, the relationship between preference and performance measures in each population was not straightforward, possibly a reflection of different levels of adaptation to hosts, or asymmetrical gene flow between different host races. Population genetic analyses by Nice and Shapiro (2001) using allozymes and mtDNA sequence data of the same taxa examined by Forister (2004, 2005) revealed little genetic differentiation among the nominal taxa, indicating recent divergence and/or ongoing gene flow for these butterflies. However, coastal *M. muiri* was found to be significantly genetically differentiated from other taxa, despite close geographic proximity. Limited gene flow due to host plant fidelity and phenological differences (Nice and Shapiro 2001) could be an explanation, as non-ecological barriers to gene flow were low.

Single host-associated populations

For butterflies from *J. ashei*-associated populations, both female oviposition preference and increased larval performance on the natal host are consistent with natural selection for increased fitness on the natal host. For populations associated with the host *J. pinchotii*, however, both the natal host and *J. ashei* were preferred as host plants for oviposition, and larvae had similar levels of performance on these two hosts relative to *J. virginiana*. In fact, after their natal host, *J. ashei*-associated butterflies both preferred and performed better on *J. pinchotii* – despite the relative geographic proximity of *J. virginiana*-associated populations. Several factors might explain the differences in adaptation to the natal host for these populations. Butterflies may have been associated with *J. ashei* longer, allowing more time for selection on preference and performance. *Mitoura* from *J. ashei*-adapted populations may have colonized areas with *J. pinchotii* more recently, and adaptation to the new host is ongoing, or populations of *Mitoura* associated with *J. pinchotii* may have had recent or ongoing gene flow with *J. ashei*-adapted butterflies. Alternatively, *J. ashei* and *J. pinchotii* may be similar in terms of their suitability as host plants for *Mitoura*. A population genetics approach would be useful in testing these alternative (although not mutually exclusive) hypotheses, and would provide the information needed to understand geographic patterns of genetic differentiation among populations and test whether gene flow is restricted based on host plant use.

Juniperus virginiana-associated populations did not exhibit a concordance between oviposition preference and larval performance. Females did not have a clear signal of preference for the natal host, and larval survival did not significantly differ among host treatments; however, larval DE was significantly higher on the natal host. One possible explanation for the lack of female preference in these populations is related to past biogeographic patterns. If these butterflies have colonized the region from the east, and have only had experience with one host plant (*J. virginiana*) in

the recent evolutionary past, then these populations have not experienced the selective pressures associated with the presence of alternate hosts, and therefore have not had a need to ‘fine-tune’ preference for their host plant (Thompson and Pellmyr 1991, Keeler and Chew 2008).

Populations in areas of host sympatry

Examining preference and performance in *Mitoura* from areas of host sympatry can provide further clues to the evolution of adaptation to each individual host. Butterflies sampled from two different locations (Junction and Independence Creek) where the host plants *J. ashei* and *J. pinchotii* are sympatric differed in their respective patterns of female oviposition preference and larval performance (Fig. 3–5). At Junction, patterns of preference and performance were similar to those found with *J. ashei*-associated populations, with a strong association of female preference and increased larval performance (for all variables measured) on *J. ashei* (followed by *J. pinchotii* and then *J. virginiana*). Butterflies at Independence Creek, however, expressed similar patterns of preference and performance as those found in *J. pinchotii*-only populations. Female preference, and larval survival and DE, did not statistically differ between the two potential hosts, *J. ashei* and *J. pinchotii*.

Evidence of specialization of *Mitoura* on their natal host allows for the possibility that distinct host races might exist in areas of host sympatry. Disruptive natural selection, and assortative mating based on host preference, would drive divergence in this scenario. Alternatively, if gene flow is occurring freely in areas of host sympatry, then these areas are predicted to resemble the population at Independence Creek, where both *J. ashei* and *J. pinchotii* were equally preferred hosts. However, if this were the case, then the prediction could further be made that females would lay roughly equal proportions of eggs on both *J. ashei* and *J. pinchotii* in preference trials. A heterogeneity G-test revealed that distinct groups were laying more eggs on one potential host than the other, and vice versa. If preference in *Mitoura* has been found to be an independently inherited (potentially dominant) trait (Forister 2005), then the observed pattern could be a result of two host races coming into secondary contact, with some initial gene flow but also the presence of individuals still expressing preference for one host over the other. Again, population genetics data would be helpful in testing the likelihood of this scenario.

If there are different host races present in areas of host plant sympatry, then the relative frequency of the hosts could influence the composition of *Mitoura* host races, with selection favoring butterflies adapted to the more frequently occurring host. The Junction site is at the easternmost edge of the range of *J. pinchotii*, where it begins to come into contact with *J. ashei*, which appears to be the more abundant host (Downey unpubl.). Conversely, Independence Creek is located closer to the center of the range for *J. pinchotii*, and the two trees appear to be in relatively equal abundance (Downey unpubl.); therefore, two distinct host races may be able to be maintained in this area. The results for the Junction population, in which there is a strong signal of adaptation to one host (*J. ashei*) despite two potential hosts being present, could be due to relative host frequency. Alternatively,

the Junction population could have been more recently colonized by *J. ashei*-adapted *Mitoura*, and incorporation of *J. pinchotii* as a suitable host is incomplete or hindered by host fidelity.

The concept of a host race is useful in distinguishing different incipient lineages and in understanding the process of divergence at a microevolutionary scale. Preference-performance relationships among the different host-associated populations examined in this study varied from a concordance between female preference and increased larval fitness on the natal host (*J. ashei*-associated populations), to roughly equivalent preference and performance on both the natal and an alternate host (*J. pinchotii*-associated populations), to a lack of oviposition preference but highest larval performance on the natal host (*J. virginiana*-associated populations). *Mitoura* are capable of using all three host plants considered in this study, have no apparent morphological differences, and in a laboratory setting, individuals from different host-associated populations are able to successfully interbreed (Downey and Nice unpubl.). In the absence of physical boundaries to migration, the patterns observed in this study may be partially explained by host fidelity and specialization driving reproductive isolation between host associated groups – host race formation – although what is taking place here appears to be in the earliest stages of divergence. *Mitoura gryneus* species are New World taxa, and their Cupressaceous hosts are more diverse in western areas of North America. Future research that examines patterns of geographic genetic differentiation of *Mitoura*, and whether these are in alignment with host plant associations, may reveal if gene flow is limited based on host plant use. Examining these patterns on a broader scale – both geographically as well as phylogenetically – will be valuable in understanding the importance of ecological interactions in driving the diversification of phytophagous insect species on their hosts.

Acknowledgements – We thank undergraduate students (N. Cavazos, B. Davidson, E. Jaramillo, D. Jasso and B. Kearns) for help in the lab, L. Downey and C. Campbell for help collecting specimens in the field, and The Nature Conservancy and Texas Parks and Wildlife for permission to access park lands. We thank J. Ott, N. Martin and M. Forister for comments on drafts of this manuscript, and B. Weckerly and J. Fordyce for advice on statistical analyses. This work was supported by grants from The American Museum of Natural History, The Southwestern Association of Naturalists, The Texas Academy of Science and the Dept of Biology at Texas State Univ.-San Marcos.

References

- Benrey, B. and Denno, R. F. 1997. The slow-growth-high-mortality hypothesis: a test using the cabbage butterfly. – *Ecology* 78: 987–999.
- Clancy, K. M. and Price, P. W. 1987. Rapid herbivore growth enhances enemy attack - sublethal plant defenses remain a paradox. – *Ecology* 68: 733–737.
- Conover, W. J. 1999. *Practical nonparametric statistics*. – Wiley.
- Courtney, S. P. and Kibota, T. T. 1990. Mother doesn't know best: host selection by ovipositing insects. – In: Bernays, E. A. (ed.), *Insect-plant interactions*. CRC Press, pp. 161–188.
- Cox, D. R. and Snell, E. J. 1989. *Analysis of binary data*. – Chapman and Hall.
- Diehl, S. R. and Bush, G. L. 1984. An evolutionary and applied perspective of insect biotypes. – *Annu. Rev. Entomol.* 29: 471–504.
- Drès, M. and Mallet, J. 2002. Host races in plant-feeding insects and their importance in sympatric speciation. – *Phil. Trans. R. Soc. B* 357: 471–492.
- Feder, J. L. 1998. The apple maggot fly *Rhagoletis pomonella*: flies in the face of conventional wisdom about speciation? – In: Howard, D. J. and Berlocher, S. H. (eds), *Endless forms: species and speciation*. Univ. Oxford Press, pp. 130–144.
- Feder, J. L. et al. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. – *Proc. Natl Acad. Sci. USA* 91: 7990–7994.
- Feder, J. L. et al. 2003. Allopatric genetic origins for sympatric host-plant shifts and race formation in *Rhagoletis*. – *Proc. Natl Acad. Sci. USA* 100: 10314–10319.
- Feeny, P. 1976. Plant apparency and chemical defense. – In: Nansel, R. L. (ed.), *Advances in phytochemistry*. Plenum Press, pp. 1–40.
- Fitzpatrick, B. M. et al. 2009. Pattern, process and geographic modes of speciation. – *J. Evol. Biol.* 22: 2342–2347.
- Fordyce, J. A. and Shapiro, A. M. 2003. Another perspective on the slow-growth/high-mortality hypothesis: chilling effects on swallowtail larvae. – *Ecology* 84: 263–268.
- Forister, M. L. 2004. Oviposition preference and larval performance within a diverging lineage of lycaenid butterflies. – *Ecol. Entomol.* 29: 264–272.
- Forister, M. L. 2005. Independent inheritance of preference and performance in hybrids between host races of *Mitoura* butterflies (Lepidoptera : Lycaenidae). – *Evolution* 59: 1149–1155.
- Forister, M. L. 2008. Experimental design and the outcome of preference-performance assays, with examples from *Mitoura* butterflies (Lycaenidae). – *J. Lepidopt. Soc.* 62: 99–105.
- 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. and Nosil, P. 2008. Comparative analyses of ecological speciation. – In: Tilmon, K. J. (ed.), *Specialization, speciation and radiation: the evolutionary biology of herbivorous insects*. Univ. of California Press, pp. 117–135.
- Funk, D. J. et al. 2002. Herbivorous insects: model systems for the comparative study of speciation ecology. – *Genetica* 116: 251–267.
- Gripenberg, S. et al. 2010. A meta-analysis of preference-performance relationships in phytophagous insects. – *Ecol. Lett.* 13: 383–393.
- Honek, A. 1993. Intraspecific variation in body size and fecundity in insects – a general relationship. – *Oikos* 66: 483–492.
- Hunter, M. D. and McNeil, J. N. 1997. Host-plant quality influences diapause and voltinism in a polyphagous insect herbivore. – *Ecology* 78: 977–986.
- Jaenike, J. 1990. Host specialization in phytophagous insects. – *Annu. Rev. Ecol. Syst.* 21: 243–273.
- Jansen, D. H. 1985. On ecological fitting. – *Oikos* 45: 308–310.
- Janz, N. and Nylin, S. 1998. Butterflies and plants: a phylogenetic study. – *Evolution* 52: 486–502.
- Janz, N. and Nylin, S. 2008. The oscillation hypothesis of host-plant range and speciation. – In: Tilmon, K. J. (ed.), *Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects*. Univ. of California Press, pp. 203–215.
- Janz, N. et al. 2006. Diversity begets diversity: host expansions and the diversification of plant-feeding insects. – *BMC Evol. Biol.* 6: 4.
- Johnson, K. D. 1981. Revision of the *Callophryina* of the world with phylogenetic and biogeographic analyses (Lepidoptera: Lycaenidae). – PhD thesis, City Univ. of New York.

- Keeler, M. S. and Chew, F. S. 2008. Escaping an evolutionary trap: preference and performance of a native insect on an exotic invasive host. – *Oecologia* 156: 559–568.
- Levins, R. and MacArthur, R. 1969. An hypothesis to explain the incidence of monophagy. – *Ecology* 50: 910–911.
- Michel, A. P. et al. 2007. The genetic structure of hawthorn-infesting *Rhagoletis pomonella* populations in Mexico: implications for sympatric host race formation. – *Mol. Ecol.* 16: 2867–2878.
- Miller, L. D. and Brown, F. M. 1981. A catalogue and checklist of the butterflies of America north of Mexico: Memoir No. 2. – *Lepidopt. Soc.*
- Moreau, C. S. et al. 2006. Phylogeny of the ants: diversification in the age of angiosperms. – *Science* 312: 101–104.
- Nice, C. C. and Shapiro, A. M. 2001. Population genetic evidence of restricted gene flow between host races in the butterfly genus *Mitoura* (Lepidoptera : Lycaenidae). – *Ann. Entomol. Soc. Am.* 94: 257–267.
- Nosil, P. 2008. Ernst Mayr and the integration of geographic and ecological factors in speciation. – *Biol. J. Linn. Soc.* 95: 26–46.
- Nosil, P. et al. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. – *Nature* 417: 440–443.
- Nylin, S. and Gotthard, K. 1998. Plasticity in life-history traits. – *Annu. Rev. Entomol.* 43: 63–83.
- Nylin, S. and Wahlberg, N. 2008. Does plasticity drive speciation? Host-plant shifts and diversification in nymphaline butterflies (Lepidoptera : Nymphalidae) during the tertiary. – *Biol. J. Linn. Soc.* 94: 115–130.
- Rundle, H. D. and Nosil, P. 2005. Ecological speciation. – *Ecol. Lett.* 8: 336–352.
- Schluter, D. 2000. *The ecology of adaptive radiation.* – Oxford Univ. Press.
- Schluter, D. 2001. *Ecology and the origin of species.* – Trends Ecol. Evol. 16: 372–380.
- Scott, J. 1992. *The butterflies of North America: a natural history and field guide.* – Stanford Univ. Press.
- Singer, M. C. et al. 1992. Distinguishing between preference and motivation in food choice – an example from insect oviposition. – *Anim. Behav.* 44: 463–471.
- Sokal, R. R. and Rohlf, F. J. 1995. *Biometry: the principles and practice of statistics in biological research.* – W.H. Freeman.
- Thompson, J. N. 1988a. Evolutionary ecology of the relationship between oviposition preference and performance of offspring in phytophagous insects. – *Entomol. Exp. Appl.* 47: 3–14.
- Thompson, J. N. 1988b. Evolutionary genetics of oviposition preference in swallowtail butterflies. – *Evolution* 42: 1223–1234.
- Thompson, J. N. and Pellmyr, O. 1991. Evolution of oviposition behavior and host preference in Lepidoptera. – *Annu. Rev. Entomol.* 36: 65–89.
- Via, S. 1999. Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. – *Evolution* 53: 1446–1457.
- Via, S. 2009. Natural selection in action during speciation. – *Proc. Natl Acad. Sci. USA* 106: 9939–9946.

Supplementary material (available online as Appendix O19290 at <www.oikosoffice.lu.se/appendix>). Appendix 1–5.