

ADAPTIVE POPULATION DIVERGENCE IN CRYPTIC COLOR-PATTERN FOLLOWING A REDUCTION IN GENE FLOW

Patrik Nosil^{1,2,3,4}

¹Zoology Department and Biodiversity Research Centre, University of British Columbia, Vancouver BC, V6T 1Z4, Canada

³Institute for Advanced Study, Wissenschaftskolleg, Berlin, 14193, Germany

⁴E-mail: patrik.nosil@wiko-berlin.de

Received November 1, 2008

Accepted February 5, 2009

Adaptive population divergence is often driven by divergent natural selection, but can be constrained by the homogenizing effect of gene flow between populations. Indeed, a common pattern in nature is an inverse correlation between the degree of adaptive phenotypic divergence between populations and levels of gene flow between populations. However, there is essentially no experimental data on whether this correlation arises because gene flow constrains adaptation or, conversely, because adaptive divergence causes barriers to gene flow (ecological speciation). Here, I report increased adaptive divergence in cryptic color pattern between a pair of *Timema* insect populations following an experimental reduction in between-population gene flow. The reduction in gene flow arose due to a natural experiment, and thus was not replicated at a second site. However, temporal replication of the trends among six generations of data, coupled with a lack of increased adaptive divergence for two other population pairs where gene flow was not manipulated (i.e., control sites), argues that the results did not arise by chance. Estimates of dispersal ability and population size further support reduced gene flow, rather than increased genetic drift, as the cause of divergence. Thus, the findings provide experimental evidence that gene flow constrains adaptation in nature.

KEY WORDS: Adaptation, dispersal, ecological speciation, experiment, standing variation.

Our understanding of evolution in nature often derives from the reconstruction of historical events (Pagel 1999) or from extrapolating the results of laboratory studies (Rice and Hostert 1993). However, long-term monitoring and experimentation in natural populations holds the potential to directly test the processes driving and constraining evolution in the wild, as exemplified by recent field studies on the roles of various factors in evolution, including behavior (Losos et al. 2004), predation (Losos et al. 2004, 2006; Nosil and Crespi 2006), climatic variability (Spiller et al. 1998; Grant et al. 2002), human disturbance (Hendry et al. 2006), frequency-dependent selection (Olendorf et al. 2006), and competition (Grant and Grant 2006). A general topic, where ex-

perimental data from the wild is lacking is the association between adaptive divergence and gene flow (Räsänen and Hendry 2008 for review). What is the role of gene flow in constraining adaptive divergence, and vice versa?

The above question is motivated by the observation that inverse correlations between the degree of adaptive phenotypic divergence between populations and levels of genetic exchange between them (i.e., gene flow) are widespread in nature (Mayr 1963; Riechert 1993a,b; Sandoval 1994a; King and Lawson 1995; Lu and Bernatchez 1998; Crespi 2000; Hendry et al. 2001; Nosil et al. 2003; Nosil 2004; Nosil and Crespi 2004; Bolnick et al. 2008; Räsänen and Hendry 2008). Although it is often assumed that this correlation arises because gene flow constrains adaptive divergence (Mayr 1963; Räsänen and Hendry 2008), there is little direct experimental evidence testing this causal interpretation (to

²Current address: Department of Ecology and Evolutionary Biology, University of Boulder, Colorado, 80309, USA

my knowledge, only one experiment has manipulated gene flow in the wild to explicitly test whether gene flow constrains adaptation; Riechert 1993a). Such evidence is required because two general, yet opposing, hypotheses exist for why inverse associations between adaptive divergence and gene flow are widespread in nature (Riechert 1993a,b; Hendry et al. 2001; Nosil and Crespi 2004; Räsänen and Hendry 2008). First, gene flow may constrain adaptive divergence. Second, reverse causality is possible, whereby adaptive divergence causes the evolution of barriers to gene flow between populations. This process of “ecological speciation” also generates an inverse association between the degree of adaptive divergence and levels of gene flow, and has gained strong support over the last decade (Funk 1998; Lu and Bernatchez 1998; Schluter 2000; Funk et al. 2006; Nosil 2007; Nosil et al. 2008). Experiments are required to disentangle whether gene flow constrains adaptive divergence, or vice versa (Räsänen and Hendry 2008).

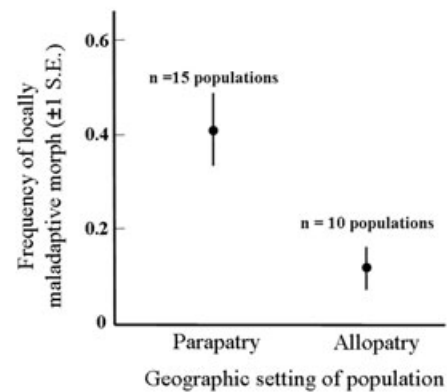
Here, multiyear field data from a natural experiment is used to test whether gene flow constrains adaptive divergence in cryptic color pattern (i.e., camouflage) between populations of *Timema cristinae* walking-stick insects living on two different host plant species (*Ceanothus spinosus* and *Adenostoma fasciculatum*). In this system, the degree of adaptive population divergence in morphological traits varies markedly among population pairs, and is inversely correlated with levels of between-host gene flow (Sandoval 1994a; Nosil et al. 2003; Nosil 2004; Nosil and Crespi 2004; Bolnick and Nosil 2007; Nosil 2008; see Fig. 1 and methods). This pattern provides the necessary preconditions to test directly whether this association arises, at least in part, because gene flow constrains adaptive divergence. Such a test was made possible by the monitoring of color-pattern morph frequencies at three different parapatric pairs of populations over a six-year period, coupled with a “natural experiment,” which reduced or eliminated gene flow between one of the three population pairs two insect generations ago. The results provide experimental evidence from nature that gene flow constrains adaptive divergence.

Methods

STUDY SYSTEM

Timema walking-sticks are wingless, herbivorous insects inhabiting Southwestern North America (Vickery 1993; Crespi and Sandoval 2000). Individuals feed and mate on the host plants upon which they rest. The current study considers *T. cristinae*, which uses two host plant species (*A. fasciculatum*: Rosaceae and *C. spinosus*: Rhamnaceae). These two host species differ strikingly in foliage and general morphology. *Ceanothus* is relatively large, tree-like, and broad-leaved. *Adenostoma* is small, bush-like, and exhibits thin, needle-like leaves.

A) Past evidence for gene flow: allopatry versus parapatry



B) Past evidence for gene flow: relative population sizes

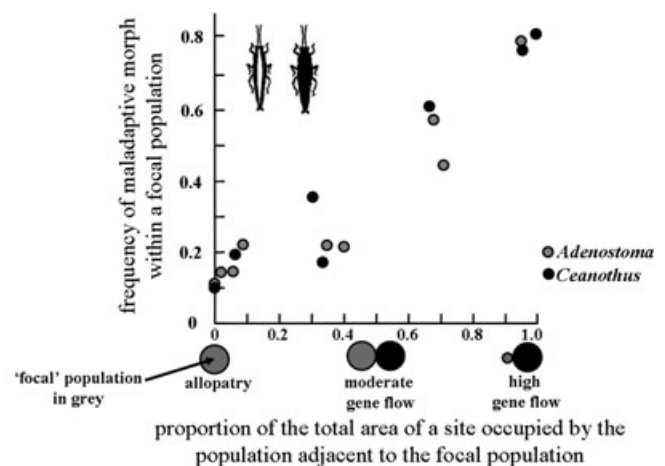


Figure 1. Previously published evidence for gene flow between populations of *Timema cristinae* living on different host plant species (*Ceanothus* vs. *Adenostoma*). (A) The mean frequency (± 1 SE) of the maladaptive color-pattern morph (i.e., striped on *Ceanothus* and unstriped on *Adenostoma*) within allopatric versus parapatric populations. (B) The mean frequency of the maladaptive color-pattern morph within a focal population as a function of the size of the population that is adjacent to the focal population (here estimated as the proportion of the total area of the site occupied by the host that is alternative to the focal population, where total area = area of focal population plus area of adjacent, neighboring population). Gene flow into the focal population increases as the size of the neighboring population increases. See text for details. Modified from Bolnick and Nosil (2007) and reprinted with permission of Blackwell Publishing.

A “population” of *T. cristinae* is defined as all of the walking-sticks collected within a homogenous patch of a single host species (Nosil et al. 2002, 2003, Nosil 2007). Patches of the two host species used by *T. cristinae* are often distributed in adjacent patches that are in geographic contact with one another. Insect populations associated with such patches are referred to as “parapatric” (Nosil et al. 2003; each parapatric population has only a single adjacent population on the alternative host).

Other host patches are separated from patches of the alternative host, usually via regions containing unsuitable hosts, by distances > 50 times the per-generation dispersal distance of *T. cristinae* (the per-generation dispersal distance is estimated at 12 m using mark–recapture methods; Sandoval 2000). Insect populations in such geographically separated patches are termed “allopatric.” A map of the populations considered in the current study is shown in Figure 2A.

ADAPTIVE DIVERGENCE DUE TO VISUAL PREDATION

Past work on *T. cristinae* demonstrated strong divergent selection between hosts on the genetically heritable trait of presence versus absence of dorsal stripe: an unstriped color pattern morph has higher survival in the face of visual predation on *Ceanothus*, whereas a striped morph has higher survival on *Adenostoma* (Sandoval 1994a,b; Nosil et al. 2003; Nosil 2004; Nosil and Crespi 2006; Nosil et al. 2006a,b; Fig. 3). Evidence for divergent selection on color-pattern stems from multiple types of experiments, including a perturbation experiment that monitored changes in morph frequency in the wild (Sandoval 1994a), predation trials with wild jays (Sandoval 1994b), and mark–recapture estimates of survival on each host species in a field experiment that manipulated the presence versus absence of visual predation (Nosil 2004). One explanation for divergent selection between hosts is that the stripe divides the body of *T. cristinae* into two thinner segments, thereby increasing crypsis when resting on the thin leaves of *Adenostoma*. In contrast, the presence of the stripe is conspicuous against the broad leaves of *Ceanothus*. In summary, divergent selection on color-pattern occurs and, consequently, populations on different hosts have diverged in morph frequency. Although fixed differences between populations in this trait are almost never observed, each morph is often more common on the host upon which it has higher survival (Figs. 1 and 3).

EVIDENCE FOR BETWEEN-HOST GENE FLOW

Past morphological, behavioral, and molecular studies have shown that: (1) gene flow occurs between adjacent parapatric populations of *T. cristinae* on different hosts and (2) that the degree of adaptive phenotypic divergence between populations is inversely correlated with levels of between-host gene flow (Sandoval 1994a, 2000; Nosil et al. 2003; Nosil 2004; Nosil and Crespi 2004; Nosil et al. 2006a,b; Bolnick and Nosil 2007). Three main types of evidence have been put forth.

The first is based upon comparing divergence between allopatric versus parapatric population pairs. For example, allopatric populations exhibit a much lower frequency of the maladaptive color-pattern morph than do parapatric populations (Fig. 1A) and thus allopatric pairs exhibit much greater adaptive divergence in color-pattern than do parapatric pairs (Fig. 3A). This pattern indicates that gene flow occurs between parapatric pairs, and that it

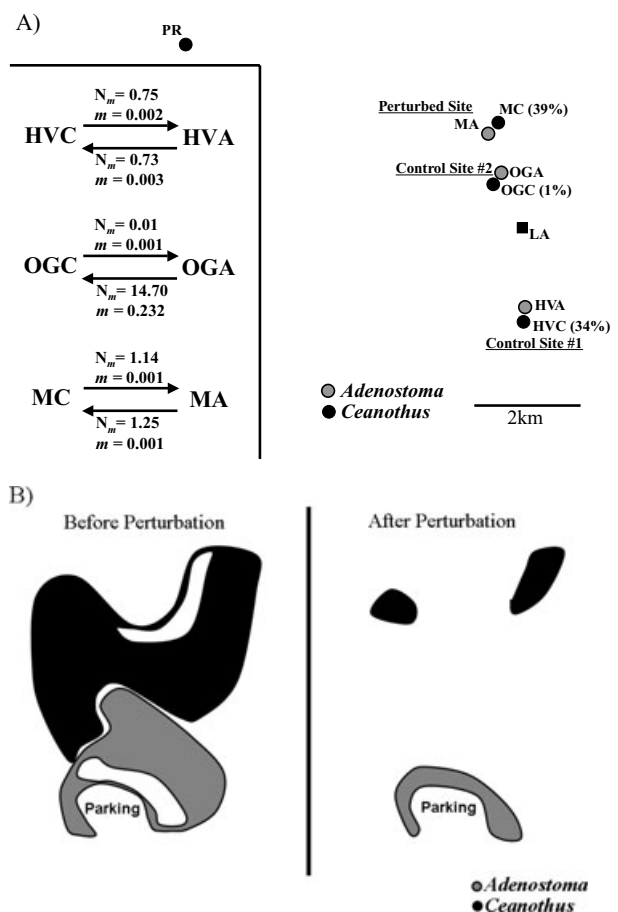


Figure 2. Maps of the study sites and information on levels of gene flow between populations on different hosts. (A) Map of all the study populations. The numbers in parentheses refer to the percentage of the total area of site that is occupied by *Ceanothus*. Also shown are migration rates into each parapatric population from their neighboring population on the alternative host. Migration rates were inferred using the coalescent-based methods of Beerli and Felsenstein (2001), applied to mitochondrial DNA sequence variation. N_m = number of migrants into a population per generation. The migration rate itself (m = proportion of population consisting of migrants) was estimated from N_m using estimates of population size (text for details). The data are from Nosil et al. (2003). (B) A schematic close-up of the perturbed site before versus after the gene flow perturbation. The figure is schematic because the actual distribution of plants after the perturbation was not mapped in detail. The minimum distance between host species following the perturbation was estimated at 36 m, with the regions between host species being occupied by bare ground. The unfilled areas within patches of each host represent areas in which the density of *T. cristinae* tended to be highest such that most individuals were captured from there, both before and after the perturbation.

may constrain divergence. Similar patterns of greater divergence between allopatric population pairs are observed for other morphological traits such as body size and shape (Nosil and Crespi 2004), for host plant feeding preference (Nosil et al. 2006a,b), and

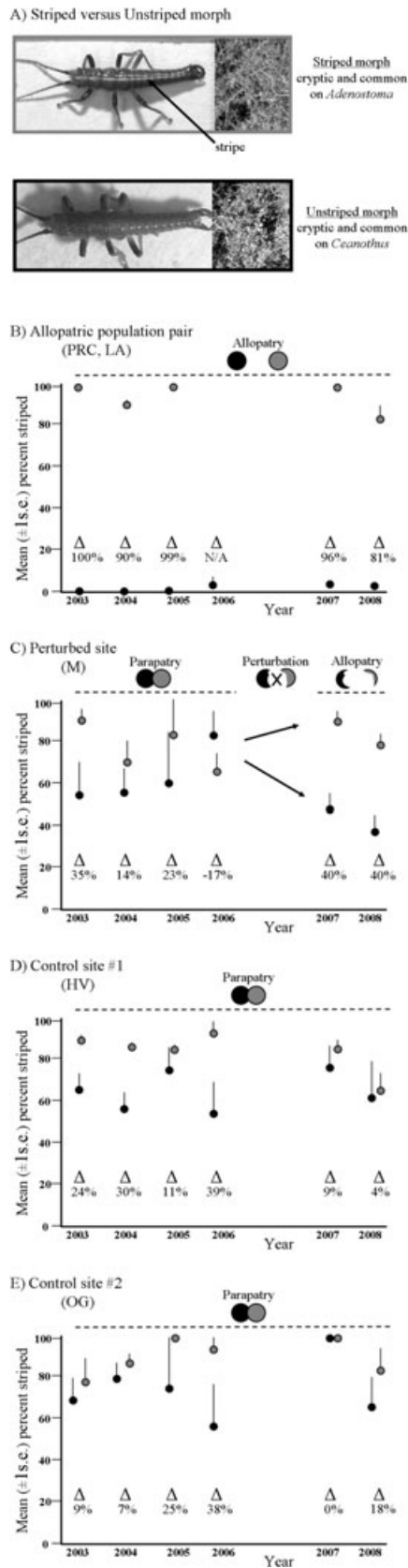


Figure 3. The frequency (mean \pm 1 SE) of the striped color-pattern morph of *Timema cristinae* on two different host plant species (black circles, *Ceanothus*; gray circles, *Adenostoma*), from

for putatively neutral molecular markers (Nosil et al. 2003). For example, Nosil et al. (2003) reported that F_{ST} inferred from mitochondrial DNA (mtDNA) sequence variation was consistently and significantly greater between geographically isolated population pairs relative to that observed between adjacent, parapatric pairs (parapatric pairs, mean $F_{ST} = 0.07$, range = 0.00–0.25, $n = 7$ pairs; geographically separated pairs, mean $F_{ST} = 0.31$, range = 0.00–0.79, $n = 129$ pairs, Mantel test, $P < 0.01$). In summary, allopatric population pairs undergo less between-host gene flow than do parapatric pairs, and allopatric pairs also exhibit greater adaptive divergence.

Second, actual levels of gene flow into parapatric populations, from their neighboring population on the alternative host, have been explicitly estimated. For example, Nosil et al. (2003) applied the coalescent-based methods of Beerli and Felsenstein (2001) to mitochondrial DNA sequence variation to estimate both N_m , the number of migrants into a population per generation, and the migration rate itself (i.e., m = proportion of population consisting of migrants, where m was estimated from N_m by inferring population size, N , from the known relationship between *T. cristinae* population size and host-plant patch size; Nosil et al. 2003 for details). These analyses revealed that substantial migration into parapatric populations was common: range of number of migrants (N_m) = 0.68–14.7, mean = 3.13; range of migration rates (m) = 0.001–0.232, mean = 0.043. Population genomic analyses based upon AFLPs also indicate ongoing gene flow between populations (Nosil et al. 2008).

Third, quantitative estimates of the correlation between levels of gene flow and the degree of adaptive divergence have been conducted (Nosil 2008 for review). For example, *T. cristinae* population size is known to be strongly and positively correlated with the size of the host plant patch occupied ($r^2 = 0.63$ and 0.53 for patches of *Ceanothus* and *Adenostoma*, respectively, see Sandoval 1994a). Thus, insect population size can be inferred from host-plant patch size (where the latter is estimated from aerial photographs, Sandoval 1994a; Nosil et al. 2003 for details). Variation in population size generates the following prediction: as the size of the population that is adjacent to a focal population increases,

2003–2008. Δ , percent divergence in morph frequency. (A) Pictures of the striped morph and the unstriped morph. (B) An allopatric population pair (for replication in other allopatric pairs see Fig. 1 or Sandoval 1994a; Nosil 2004; Bolnick and Nosil 2007), (C) the perturbed site (i.e., a formerly parapatric site that was made allopatric). (D, E) two control sites that remained parapatric during the entire six-year monitoring period. In some instances, standard errors are not highly visible because the datapoint overlaps them. Divergence in morph frequency (Δ) increased in 2007 and 2008 only for the perturbed site where gene flow was experimentally reduced.

the level of gene flow into the focal population from the adjacent population increases. Supporting this prediction, the proportion of the total area of a site that is occupied by the alternative host is positively correlated with the level of mtDNA gene flow into a focal population (Spearman rank correlation, $\rho = 0.86$ and 0.62 when using N_m and m as estimates of mtDNA gene flow, Nosil et al. 2003). A number of studies using relative population sizes and molecular data to estimate gene flow have shown a positive relationship between the level of gene flow into a population and the level of maladaptation within the population (Nosil 2008 for review). Figure 1B depicts such a relationship for the frequency of maladaptive color-pattern morphs (data from Bolnick and Nosil 2007). In turn, an inverse correlation exists between levels of between-population gene flow and the degree of adaptive divergence between populations in numerous traits including morph frequency, body size, body shape, host plant feeding preference, and quantitative measurements of color (Sandoval 1994a; Nosil and Crespi 2004; Nosil et al. 2006a,b; Nosil 2008 for review).

Importantly for the current study, past molecular and morphological data provide evidence for gene flow between all the specific parapatric population pairs examined here. For example, the coalescent-based methods from Nosil et al. (2003) described above revealed nonzero gene flow into each of the parapatric populations examined here (mean number of migrants $N_m = 3.10$ and mean migration rate $m = 0.04$). These data, including population-specific migration rates, are depicted in Figure 2A. The degree of divergence in morph frequencies supports the molecular evidence for gene flow: all the parapatric pairs examined here exhibit substantially weaker divergence in morph frequencies than do allopatric population pairs (Fig. 3). In summary, although there is partial reproductive isolation between populations of *T. cristinae* using different host plants (Nosil 2007 for review), this reproductive isolation is far from complete and there is abundant evidence for substantial levels of between-host gene flow.

NATURAL EXPERIMENT: DESCRIPTION

Morph frequencies were monitored at three sites, each comprising two adjacent populations on different hosts, from 2003 to 2008 (Fig. 2A). The population pairs at two sites (HV and OG) remained parapatric during the six-year monitoring period (control sites 1 and 2 hereafter, $n = 1062$ and 190 individuals, respectively). At the other site (M, “perturbed site” hereafter, $n = 316$), the parapatric population pair was in essence made allopatric two insect generations ago. Specifically, road crews cut down most plant individuals of both host species around fall 2006, particularly in areas within the site where the different host species were in direct contact. The remaining plant individuals from the different hosts became geographically separated from each other by regions of bare ground that spanned distances of at least 36 m, which is three times the small (12 m) per generation dispersal

distance of these wingless insects (Sandoval 2000). Notably, the minimum distance between host species prior to the perturbation was essentially zero, with the plant individuals of different species growing directly adjacent to one another in the region in which the two species were in contact. The geographic setting of this natural experiment is depicted in Figure 2B.

NATURAL EXPERIMENT: EVIDENCE FOR REDUCED GENE FLOW

The increased physical distance between different species of hosts at the perturbed site following the perturbation very likely generated a strong barrier to between-host dispersal. Because, as argued above, substantial between-host gene flow occurs, such a reduction in dispersal would result in a reduction in gene flow. Specifics are as follows.

Both mark–recapture and molecular data from *T. cristinae* suggest very limited dispersal between regions unoccupied by *Ceanothus* or *Adenostoma*. For example, Nosil (2004) used mark–recapture methods to estimate the lifetime adult survival of *T. cristinae* released onto experimental bushes of each host species in the wild. Each experimental bush was cleared of all unmarked *T. cristinae* prior to the onset of the experiment and was separated from all other experimental and nonexperimental bushes by a minimum distance of 5 m (by regions of bare ground). Two lines of evidence support extremely limited movement between bushes, despite the relatively small distances between them. First, movement between experimental bushes was never observed (i.e., individuals were never recaptured on bushes other than the one onto which they were initially released). Second, movement from nonexperimental bushes onto experimental ones was also never observed (i.e., unmarked individuals were never captured on the experimental bushes). These results suggest very limited (or even zero) dispersal across areas without *Ceanothus* or *Adenostoma*. Because such an area, of at least 36 m in length, was created at the perturbed site, a very strong barrier to between-host dispersal was likely generated. Additional evidence for limited movement across regions without *Ceanothus* or *Adenostoma* stems from the observation that populations separated by regions of unsuitable hosts by less than a few kilometers regularly exhibit significant neutral genetic divergence in mtDNA, nuclear DNA sequences, and AFLPs (Nosil et al. 2002, 2003, 2008).

Moreover, movement even within relatively contiguous patches of suitable hosts appears limited. Sandoval (2000) conducted a mark–recapture study in *T. cristinae* within a 150 m² patch that was predominated by *Ceanothus*, with the explicit intent of studying dispersal ability. The mean weekly dispersal was estimated at 2 m and the maximum weekly dispersal at 8 m. Nosil (2004) estimated the weekly survival probability of each morph on the host upon which it is more cryptic as roughly 85% (Fig. 2 of Nosil 2004). With this information, the proportion of individuals

surviving long enough to disperse between hosts, before versus after the perturbation, can be estimated. In these calculations, I assume dispersal between hosts to involve a movement of at least 2 m before the perturbation versus a movement of at least 36 m after the perturbation.

A highly conservative approach is to assume that every single week every single individual moves the maximum distance known in *T. cristinae* (8 m) and does so in a straight line toward the opposite host. In this case, when considering weekly dispersal, only one week would be required to move between hosts prior to the perturbation. Thus, 85% of individuals would survive long enough for between-host dispersal. In contrast, five weeks would be required to move the 36 m between hosts following the perturbation. In this case, only 44.4% (0.85^5 , assuming independent survival probabilities among weeks) of individuals would survive long enough for between-host dispersal. Thus, dispersal following the perturbation would be roughly halved relative to pre-perturbation dispersal ($44/85 = 52\%$). If the same calculations are applied using the arguably more realistic criteria of individuals dispersing the mean distance, dispersal following the perturbation would be less than 7% its original level. In this case, it would require 18 weeks to disperse 36 m and only 5.4% (0.85^{18}) of individuals would be expected to survive that long ($5.4/85 = 6.4\%$). Similar results emerge if the actual distribution of dispersal distances is estimated. For example, if one multiplies the distance moved by each of the 54 individuals recaptured in the Sandoval (2000) experiment in a single week by 16 (representing the full life span of *T. cristinae*) and makes the extremely conservative assumption of zero mortality, still only 46.2% of the individuals would move 36 m or more in their lifetime. In contrast, all individuals would be capable of moving the few meters required for between-host dispersal prior to the perturbation.

Thus, even using conservative estimates that either applied the maximum dispersal distance to all individuals or completely ignored the increased probability of mortality through time, dispersal following the perturbation is roughly halved. This estimate is likely even more conservative than it appears, because bare ground would need to be covered such that insects may behaviorally choose to not cover such ground, or may suffer lower than 85% weekly survival during such movement. Moreover, all calculations are based upon adult movement, and nymphs are smaller (i.e., less physically capable of movement), may be generally less active (e.g., do not need to search for mates) and may suffer higher mortality than adults. Finally, all the calculations used the minimum post-perturbation distance between host species (36 m). Thus, between-host dispersal was likely reduced to far less than half, perhaps to 7% of the original level, as suggested by calculations using mean dispersal distance. In fact, between-host dispersal may have even ceased after the perturbation, as evidenced by data on lack of movement across bare ground (Nosil 2004).

The natural history of *T. cristinae* allows the timing of reductions in gene flow, relative to the life-history of the insects, to be inferred. *Timema cristinae* is univoltine with nonoverlapping generations: individuals emerge from egg diapause around late February and are generally present from March to June of that year, after which the next generation of eggs has been laid, and these eggs remain in diapause until the following spring (i.e., *T. cristinae* is not found in the field from roughly late June onwards) (Sandoval 1994a,b, 2000; Nosil et al. 2003). Thus, gene flow between *Timema* on different hosts at the perturbed site, documented in the past, has likely been strongly reduced for two (2007 and 2008) insect generations. When referring to the perturbation, I hereafter use the more conservative term “reduction in gene flow,” although the term “cessation of gene flow” may be appropriate. In contrast, there is no reason to suspect that gene flow between populations at the two control sites changed systematically in 2007 and 2008.

COLLECTION OF SPECIMENS

Specimens were collected between January and July of each year using sweep nets. The control sites were used as such because they were the only sites, other than the perturbation site, where both hosts were sampled in every year from 2003 to 2008. Specimens were scored by eye for the presence versus absence of the stripe (a highly repeatable measurement; Sandoval 1994a,b; Nosil 2004), and were not returned to the wild.

Insects were sampled throughout the entire patch both before and after the perturbation. Thus, preperturbation collections do not represent primarily individuals sampled from the actual area in which the two host species were in closest geographic contact. In fact, the majority of individuals captured were from similar areas of the site both before and after the perturbation, as depicted in Figure 2B. Raw data on morph frequencies, along with sample sizes, for each population, year, and month are provided in the Supporting Information (Table S1).

STATISTICAL ANALYSES

Logistic regression was used to analyze whether morph (striped or unstriped) was dependent upon host species, collection month, collection year, sample site (perturbed, control #1, control #2), and all possible interactions between these factors. Following the detection of a significant host \times year \times site interaction (LR = 25.34, $df = 10$, $P = 0.005$), each site was analyzed separately, to test which individual sites exhibit a significant host \times year interaction. The significance of differences between hosts for individual years within each site was assessed using the same analysis, but with the “year” term removed. In all cases, the reported results stem from a reduced logistic regression model derived using backward elimination (the initial model included all factors and interaction terms, but then removed all terms for which the significance of

$-2 \log$ likelihood ratio was > 0.10 , repeating this elimination step until all terms were either above or below the 0.10 threshold). Log likelihood ratio of -2 is referred to as LR hereafter.

Because interactions between “year” and other factors could be influenced by interannual variation that was not due to the perturbation, nested logistic regression analyses were run that explicitly analyzed the term “before versus after perturbation” (simply “before/after” hereafter). In these analyses, year was nested within before/after and month was nested within year. The first analysis tested the site \times host \times before/after interaction in a model that included this interaction and the before/after term (with year and month nested within it). Following the detection of a highly significant interaction (indicative of the effects on morph of host and before/after varying among sites), separate analyses were conducted within each of the three sites. Each site-specific analysis included the before/after term (again with year and month nested within it), the main effects of host, and the interaction between before/after and host (the latter being the term of interest reported in the main text). These nested logistic regression models were implemented in R (R Development Core Team 2008), and z -values are reported (regression coefficient divided by the standard error).

ESTIMATING SELECTION

Standard population genetic theory was used to estimate the strength of selection and relative fitness of each phenotype on each host required to generate the observed change in morph frequency between the generations directly before versus after the perturbation (i.e., between the 2006 and 2007 generations) (Futuyma 1998). I assume single-locus control of the color pattern with the striped phenotype being homozygous recessive for the allele causing presence of the stripe (consistent with past data showing dominance of the allele for lack of stripe) (Sandoval 1994a,b; Nosil et al. 2006b).

Thus, percent striped captured in the field is equal to q^2 in the Hardy–Weinberg equation, thereby allowing p to be inferred (where $1 - q = p$). The change in the frequency of p (Δp) between 2006 (p) and 2007 (p') was used to estimate the strength of selection (s) according to the following well-known equations. On *Ceanothus*, there is selection against the recessive allele such that

$$\Delta p = p' - p = \frac{spq^2}{1 - sq^2} \quad (1)$$

and thus,

$$s = \frac{\Delta p}{q^2(p + \Delta p)} \quad (2)$$

On *Ceanothus*, there is selection against the striped phenotype and thus the relative fitness of each morph was estimated using the following selection model: $w_{AA} = 1$, $w_{Aa} = 1$, $w_{aa} = 1 - s$

(where the unstriped morph is either AA or Aa and the striped morph aa).

On *Adenostoma*, there is selection against the dominant allele such that

$$\Delta p = p' - p = \frac{-spq^2}{1 - s + sq^2} \quad (3)$$

and thus,

$$s = \frac{-\Delta p}{\Delta p(q^2 - 1) + pq^2}. \quad (4)$$

On *Adenostoma*, there is selection against the unstriped phenotype such that the following selection model was used to estimate relative fitness: $w_{AA} = 1 - s$, $w_{Aa} = 1 - s$, $w_{aa} = 1$.

Results

Following the reduction in gene flow at the perturbed site, increased adaptive divergence was rapidly observed. In the generation immediately following the perturbation (2007), morph frequencies at the perturbed site were more divergent between hosts than within in any of the preceding 4 years (Fig. 3B). This pattern was replicated in the second year following the perturbation (2008). Differences between hosts following the perturbation were significant ($P < 0.001$ in each year) and in the direction predicted by natural selection.

Although much has been learned from natural experiments in the wild, a limitation is that they are often not highly spatially or temporally replicated. For example, the perturbation reported here was not replicated at a different site. However, two major lines of reasoning, based upon replication of the results among time periods and between hosts, indicate that divergence at the perturbed site results from a reduction in gene flow, and is unlikely to have arisen by chance. First, at the perturbed site, the difference in morph frequency between hosts is greater in each of the two years following the perturbation relative to differences within any of the preceding 4 years (Fig. 1). Given the a priori expectation that divergence should increase following reduction of gene flow, the probability of this pattern arising by chance is $[(2!)(4!)/(6!)]/2 = 0.033$. Thus, temporal replication of the data provides statistical support for the conclusion that gene flow constrained divergence. A key point is that this statistical result is not influenced by the number of individuals or populations examined, relying instead on the temporal replication of data from both host plants at the perturbed site (Nosil et al. 2006b).

Second, data from the other two parapatric population pairs (“control sites”) demonstrate that the pattern of increased divergence between hosts in 2007 and 2008 is unique to the perturbed site (Fig. 3). Specifically, the dependence of morph (striped vs. unstriped) on an interaction between host and year varied significantly among sites (logistic regression analyses, host \times year \times site

interaction, $LR = 25.34$, $df = 10$, $P = 0.005$). Thus, morph was dependent on an interaction between host and year at the perturbed site (host \times year, $LR = 13.32$, $df = 5$, $P = 0.021$), but not at either control site (control site 1, host \times year, $LR = 8.58$, $df = 4$, $P = 0.073$; control site 2, host \times year interaction not retained in the final model, $P > 0.10$; note that significance aside, trends in the control sites were in the opposite direction to those at the perturbed site). All the terms retained in the reduced logistic regression in these site-specific models, with associated likelihood differences, and significance values, were as follows: Perturbed site (M): host, $LR = 16.17$, $df = 1$, $P < 0.001$; year, $LR = 16.92$, $df = 5$, $P = 0.005$, month \times year, $LR = 7.56$, $df = 2$, $P = 0.023$; host \times year, $LR = 13.32$, $df = 5$, $P = 0.021$; Control site #1 (HV): host, $LR = 10.49$, $df = 1$, $P = 0.001$; month, $LR = 18.76$, $df = 3$, $P < 0.001$; host \times month, $LR = 20.29$, $df = 5$, $P = 0.001$; host \times year, $LR = 8.58$, $df = 4$, $P = 0.073$; Control site #2 (OG): host, $LR = 6.93$, $df = 1$, $P = 0.008$.

Similar results were observed in analyses using the binomial factor “before/after perturbation” as the time factor (with “year” nested within “before/after” and “month” nested within “year”; analysis including site: site \times host \times before/after interaction, $z = -4.27$, $P < 0.001$; site-specific analyses: host \times before/after interaction, perturbed site, $z = -2.60$, $P = 0.009$; control site 1, $z = 0.55$, $P = 0.58$, control site 2, $z = -0.01$, $P = 0.99$). The significant results observed here indicate that there was sufficient power to test the hypotheses at hand, despite somewhat limited sample sizes in some months and years.

Population genetic theory allows inferences on the strengths of selection and relative fitness required to generate the observed evolutionary response between the generations directly before versus after the perturbation. On *Ceanothus*, the strength of selection against the recessive (i.e., striped) allele is estimated as $s = 0.84$. The relative fitness of the recessive phenotype is thus $w_{aa} = 0.16$, making the dominant phenotype 6.3 times more fit than the recessive phenotype. Conversely, on *Adenostoma*, the strength of selection against the dominant (unstriped) allele is estimated as $s = 0.77$. The relative fitness of the dominant phenotype is thus $w_{AA} = w_{Aa} = 0.23$, making the recessive phenotype 4.3 times more fit. These calculations assume that gene flow ceased following the perturbation, and thus may underestimate the strength of selection required to generate the observed change in morph frequency. However, the fact that the direction of divergence reversed between the years directly before versus after the perturbation suggests that selection strength might be slightly overestimated.

Discussion

I report here increased adaptive divergence between a parapatric population pair following an experimental reduction in gene flow.

Temporal replication of the data, coupled with a lack of increased adaptive divergence at two control sites, argue that the results did not arise by chance. Notably, there was some spatial replication of the data because morph frequencies at the perturbed site in the year following the perturbation changed on both (rather than a single) hosts. Furthermore, the control sites were spatially replicated. Finally, an important point is that the results observed here are consistent with past experimental and molecular data in this system, which provided indirect, correlative evidence that gene flow constrains divergence in morph frequencies (Sandoval 1994a,b; Nosil 2004; Nosil and Crespi 2004, 2006). Thus, the patterns observed here support a priori predictions. Although replication in additional perturbed sites would clearly be informative, the collective arguments indicate that the patterns observed arose because gene flow constrains adaptation.

I am aware of only a single other study that manipulated gene flow in the wild, and similar to the results of the current study, it concluded that gene flow constrains adaptive divergence (Riechert 1993a). This study examined divergent behavioral phenotypes of the desert spider *Agelenopsis aperta*. Specifically, this species has an “aridlands” phenotype that exhibits high aggressiveness in competitive interactions over energy-based territories and a lack of discrimination among potential prey types and a “riparian” phenotype with low aggressiveness toward conspecifics and discrimination of prey profitability. These behavioral differences have been shown to be heritable. Riechert (1993a) identified a woodland population that exhibited apparently maladaptive behavior and tested whether gene flow contributed to this maladaptation. Experimental manipulation of gene flow demonstrated that one generation of predation pressure in the absence of gene flow is sufficient to cause a marked shift in spider behavior toward the expected “riparian” phenotype. Thus, in both the spider and the walking-stick systems, it appears that gene flow constrains adaptation and that selection from predators contributes to adaptive divergence when gene flow is reduced (see also Riechert 1993b).

In the current study, the 20–30% increase in divergence following the perturbation, compared to average yearly differentiation observed prior to it, is reasonable given past data indicating that selection on color pattern is strong (s is at least 0.50) (Nosil 2004; Bolnick and Nosil 2007), the trait is highly heritable (Sandoval 1994a,b), and the preperturbation morph frequencies were not near fixation (i.e., genetic variation was present). The population genetic theory implemented here revealed further evidence for strong selection, illustrating how visual predators can be a potent source of selection and an important cause of evolutionary diversification (Reimchen 1979; Mallet and Barton 1989; Reimchen and Nosil 2002, 2004; Mappes et al. 2005; Vamosi 2005 for review). Indeed, past studies have demonstrated that selection exerted by visual predators can cause or maintain

divergence in the face of gene flow (King and Lawson 1995; Merilaita 2001; Hoekstra et al. 2004; Harper and Pfennig 2008). An unanswered question in the current study is why divergence in the second generation following the perturbation remained similar to that observed in the first generation, rather than increasing further. Some possibilities include weakening of selection (e.g., if visual predators were less abundant) or an increase in gene flow. More generally, for all the sites considered here variation among years in selection strength, as well as sampling error, could contribute to differences among years in the level of adaptive divergence observed.

Two additional factors warrant discussion. First, genetic drift might potentially affect morph frequencies, given that population sizes at the perturbed site were likely reduced following the perturbation. Specifically, host plant patch size is positively correlated with *T. cristinae* population size (Sandoval 1994a). Thus, *T. cristinae* population sizes at the perturbed site were likely reduced following the perturbation, potentially increasing the opportunity for genetic drift. Although direct data on population size following the perturbation are not available, numerous individuals were captured following the perturbation, indicating that the drastic reductions in population size associated with extreme bottlenecks did not occur. Additionally, indirect estimates of population size can be generated using the known relationship between host plant patch size and *T. cristinae* population size. Specifically, Sandoval (1994a) cleared all the insects from 26 host patches of varying size, thereby estimating the relationship between population size (the number of insects captured in a patch) and host plant patch size. For *Adenostoma*, this relationship was estimated as $y = 2.27 + 3.4x$, where y = number of individuals and x = patch size in meters squared. Prior to the perturbation, the area of the *Adenostoma* patch at the perturbed site was 54,360 m² (reported in Sandoval 1994a), yielding a population size estimate of 184,846 individuals. Thus, even with a 95% reduction in population size, the population would be estimated at 9242 individuals. Population size estimates for 99, 99.5, and 99.9% reductions in size are 1849, 924, and 185 individuals, respectively. Estimates for *Ceanothus* are similar ($y = 1.6 + 4.1x$, patch size = 34,754, yielding a pre-perturbation population size of 142,493, and 7125, 1425, 712, and 142 individuals for the same reductions noted above). Thus, it would take extremely severe reductions in population size to reduce population size below 1000 individuals. Although a quantitative estimate of the degree to which patch size was decreased following the perturbation is not available, many plants did remain (see Fig. 2B). A caveat is that the patch sizes used in the above calculations extrapolate beyond the maximum patch size examined in Sandoval (1994a). Another consideration is that the estimates above are of population size, rather than effective population size. Nonetheless, the collec-

tive arguments suggest reasonable population sizes even after the perturbation.

More important, even if genetic drift was acting, it is not expected to produce patterns such as those reported here. Namely, drift is not expected to produce a correlation between host plant species used and morph frequency (Schluter 2000). Likewise, drift is not expected to generate concerted changes in morph frequency, in the direction predicted by natural selection, following a reduction in gene flow (Futuyma 1998; Schluter 2000; Räsänen and Hendry 2008). Thus, although some effect of drift on morph frequencies cannot be excluded, drift is unlikely to explain the increased adaptive divergence observed at the perturbed site.

Second, assortative mating might affect morph frequencies, but is also unlikely to contribute strongly to the results: although there is some assortative mating between individuals from different hosts, it does not arise via assortative mating by color-pattern morph (i.e., some trait other than color-pattern underlies the between-host assortative mating) (Nosil et al. 2002; Nosil and Crespi 2004; Nosil et al. 2007). Moreover, despite reproductive isolation between populations on different hosts, there is strong evidence for gene flow between hosts (Nosil 2007, 2008).

The main conclusion is that gene flow constrained adaptive divergence in nature, at least for the trait considered here. The results illustrate how adaptive evolution following the cessation of gene flow can be both marked and extremely rapid. The observed rapid evolutionary response was likely made possible because gene flow maintained genetic variation within populations, and is consistent with theory indicating that rapid adaptation is possible when evolution proceeds from standing genetic variation, rather than having to wait for the fortuitous emergence of beneficial mutations (Barrett and Schluter 2008 for review). In sum, the interplay between selection, genetics, and geography reliably predicted the rate and timing of adaptive evolution in the wild. Interestingly, the reduction of gene flow considered here was induced by road crews, suggesting that human disturbance can unknowingly and quickly affect the evolution of animal populations (Hendry et al. 2006). A final point is that the current study examined the role of gene flow in constraining divergence, but did not consider the alternative casual association of adaptive divergence constraining gene flow. Future research could usefully focus on this alternative casual association, as well as potential feedbacks between different causal pathways (Riechert 1993a; Hendry et al. 2001; Nosil and Crespi 2004; Räsänen and Hendry 2008). Such work could be conducted in *T. cristinae*, where there is now evidence that gene flow constrains adaptation (Sandoval 1994a; Nosil and Crespi 2004; Bolnick and Nosil 2007; this study), as well as is indirect correlative evidence from experiments and molecular data that adaptive divergence reduces gene flow (Nosil et al. 2002, 2003, 2008).

ACKNOWLEDGMENTS

I thank D.J. Funk, B.J. Crespi, C.P. Sandoval, J. Mallet, and D. Schluter for discussion. J. Mallet and H. Collin helped with the selection estimates. Comments on an earlier version of this manuscript from C. Nice, A. Hendry, and five anonymous reviewers greatly improved the final version. I am particularly indebted to C. P. Sandoval, for introducing me to *Timema* walking-stick insects and for providing the raw dispersal data from Sandoval (2000). Funding was provided by the Natural Sciences and Engineering Research Council of Canada and Wissenschaftskolleg zu Berlin.

LITERATURE CITED

- Barrett, R. D. H., and D. Schluter. 2008. Adaptation from standing genetic variation. *Trends Ecol. Evol.* 23:38–44.
- Beerli, P., and J. Felsenstein. 2001. Maximum-likelihood estimation of a migration matrix and effective population sizes in *n* subpopulations by using a coalescent approach. *Proc. Natl. Acad. Sci. USA* 98:4563–4568.
- Bolnick, D. A., and P. Nosil. 2007. Natural selection in populations subject to a migration load. *Evolution* 61:2229–2243.
- Bolnick, D. I., E. Caldera, and B. Matthews. 2008. Migration load in a pair of ecologically divergent lacustrine stickleback populations. *Biol. J. Linn. Soc.* 94:273–287.
- Crespi, B. J. 2000. The evolution of maladaptation. *Heredity* 84:623–629.
- 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.
- Futuyma, D. J. 1998. *Evolution*. Sinauer, MA.
- 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., P. Nosil, and W. Etges. 2006. Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. *Proc. Natl. Acad. Sci. USA* 103:3209–3213.
- Grant, P. R., and B. R. Grant. 2002. Unpredictable evolution in a 30-year study of Darwin's finches. *Science* 296:707–711.
- . 2006. Evolution of character displacement in Darwin's finches. *Science* 313:224–226.
- Harper, G. R. Jr., and D. W. Pfennig. 2008. Selection overrides gene flow to break down maladaptive mimicry. *Nature* 451:1103–1106.
- Hendry A. P., T. Day, and E. B. Taylor. 2001. Population mixing and the adaptive divergence of quantitative traits in discrete populations: a theoretical framework for empirical tests. *Evolution* 55:459–466.
- Hendry, A. P., P. R. Grant, B. R. Grant, H. A. Ford, M. J. Brewer, and J. Podos. 2006. Possible human impacts on adaptive radiation: beak size bimodality in Darwin's finches. *Proc. R. Soc. Lond. B.* 273:1887–1894.
- Hoekstra, H. E., K. E. Drumm, and M. W. Nachman. 2004. Ecological genetics of adaptive color polymorphism in pocket mice: geographic variation in neutral and selected genes. *Evolution* 58:1329–1341.
- King, R. B., and R. Lawson. 1995. Color-pattern variation in Lake Erie water snakes: the role of gene flow. *Evolution* 49:885–896.
- Losos, J. B., T. W. Schoener, and D. A. Spiller. 2004. Predator-induced behavior shifts and natural selection in field-experimental lizard populations. *Nature* 432:505–508.
- Losos, J. B., T. W. Schoener, R. B. Langerhans, and D. A. Spiller. 2006. Rapid temporal reversal in predator-driven natural selection. *Science* 314:1111.
- Lu, G. Q., and L. Bernatchez. 1998. Correlated trophic specialization and genetic divergence in sympatric lake whitefish ecotypes (*Coregonus clupeaformis*): support for the ecological speciation hypothesis. *Evolution* 53:1491–1505.
- Mallet, J., and N. H. Barton. 1989. Strong natural selection in a warning-color hybrid zone. *Evolution* 43:421–431.
- Mappes J., N. Marples, and J. A. Endler. 2005. The complex business of survival by aposematism. *Trends Ecol. Evol.* 20:598–603.
- Merilaita, S. 2001. Habitat heterogeneity, predation and gene flow: colour polymorphism in the isopod *Idotea baltica*. *Evol. Ecol.* 15:103–116.
- Mayr, E. 1963. *Animal species and evolution*. Harvard Univ. Press, Cambridge, MA.
- Nosil, P. 2004. Reproductive isolation caused by visual predation on migrants between divergent environments. *Proc. R. Soc. Lond. B* 271:1521–1528.
- . 2007. Divergent host-plant adaptation and reproductive isolation between ecotypes of *Timema cristinae* walking-sticks. *Am. Nat.* 169:151–162.
- . 2008. Ernst Mayr and the integration of geographic and ecological factors in speciation. *Biol. J. Linn. Soc.* 95:26–26.
- Nosil, P., and B. J. Crespi. 2004. Does gene flow constrain trait divergence or vice-versa? A test using ecomorphology and sexual isolation in *Timema cristinae* walking-sticks. *Evolution* 58:101–112.
- . 2006. Experimental evidence that predation promotes divergence in adaptive radiation. *Proc. Natl. Acad. Sci. USA* 103:9090–9095.
- Nosil, P., B. J. Crespi, and C. P. Sandoval. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. *Nature* 417:440–443.
- . 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. *Proc. R. Soc. Lond. B* 270:1911–1918.
- . 2006a. The evolution of host preferences in allopatric versus parapatric populations of *Timema cristinae*. *J. Evol. Biol.* 19:929–942.
- Nosil, P., B. J. Crespi, C. P. Sandoval, and M. Kirkpatrick. 2006b. Migration and the genetic covariance between habitat preference and performance. *Am. Nat.* 167:E66–E78.
- Nosil, P., B. J. Crespi, R. Gries, and G. Gries. 2007. Natural selection and divergence in mate preference during speciation. *Genetica* 129:309–327.
- Nosil P., S. P. Egan, and D. J. Funk. 2008. Heterogeneous genomic differentiation between walking-stick ecotypes: 'isolation-by-adaptation' and multiple roles for divergent selection. *Evolution* 62:316–336.
- Olendorf, R., H. F. Rodd, D. Punzalan, A. E. Houde, C. Hurt, D. N. Reznick, and K. Hughes. 2006. Frequency dependent survival in natural guppy populations. *Nature* 441:633–636.
- Pagel, M. 1999. Inferring the historical patterns of biological evolution. *Nature* 401:877–884.
- R Development Core Team. 2008. (R Foundation for Statistical Computing, Vienna, Austria).
- Räsänen, K., and A. P. Hendry. 2008. Disentangling interactions between adaptive divergence and gene flow when ecology drives diversification. *Ecol. Letts.* 11:624–636.
- Reimchen, T. E. 1979. Substratum heterogeneity, crypsis, and colour polymorphism in an intertidal snail. *Can. J. Zool.* 57:1070–1085.
- Reimchen, T. E., and P. Nosil. 2002. Temporal variation in divergent selection on spine number in a population of threespine stickleback. *Evolution* 56:2472–2483.
- . 2004. Variable predation regimes predict the evolution of sexual dimorphism in a population of threespine stickleback. *Evolution* 58:1274–1281.
- Rice, W. R., and E. E. Hostert. 1993. Laboratory experiments in speciation: what have we learned in 40 years? *Evolution* 47:1637–1653.
- Riechert, S. E. 1993a. Investigation of potential gene flow limitation of behavioral adaptation in an aridlands spider. *Behav. Ecol. Sociobiol.* 32:355–363.

- . 1993b. The evolution of behavioral phenotypes: lessons learned from divergent spider populations. *Adv. Anim. Behav.* 22:130–134.
- Sandoval, C. P. 1994a. The effects of relative geographic scales of gene flow and selection on morph frequencies in the walking stick *Timema cristinae*. *Evolution* 48:1866–1879.
- . 1994b. Differential visual predation on morphs of *Timema cristinae* (Phasmatodeae: Timemidae) and its consequences for host range. *Biol. J. Linn. Soc.* 52:341–356.
- . 2000. Presence of a walking-stick population (Phasmatoptera: Timematodea) after a wildfire. *Southwest. Nat.* 45:19–25.
- Schluter, D. 2000. *The ecology of adaptive radiation*. Oxford Univ. Press, Oxford.
- Spiller, D. A., J. B. Losos, and T. W. Schoener. 1998. Impact of a catastrophic hurricane on island populations. *Science* 281:695–697.
- Vamosi, S. M. 2005. On the role of enemies in divergence and diversification of prey: a review and synthesis. *Can. J. Zool.* 83:894–910.
- Vickery, V. R. 1993. Revision of *Timema* Scudder (Phasmatoptera: Timematodea) including three new species. *Can. Entomol.* 125:657–692.

Associate Editor: C. Nice

Supporting Information

The following supporting information is available for this article:

Table S1. Raw data on the frequency of the striped morph within each population, divided by collection year and month.

Supporting Information may be found in the online version of this article.

(This link will take you to the article abstract).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Additional results and discussion can be found in a document at <http://www.repository.naturalis.nl/record/289893>.