The Means of Signal Divergence Early in a Host Shift

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ABSTRACT: We used a "quasi-natural" selection experiment and subsequent reciprocal transplants to assess the means of divergence in mating signals early in a host shift. We worked with a member of the *Enchenopa binotata* complex of treehoppers (Hemiptera: Membracidae), where speciation results from host plant shifts and involves remarkable signal-preference codivergence. We shifted treehoppers from a natural population on one host plant to three different novel host species under conditions of allopatry and sympatry. After five generations, we conducted reciprocal transplants that manipulated oviposition and development hosts. We found tentative evidence of signal divergence fueled by standing genetic variation and strong evidence of signal divergence through overall plasticity and evolution in the form of plasticity, resulting in signal differences between treehoppers on novel and ancestral hosts. These results suggest that signal divergence (and consequently assortative mating) may arise early in a host shift from multiple means. Together with a prior analysis of the adaptive consequences of these experimental host shifts, our findings indicate that adaptation/specialization and divergence in sexual traits may originate independently and in parallel or with divergence in sexual traits leading. Thus, ecological specialization may be facilitated by sexual divergence rather than being the initiating factor.

Keywords: developmental plasticity, genetic accommodation, novel environment, speciation with gene flow, sympatric speciation, vibrational signaling.

Introduction

Speciation—the process that creates ecologically distinct and reproductively isolated organismal forms—generally entails divergence between populations that live in different environments (Coyne and Orr 2004). This means that speciation frequently involves not only different selection regimes but also different environmental conditions for development and trait expression. Thus, the variants on which selection acts early in speciation may stem not only from standing genetic variation in the populations but also from developmental plasticity.

Environmental causes of variation may be at least as relevant as standing genetic variation early in speciation. This is because environmental inputs into trait expression can affect many individuals at once, offering a broader target for the creation of new variants than mutation, and the resulting variants may expose to selection otherwise hidden genetic variation in developmental mechanisms (West-Eberhard 2003, 2005; Barrett and Schluter 2008; Le Rouzic and Carlborg 2008). Furthermore, speciation may involve change in the form of plasticity itself. This may entail novel forms of plasticity (through the process termed "genetic accommodation") or novel fixed (nonplastic) trait forms originally reached through plasticity (through the process of genetic assimilation; West-Eberhard 2003, 2005; Suzuki and Nijhout 2006; Gerhart and Kirschner 2007; Pfennig et al. 2010; Moczek et al. 2011; Renn and Schumer 2013; Schlichting and Wund 2014; Ghalambor et al. 2015; Levis and Pfennig 2016).

Plasticity may make a further contribution to early speciation. When present in sexual traits such as advertisement signals and mate preferences, it may produce early reproductive isolation and even alter the form or strength of selection on sexual communication (Bailey and Moore 2012; Rebar and Rodríguez 2015; Desjonquères et al. 2023).

However, there is also the possibility that plasticity may hinder evolution and divergence. Adaptive adjustment in phenotypes, although favorable for population persistence

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(West-Eberhard 2003; Yeh and Price 2004), may reduce the genetic response to selection (Price et al. 2003; Ghalambor et al. 2007; Pfennig et al. 2010). The genetic variants exposed by plasticity may be deleterious (Moczek et al. 2011). And some patterns of plasticity in sexual traits may counter reproductive isolation rather than promote it—for example, if they lead to disassortative mating. But note that the form of plasticity may be maladaptive and yet promote adaptive evolution (Ghalambor et al. 2015).

Understanding the early course of speciation therefore requires analyzing the extent to which divergence involves standing genetic variation, plasticity, and evolution in the form of plasticity. Here, we report on a "quasi-natural" selection experiment (Fry 2003) and reciprocal transplant experiment designed to conduct this analysis. We focused on divergence in advertisement signals because of its potential impact on reproductive isolation.

We worked with a sap-feeding insect that communicates with plant-borne vibrational signals, a member of the *Enchenopa binotata* complex of treehoppers (Hemiptera: Membracidae; fig. 1). Speciation in this clade occurs mainly in sympatry, is a consequence of shifts to novel host plants, and involves host plant specialization through behavioral and physiological mechanisms (Wood and Guttman 1983; Wood 1993; Lin and Wood 2002; Rodríguez et al. 2004, 2006; Cocroft et al. 2008, 2010; Hsu et al. 2018) as well

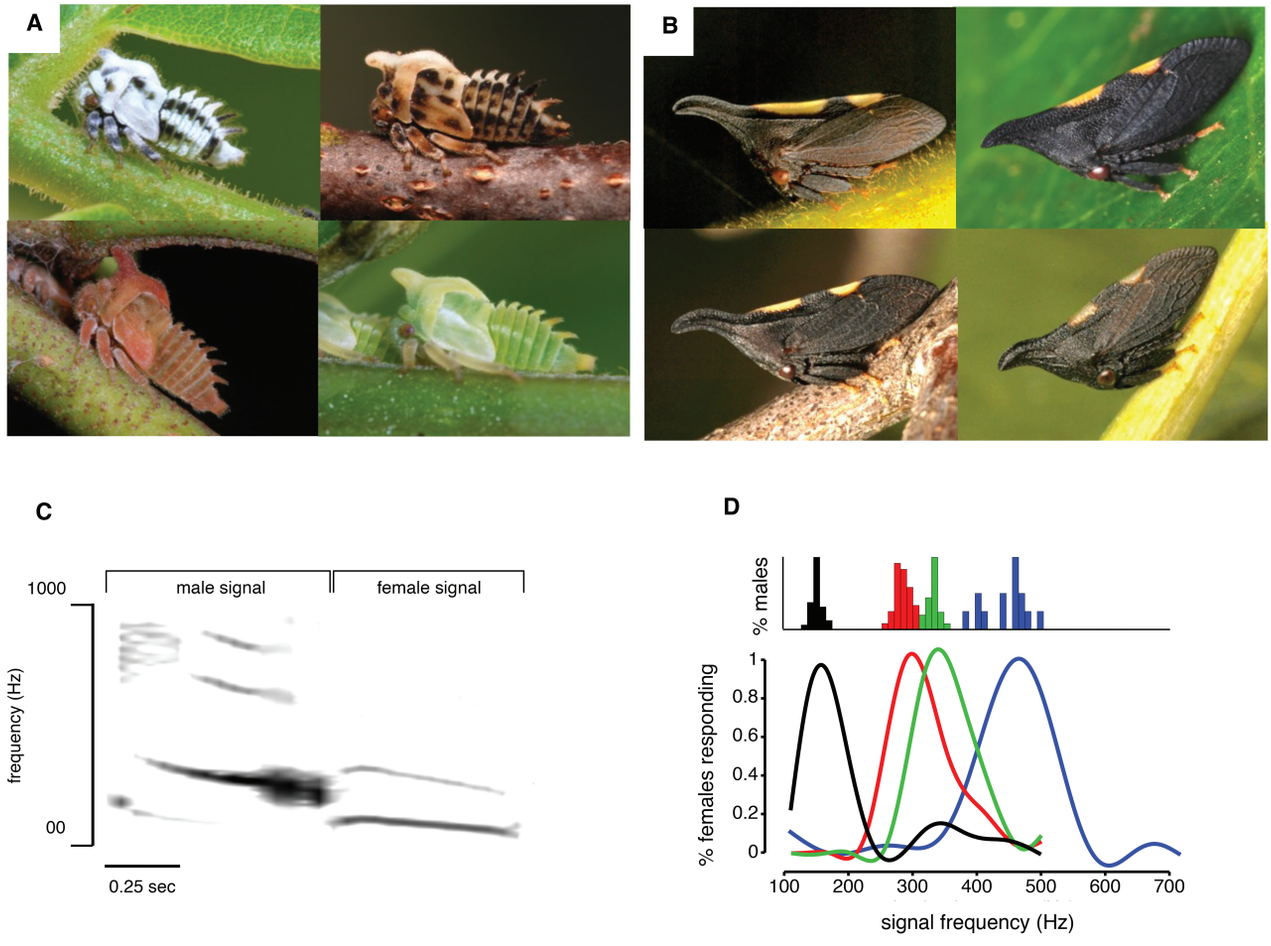

Figure 1: Key aspects of the natural history of *Enchenopa binotata* treehoppers (Cocroft et al. 2008). *A*, Nymph coloration differs among host-associated species (hosts, clockwise from upper left: *Juglans nigra*, *Ptelea trifoliata*, *Robinia pseudoacadia*, and *Viburnum rufidulum*). *B*, Adults are similar among host-associated species (host, clockwise from upper left: *Cercis canadensis*, *Juglans nigra*, *Viburnum rufidulum*, and *Ptelea trifoliata*). *C*, Mate searching *E. binotata* males fly from plant to plant producing advertisement signals, and receptive females produce their own signals in response, establishing a duet that continues until mating begins; females express their mate preferences through selective duetting (Rodríguez et al. 2004, 2006; Rodríguez and Cocroft 2006). Here, we show a male-female duet of the *E. binotata* species from the host plant *Ptelea trifoliata*. *D*, Signal-preference correlation among host-associated species. The upper histogram shows the distribution of signal frequency for four species, and the lower curves show the female preferences for frequency in the same four species (hosts: *Cercis canadensis* [black], *Viburnum rufidulum* [red], *Ptelea trifoliata* [green], *Celastrus scandens* [blue]). Redrawn from Rodríguez et al. (2006) with permission.

as remarkable signal-preference coevolution mainly involving the dominant frequency of male advertisement signals (Rodríguez et al. 2004, 2006, 2013*a*; Cocroft et al. 2010).

We took *E. binotata* treehoppers from a population on its natural host and shifted them to three different novel host plant species (Rodríguez et al. 2021; see below). This created three independent experimental host shifts, each establishing treehopper lines that were selected on either the ancestral or the novel host. To test the role of gene flow in signal divergence, we conducted each shift under conditions of allopatry (only one host species present, whether ancestral or novel) and sympatry (ancestral and novel hosts present; see below). The "shift" to the ancestral host under allopatry constitutes a control (Fry 2003) that allows testing for changes unrelated to colonization of novel hosts. Because we wanted to examine speciation at an early stage while allowing for some evolution, we allowed five generations of selection subsequent to the host shifts.

The treehoppers were philopatric on their ancestral/novel hosts for mating and oviposition: averaging across the different host shifts, at least 59%–78% of adults mated and 63%–73% of females oviposited on their natal plant, and in at least 48%–67% of mating pairs the male and female were from the plant where they mated (Stearns et al. 2013). Thus, host shifts in sympatry allowed for dispersal and gene flow involving only adults. All nymphs developed on their assigned plants, as they almost never leave their plant (Cocroft et al. 2008) and would have been unlikely to reach the other plant had they done so.

We have reported on the adaptive consequences of this selection-reciprocal transplant experiment in a separate article, focusing on performance on the different hosts as measured by female fecundity (Rodríguez et al. 2021). In brief, after the five generations of selection and despite considerable genetic variation in performance across the ancestral and novel hosts in the source population (Tilmon et al. 1998), either there was no adaptation to the novel hosts or there was adaptation but without specialization—that is, without performance trade-offs between the ancestral and novel host (Rodríguez et al. 2021). Adaptation was more likely in sympatry, whereas extinction on novel hosts was more likely in allopatry. We concluded that early in speciation, adaptation to novel environments does not necessarily bring about performance trade-offs in ancestral environments. This suggests that adaptation (and eventual specialization) and reproductive isolation may originate independently and in parallel, with additional causes of specialization and assortative mating perhaps being important.

Here, we report the results of the selection-reciprocal transplant experiment for divergence in advertisement signals, measured from adult males reared from the nymphs we used in the prior study to estimate female fecundity (Rodríguez et al. 2021). We manipulated the oviposition host (henceforth, "egg host") and developmental host (henceforth, "nymph host") of treehoppers from the different shifted lines. Thus, the experiment manipulated inputs into development stemming from the early developmental environment of just-eclosed nymphs (egg host), the developmental environment of nymphs, and possible inputs from maternal effects due to the environment at the time of egg laying (Moore et al. 1997).

We used the selection-reciprocal transplant experiment to test a suite of hypotheses that analyze the contributions from standing genetic variation, plasticity, and evolution in the form of plasticity to signal divergence at the beginning of a host shift. The hypotheses and predictions are defined by scenarios contrasting differences among treehoppers from ancestral and novel lines that developed on ancestral versus novel hosts (table 1; fig. 2).

We also test the role of gene flow in these processes. Gene flow is broadly expected to oppose divergence unless selection is strong or robust mechanisms of assortative mating are already in place (Coyne and Orr 2004; Kopp et al. 2018). If so, signal divergence should be more likely under allopatry. However, gene flow may promote divergence through a rescue-like effect by supplementing genetic variation for selection to act on (Holt and Barfield 2011; Eriksson et al. 2014; Tomasini and Peischl 2020). If so, signal divergence should be more likely under sympatry. As we noted above, gene flow was possible in the sympatric shifts, as both some mating and some adult dispersal occurred across plants (Stearns et al. 2013; Rodríguez et al. 2021). We therefore focus on the contrast between conditions of sympatry (gene flow between hosts is possible) and allopatry (gene flow between hosts is not possible).

Finally, we integrate this and the prior study (Rodríguez et al. 2021) to address the nature of the relationship between adaptation and signal divergence early in the process of speciation.

Methods

The data we report here were collected as part of a collaboration between Tom Wood, Rex Cocroft, Randy Hunt, Kelley Tilmon, Frank Stearns, Rob Snyder, and Michael Cast during the period 2000–2002. Tom unfortunately passed away in 2002 but was the initiator of the experiment and integral in the study design and carrying out experimental work.

Selection and Reciprocal Transplant Experiments

We provide a detailed account of the selection experiment in Rodríguez et al. (2021). In brief, we collected a large sample (28,000) of mated females from an *Enchenopa binotata* natural population on the host plant *Viburnum*

Table 1: Hypotheses that analyze the contributions from standing genetic variation, plasticity, and evolution in the form of plasticity to signal divergence at the beginning of a host shift

Hypothesis	Prediction	Implication for signal divergence and potential consequence for assortative mating	Scenario in figure 1
Divergence through standing genetic variation only	Signals will differ only across treehoppers from different selection lines, regardless of developmental host	Signal differences appear gradually Assortative mating possible, likely sustained across hosts	A
Divergence through plasticity only	Signals will differ only across insects that develop on ancestral versus novel hosts, regardless of selection line	Signal differences across hosts appear immediately Assortative mating possible, depending on whether differences remain upon return to ancestral host	B
Divergence through evolution in the form of plasticity[a]	Signal differences across selection lines will vary according to developmental host, due to differences in the plastic response	Signal differences may appear immediately, quickly become sustained across hosts Assortative mating possible, likely sustained across hosts	C
Null	No change in signals across selection lines or developmental hosts	No divergence No assortative mating	D

Note: Stated are predictions in terms of scenarios for differences among treehoppers from ancestral and novel lines that developed on ancestral versus novel hosts in the selection and reciprocal transplant experiments.

[a] Note that evolution in the form of plasticity may involve genetic accommodation, which would be evinced with scenarios such as that in figure 2*C*, or genetic assimilation, which if very rapid could look like the scenario in figure 2*A*. We consider the latter unlikely, however.

lentago (Adoxaceae). These constitute the ancestral source population and ancestral host, respectively. We assigned these mated females to one of three novel host plant species: *V. lantana*, *V. prunifolium*, and *V. utile*. Of these, *V. lantana* and *V. utile* are nonnative species that are not used by any other member of the *E. binotata* complex. By contrast, *V. prunifolium* is used by our study species (Cocroft et al. 2010; Hsu et al. 2018), although not at the site of the source population, and is both more closely related and more phenotypically similar to the ancestral host (*V. lentago*; Clement et al. 2014; Rodríguez et al. 2021).

We introduced the mated females to 28 screened enclosures at an outdoor facility at the University of Delaware (1,000 females/enclosure). Each enclosure was

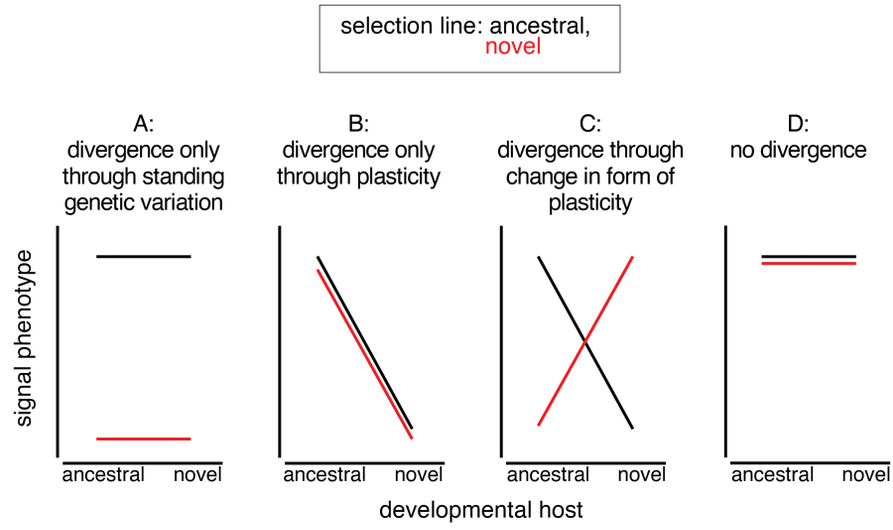

Figure 2: Scenarios for signal divergence in the host shift (ancestral and novel lines in black and red, respectively) and reciprocal transplant experiment (*x*-axis: ancestral and novel developmental hosts). Scenarios are as follows: *A*, divergence through standing genetic variation only; *B*, divergence through developmental plasticity only; *C*, divergence through evolution in the form of plasticity; *D*, no divergence.

approximately 2.5 m tall, 2 m wide, and 9 m long and contained two trees spaced approximately 7 m apart, which is within the adult treehoppers' "cruising range" (sensu Mayr 1942). The tree combinations in each enclosure defined the shifts and conditions of either sympatry (shift 1: *V. lentago*–*V. lantana*; shift 2: *V. lentago*–*V. prunifolium*; shift 3: *V. lentago*–*V. utile*) or allopatry (each enclosure with two trees of the same species; ancestral "control": *V. lentago*–*V. lentago*; shift 1: *V. lantana*–*V. lantana*; shift 2: *V. prunifolium*–*V. prunifolium*; shift 3: *V. utile*–*V. utile*). Within each shift, we sought to minimize causes of variation due to individual differences between conspecific trees by using clones propagated from suckers obtained from a single individual plant for each plant species. The experiment started with four enclosures (replicates) for each tree combination. The allopatric shift 3 (*V. utile*–*V. utile*) populations became extinct within four generations. We lost an additional one or two enclosures per combination due to causes unrelated to selection (e.g., death of trees, collapse of enclosure roofs).

We conducted the reciprocal transplant rearing experiment after the five generations of selection. As *E. binotata* have one generation per year (Wood 1980), this corresponds to 5 years after the start of the shifts. We manipulated the egg-laying host for females and the development host for the nymphs that eclosed from those eggs (fig. 3). We collected a sample of mated females from each tree in each enclosure ($n = 10$–30 females/enclosure) at the beginning of the oviposition season in the fall and assigned them either to the tree on which they were collected or to the other tree in that enclosure. For example, in each enclosure for sympatric shift 2 (*V. lentago*–*V. prunifolium*), we took females from *V. lentago* and placed them in sleeve cages on stems of *V. lentago* and *V. prunifolium* plants, and we took females from *V. prunifolium* and sleeved them onto *V. prunifolium* and *V. lentago*. And in each enclosure for allopatric shift 2 (*V. prunifolium*–*V. prunifolium*), we took females from either of the *V. prunifolium* trees and sleeved onto the same or the other tree.

The following year we assigned half of the eclosing nymphs to the plant on which they were born and the other half to the other plant in the enclosure (fig. 3). For example, in each enclosure for shift 1 (*V. lentago*–*V. lantana*), there

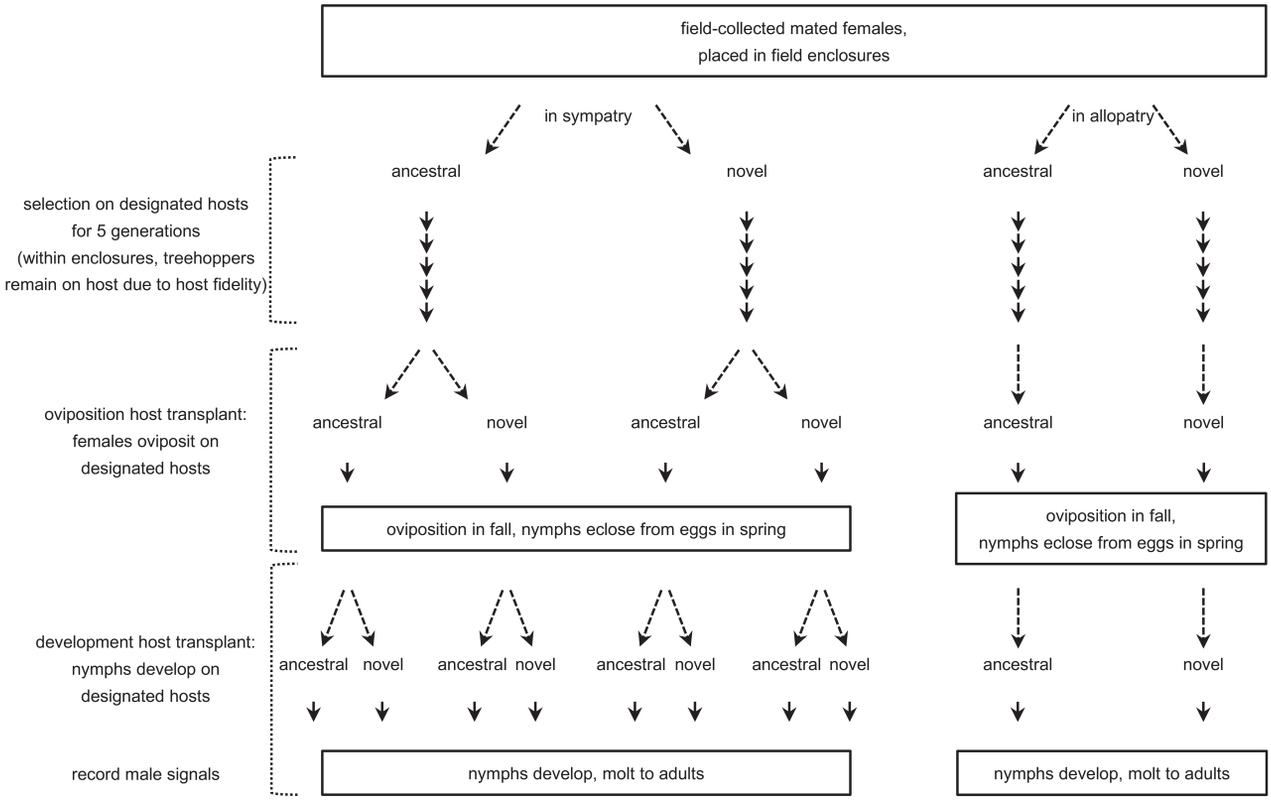

Figure 3: Outline of the host shift experiment. We collected a large sample of mated females and shifted them to novel hosts in conditions of sympatry (*left*) or allopatry (*right*). After five generations of selection, we conducted a rearing experiment involving reciprocal transplantation for oviposition and then again for development. Note that in allopatry, the oviposition host was the host on which the treehoppers had been on for the five generations of the host shift.

was a second reciprocal transplant with nymphs born on *V. lentago* divided and then placed on *V. lentago* and *V. lantana* (and vice versa). We reared the nymphs to the adult stage to allow us to record male advertisement signals (fig. 3).

Signal Recording and Analysis

When males became sexually active (~2 weeks after the adult molt), we recorded their advertisement signals with laser vibrometers at the University of Missouri and at Indiana University Southeast. Reproductively mature males were shipped overnight from the University of Delaware to the University of Missouri and Indiana University Southeast, with individuals from each cage and line split between the two laboratories. Once in the lab, individuals were maintained in sleeve cages on potted host plants of the same species on which they developed as nymphs. To record a male, we placed him on the stem of a potted plant. Their natural "call-fly" behavior induces them to signal shortly after being placed on a plant (Cocroft et al. 2008). To avoid introducing additional inputs into signal variation, we recorded males on potted host plants of the species on which they developed as nymphs. Note that as *E. binotata* male signals are mainly pure tones (figs. 2*C*, 4), differences in the signal-transmission features of recording plants introduce little variation into measurements of their signals beyond a reduced likelihood to signal and production of shorter signals when males are placed on a distantly related host (Sattman and Cocroft 2003; Cocroft et al. 2006; Rodríguez et al. 2008).

We focused the beam of the laser vibrometers on a small piece of reflective tape (~2 mm^2) that we attached to the stem of the recording plant, near the male's position. At the University of Missouri, we filtered (Krohn-Hite 3202; high pass at 60 Hz) the output of the laser vibrometer (CLV 1000 sensor head, CVL M030 decoder module; Polytec, Auburn, MA) and recorded it on a Macintosh G4 computer with the program SoundEdit (Macromedia, San Francisco, CA) at a sampling rate of 44.1 kHz. At Indiana University Southeast, we sent the output of the laser vibrometer (Polytec OFV 353, OFV 2602 decoder module) to a Macintosh G4 with an Audiomedia III (Digidesign) digital interface and recorded it with the program Peak (ver. 3.0; BIAS, Petaluma, CA). We measured the air temperature near the male's position (mean ± SD = 24.1°C ± 1.2°C). We analyzed the recordings with SoundEdit 16 (Macromedia), taking seven signal measurements for each male: the temporal signal traits signals/bout, signal interval, whine length, number of pulses, and pulse rate and the spectral signal traits dominant frequency and frequency sweep (fig. 4).

Our total sample size was 347 recorded males. There were 2–74 males/replicate (mean ± SD = 35 ± 20.3) and 1–74 males/line × egg host × nymph host combination (mean ± SD = 13.3 ± 16). Replicate numbers were two enclosures each for sympatric shifts 1 and 2, three enclosures for sympatric shift 3, and one enclosure for each of the allopatric shifts. Details are provided in figure 5.

Statistical Analysis

We conducted all analyses with linear mixed models in JMP Pro (ver. 17.0.0). The data included different signal traits (fig. 4) that may be correlated with each other, potentially increasing the risk of spurious significance (Rice 1989). However, we were interested in all signal traits because they are associated with differently shaped mate preference functions (Rodríguez et al. 2006; Sullivan-Beckers and Cocroft 2010) and may exhibit different patterns of developmental and social plasticity (Rodríguez et al. 2008; Rebar and Rodríguez 2016; Desjonquères et al. 2019*a*, 2019*b*, 2021). Thus, different signal traits may make different contributions to reproductive isolation. Furthermore, corrections for multiple comparisons compromise statistical power (Moran 2003).

We dealt with these problems in two ways. First, we began our analyses by using a principal component analysis (PCA) to summarize variation in the original signal traits. This yielded two axes with eigenvalues more than 1 (2.86 and 1.25) that together accounted for only 58.7% of the total variation (40.9% and 17.8%, respectively). The signal traits with the highest loadings for PC1 were whine length (−0.51), pulse rate (0.54), and dominant frequency (0.50)—that is, those involved in the strongest pattern of signal-preference coevolution and associated with the strongest mate preferences in the *E. binotata* complex (Rodríguez et al. 2006; Cocroft et al. 2010). The other signal traits had loadings with absolute values between 0.12 and 0.34. This analysis thus suggests that the risk of spurious significance due to correlated traits in our data is low. Indeed, of the 21 correlations between these signal traits, 18 had values of $|r| < 0.35$, and seven had values of $|r| < 0.10$. There were only three strong correlations: between whine length and pulse rate ($r = -0.73$), between whine length and dominant frequency ($r = -0.67$), and between pulse rate and dominant frequency ($r = 0.69$). However, those are also among the signal traits of high interest because of their potential contributions to reproductive isolation due to the strength of the corresponding mate preferences (see above). We therefore retained them in the analyses. Nevertheless, to be cautious about the risk of spurious significance, we conducted preliminary tests with signal PC1 as the dependent variable, then assessed patterns across signal traits only when significance in this initial test pointed to a pattern that might be interesting to explore. Second, we used a "table-wide" criterion by noting whether significant

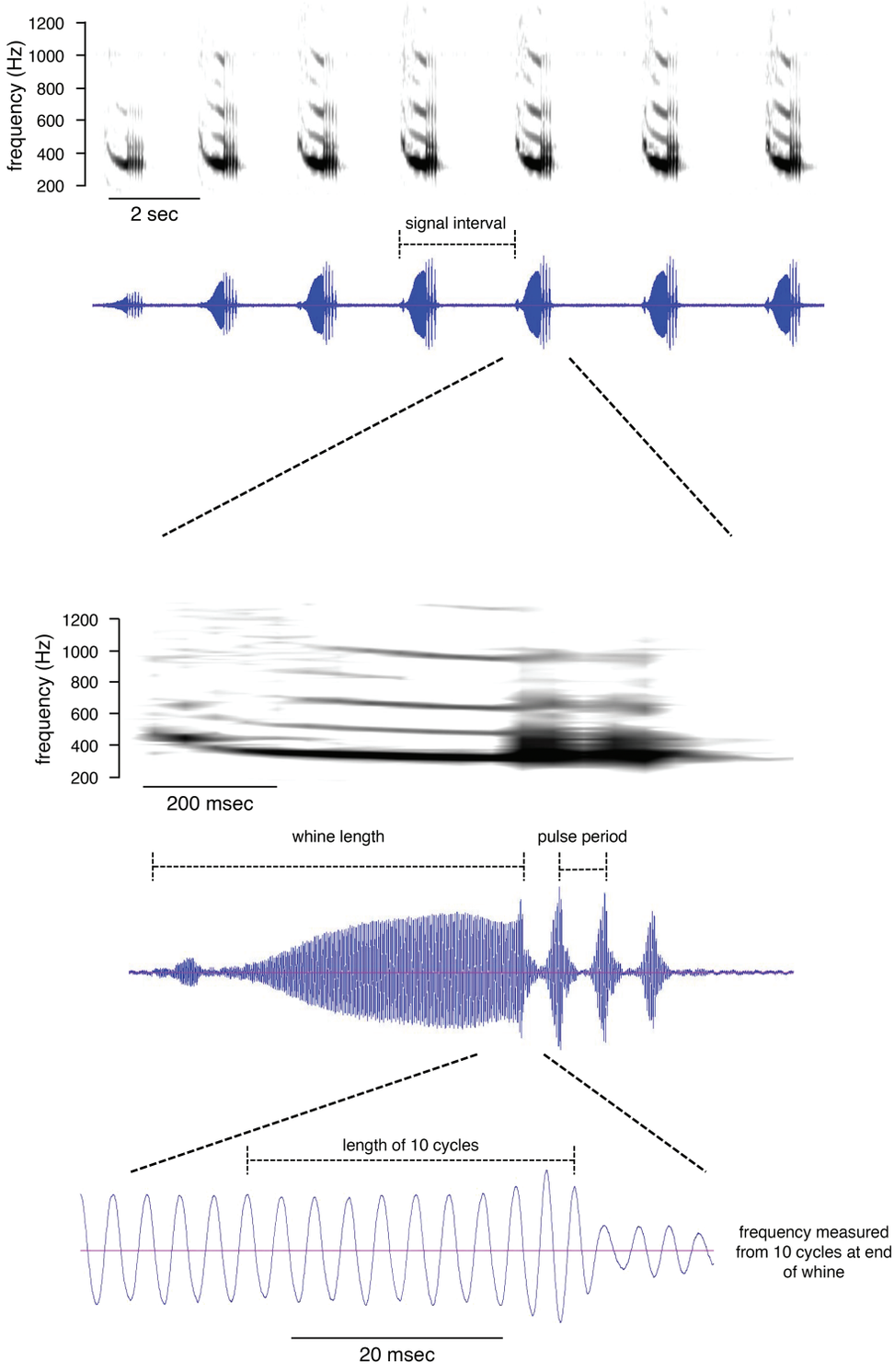

Figure 4: *Enchenopa* male signal traits measured to test for divergence due to the host shifts and reciprocal transplant experiments. *Enchenopa* males produce signals in bouts. We counted the number of signals/bout and measured the interval between landmark signals on the bout. The signals consist of a pure tone that sweeps down in frequency, followed by a series of pulses. We measured the following traits on a landmark signal on the bout: whine length, number of pulses, pulse rate, frequency at the end of the whine (measured from the length of 10 cycles in the wave form at the highest amplitude position of the main component of the signal, thus defining its dominant frequency), and difference in frequency from the beginning and end of the whine, which defines the signal's frequency sweep.

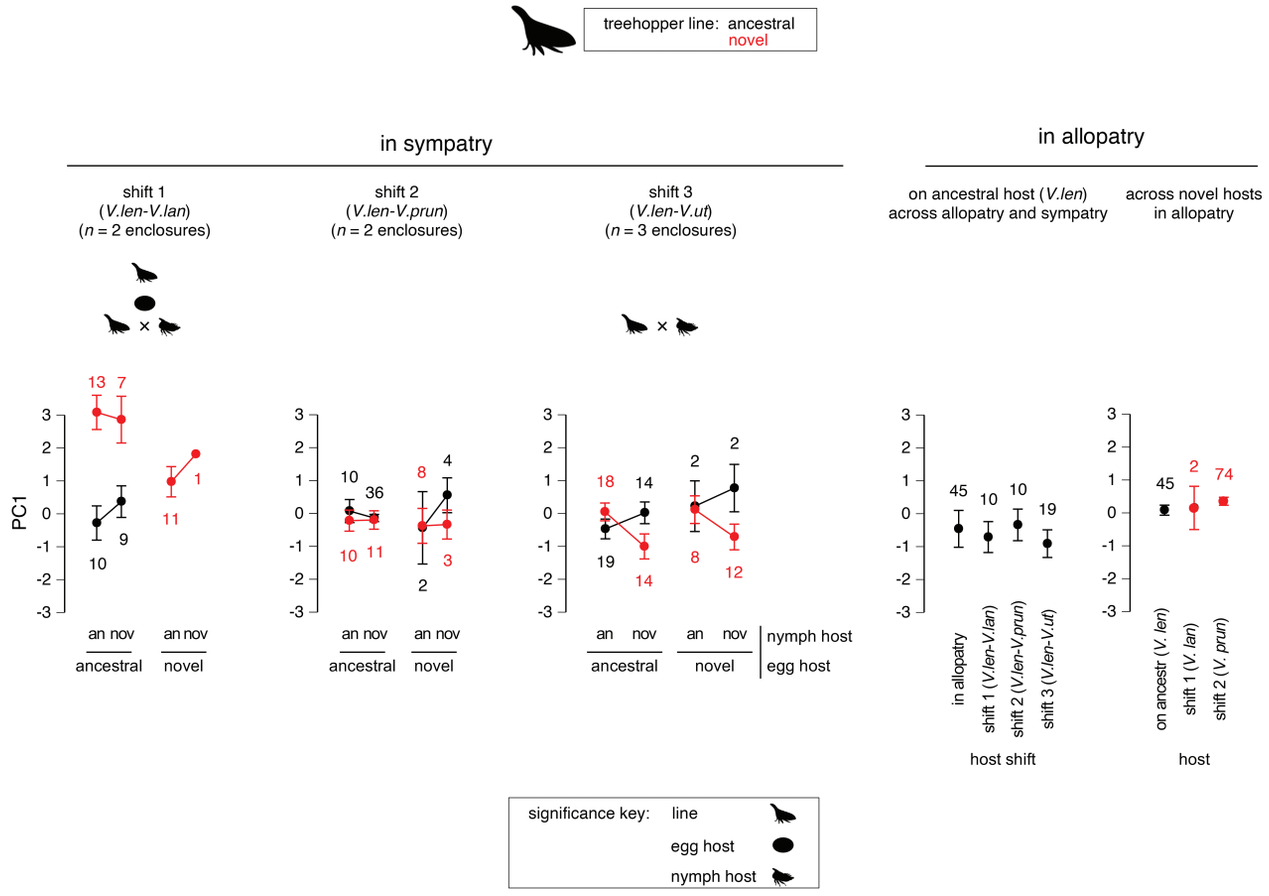

Figure 5: Variation in male advertisement signals in *Enchenopa* across host shifts and reciprocal transplant experiments. Here, we show the results with the synthetic variable generated by principal component analysis (PCA) to summarize variation across the seven signal traits we measured (signal PC1). In sympatry, the *x*-axis shows the egg and nymph hosts in the reciprocal rearing experiment. In allopatry, the egg and nymph hosts were identical. Symbols show least square mean ± SE values obtained from the linear mixed models (see text)—except for sympatric shift 1 (*V. lentago*–*V. lantana*), for which we show raw means because the statistical model for this shift was different from the other shifts (see text). The silhouettes indicate significant or marginally significant terms from table 2 (treehopper line, egg host, nymph host, and interactions). We also show the sample size of replicates (enclosures) for the sympatric host shifts. All allopatric host shifts had one enclosure. Numbers next to each symbol are the sample size of recorded males for that line × egg host × nymph host combination.

and marginally significant terms were widespread or sporadic, with the former case indicating real effects and the latter having a higher risk of spurious significance (Moran 2003).

Signal Divergence in Sympatry. We conducted the following analysis with data from the sympatric shifts only. In all models, the explanatory variables were line (ancestral, novel; i.e., the host plant onto which treehoppers had been shifted; oviposition host plant (egg host); developmental host plant (nymph host); the two- and three-way interactions between line, egg host, and nymph host; a random term for enclosure identity; and the air temperature near the male during recording.

In these models, the main term for line tests for divergence across ancestral and novel host fueled by standing genetic variation, regardless of plasticity (e.g., fig. 2*A*). The main terms for egg and nymph host, as well as their interaction, test for overall plasticity across ancestral and novel hosts, regardless of genetic divergence (e.g., fig. 2*B*). The term for egg host could indicate inputs from the early developmental environment of just-eclosed nymphs and/or inputs from maternal effects (due to variation in females' environment at the time of egg laying). The term for nymph host would indicate inputs from the developmental environment of nymphs. The interaction terms containing line (i.e., line × egg host, line × nymph host, and the three-way interaction) test for evolution in the

form of plasticity (e.g., fig. 2*C*) in response to inputs from the egg or the nymph host (two-way interactions) or both (three-way interaction).

The above test for divergence fueled by standing genetic variation (line term) could be confounded with two other means of divergence. First, the design of the selection experiment cannot distinguish a response to selection fueled by standing genetic variation from very rapid genetic assimilation. We consider the latter to be unlikely, however. Second, it could be confounded with maternal effects. To help make this distinction, we use the terms for egg host and its interaction with nymph host (which could point to a role from the manipulation of the maternal oviposition environment) to assess the likelihood of a role for maternal effects.

Finally, we noted whether each term was significant for each of the seven signal traits. We then tested for differences in the likelihood of detecting the potential means of signal divergence (according to which terms were significant) across the experimental host shifts and signal traits. For each of the four possible means of divergence (standing genetic variation, plasticity, evolution in the form of plasticity, potential maternal effects), we used a logistic regression model with whether each term was significant (1/0) as the dependent variable and host shift and signal trait as the explanatory variables.

Signal Divergence in Allopatry. We tested for differences between allopatric lines (shifts to different novel hosts) using a model with line and temperature at the time of recording as explanatory terms. We also tested for differences between ancestral-line treehoppers across host shifts, using a model with condition (allopatry/sympatry), temperature, and enclosure (random term) as explanatory terms.

Results

Signal Divergence in Sympatry

In the preliminary analysis with signal trait PC1, the terms for line, egg host, and the line × nymph host interaction were significant or marginally significant for shift 1; the line × nymph host interaction was significant for shift 3; and there were no significant effects (besides temperature) for shift 2 (table 2). This analysis, free of the risk of spurious significance due to testing correlated traits, thus indicates signal divergence fueled by standing genetic variation (line term), plasticity (egg and nymph host terms), and evolution in the form of plasticity (line × egg or nymph host interactions) in two of the three host shifts (fig. 5). Data underlying figure 5 have been deposited in the Harvard Dataverse repository (https://doi.org/10.7910/DVN/DYHRRJ; Rodríguez et al. 2025). These results span the scenarios in figure 2.

We then explored the patterns across the original signal traits (tables 3–9; figs. 6, 7). We summarize these results in table 10. The likelihood of signal divergence

Table 2: Test for signal divergence in the *Enchenopa* sympatric host shifts

	Shift 1 (*V. lentago*–*V. lantana*)[a]		Shift 2 (*V. lentago*–*V. prunifolium*)[b]		Shift 3 (*V. lentago*–*V. utile*)[c]	
Term	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*
Line	1, 34.61	**3.24, .081**	1, 67	.59, .44	1, 57.67	2.24, .14
Egg host	1, 36.82	**5.03, .031**	1, 67	.0001, .99	1, 61.84	2.14, .15
Nymph host	1, 26.19	2.70, .11	1, 67	.32, .57	1, 63.46	.47, .50
Line × egg host	…	…	1, 67	.09, .76	1, 64.96	.76, .39
Line × nymph host	1, 36.5	**3.71, .062**	1, 67	.21, .65	1, 33.39	**3.66, .064**
Egg × nymph host	…	…	1, 67	.61, .44	1, 64.93	.05, .83
Line × egg × nymph host	…	…	1, 67	.57, .45	1, 64.72	.02, .90
Temperature	1, 20.17	**83.28, <.0001**	1, 67	**96.4, <.0001**	1, 26.83	**52.4, <.0001**
Enclosure	95% CI = −.07 to .03		95% CI = −.04 to −.02		95% CI = −.21 to .27	

Note: The dependent variable is the first axis generated by the principal component analysis of the seven signal traits we measured. Terms: line tests for signal differences across ancestral and novel hosts due to overall genetic divergence, regardless of plasticity; egg host and nymph host (and their interaction) test for signal differences due to overall plasticity, regardless of genetic divergence; the interaction terms containing line test for evolutionary change in the form of plasticity. We report *F* ratios and *P* values for the fixed terms and 95% confidence intervals (CIs) for the variance component of the random term. Significant and marginally significant terms are in boldface.

[a] We were not able to include interactions with the term for egg host because there were no data for treehoppers from the ancestral female line on the novel egg host (fig. 5). We were able to test the line × nymph host interaction, however, because we have data for both nymph hosts for this line through the one egg host.

[b] Excluding the nonsignificant three-way interaction yielded similar results: all main terms and two-way interactions remained nonsignificant ($P \geq .27$), and temperature remained significant ($F = 96.7$, $P < .0001$).

[c] Excluding the nonsignificant three-way interaction yielded similar results: all main terms and two-way interactions that were nonsignificant remained so ($P \geq .13$), the marginally significant female line × nymph host interaction became significant ($F = 7.08$, $P = .011$), and temperature remained significant ($F = 52.8$, $P < .0001$).

Table 3: Test for divergence in the signal trait signals/bout (fig. 6) in the *Enchenopa* sympatric host shifts

	Shift 1 (*V. lentago*–*V. lantana*)		Shift 2 (*V. lentago*–*V. prunifolium*)[a]		Shift 3 (*V. lentago*–*V. utile*)[b]	
Term	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*
Line	1, 42.43	**2.90, .096**	1, 76	2.04, .16	1, 55.88	.49, .49
Egg host	1, 42.34	**3.19, .081**	1, 76	2.64, .11	1, 76.59	.71, .40
Nymph host	1, 42.99	.06, .82	1, 76	2.59, .11	1, 76.79	.13, .72
Line × egg host	…	…	1, 76	.90, .35	1, 61.61	.53, .47
Line × nymph host	1, 42.64	.77, .38	1, 76	1.32, .25	1, 10.39	.15, .71
Egg × nymph host	…	…	1, 76	.002, .96	1, 77.83	.38, .54
Line × egg × nymph host	…	…	1, 76	.03, .86	1, 50.29	2.65, .11
Temperature	1, 41.22	**5.52, .024**	1, 76	2.55, .11	1, 25.28	**14.1, .0009**
Enclosure	95% CI = −.28 to .37		95% CI = −.04 to −.02		95% CI = −.09 to .03	

Note: Table composition is as for table 2. CI = confidence interval.

[a] Excluding the nonsignificant three-way interaction yielded similar results: most terms that were nonsignificant remained so ($P \geq .11$), and nymph host became marginally significant ($F = 3.59$, $P = .062$).

[b] Excluding the nonsignificant three-way interaction yielded similar results: all terms that were nonsignificant remained so ($P \geq .18$), and temperature remained significant ($F = 8.84$, $P = .006$).

fueled by standing genetic variation (as per fig. 2*A*) did not vary significantly across shifts (table 10). But the likelihood of overall plasticity in signal traits (as per fig. 2*B*) varied significantly across shifts, involving the most traits in shift 1 (*V. lentago*–*V. lantana*; table 10). The likelihood of evolution in the form of plasticity (as per fig. 2*C*) also varied significantly across shifts, involving the most signal traits in shift 3 (*V. lentago*–*V. utile*; table 10). Finally, the likelihood of potential inputs from maternal effects did not vary significantly across shifts (table 10).

Interestingly, signal traits differed significantly in the likelihood of showing effects due to overall plasticity, evolution in the form of plasticity, and the potential for maternal effects; however, they did not differ in the likelihood of showing an effect due to standing genetic variation (table 10). Dominant frequency (the most distinct adult phenotype in the *Enchenopa binotata* complex, and the signal trait associated with the strongest female preferences; Rodríguez et al. 2006; Cocroft et al. 2010; fig. 4) was involved in only two of these divergence events: plasticity in shift 1 (*V. lentago*–*V. lantana*) and evolution in the form of plasticity in shift 3 (*V. lentago*–*V. utile*; table 10). Other signal traits associated with medium-strength mate preferences (whine length, pulse number, pulse rate; fig. 4) were involved more prevalently (table 10).

In the above tests, the term for line asks whether signals diverged on average across ancestral and novel host lines, regardless of plasticity. This does not quite reflect what one would observe in nature if there was signal evolution among insects on different host species, which would instead involve "ancestral-line" treehoppers on the ancestral host and "novel-line" treehoppers on the novel host.

Table 4: Test for divergence in signal interval (fig. 6) in the *Enchenopa* sympatric host shifts

	Shift 1 (*V. lentago*–*V. lantana*)		Shift 2 (*V. lentago*–*V. prunifolium*)[a]		Shift 3 (*V. lentago*–*V. utile*)[b]	
Term	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*
Line	1, 43	.09, .76	1, 75	.55, .46	1, 76.11	**3.58, .062**
Egg host	1, 43	.75, .39	1, 75	.11, .74	1, 74.99	.11, .74
Nymph host	1, 43	.32, .58	1, 75	2.62, .11	1, 75.17	.50, .48
Line × egg host	…	…	1, 75	.06, .81	1, 75.58	**7.94, .0062**
Line × nymph host	1, 43	2.13, .15	1, 75	.40, .53	1, 76.89	**9.71, .0026**
Egg × nymph host	…	…	1, 75	.04, .84	1, 75.29	**3.58, .062**
Line × egg × nymph host	…	…	1, 75	.02, .88	1, 75.54	.61, .44
Temperature	1, 43	2.26, .14	1, 75	1.59, .21	1, 76.98	**32.7, <.0001**
Enclosure	95% CI = −.008 to −.003		95% CI = −.014 to −.008		95% CI = −.22 to .58	

Note: Table composition is as for table 2. CI = confidence interval.

[a] Excluding the nonsignificant three-way interaction yielded similar results: most terms that were nonsignificant remained so ($P \geq .21$), and nymph host became marginally significant ($F = 3.60$, $P = .062$).

[b] Excluding the nonsignificant three-way interaction yielded similar results: terms that were nonsignificant remained so ($P \geq .18$), terms that were significant remained so ($F = 3.65$), female line remained marginally significant ($F = 3.65$, $P = .06$), egg host × nymph host remained marginally significant ($F = 3.02$, $P = .086$), and temperature remained significant ($F = 33.5$, $P < .0001$).

Table 5: Test for divergence in signal whine length (fig. 6) in the *Enchenopa* sympatric host shifts

	Shift 1 (*V. lentago*–*V. lantana*)		Shift 2 (*V. lentago*–*V. prunifolium*)		Shift 3 (*V. lentago*–*V. utile*)[a]	
Term	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*
Line	1, 42.73	2.38, .13	1, 76	.42, .52	1, 78	1.97, .16
Egg host	1, 42.3	**3.83, .057**	1, 76	1.14, .29	1, 78	1.45, .23
Nymph host	1, 42.99	**5.05, .03**	1, 76	**4.85, .031**	1, 78	.02, .88
Line × egg host	…	…	1, 76	.18, .67	1, 78	2.75, .10
Line × nymph host	1, 42.58	2.77, .10	1, 76	**4.61, .035**	1, 78	**4.87, .03**
Egg × nymph host	…	…	1, 76	.12, .73	1, 78	.05, .82
Line × egg × nymph host	…	…	1, 76	**4.93, .029**	1, 78	.03, .86
Temperature	1, 41.96	**51.69, <.0001**	1, 76	**98.9, <.0001**	1, 78	**49.0, <.0001**
Enclosure	95% CI = −.001 to .001		95% CI = −.00014 to .00007		95% CI = -9.7×10^{-5} to -5.0×10^{-5}	

Note: Table composition is as for table 2. CI = confidence interval.

[a] Excluding the nonsignificant three-way interaction yielded similar results: terms that were nonsignificant remained so ($P \geq .10$), female line × nymph host remained significant ($F = 7.44$, $P = .008$), and temperature remained significant ($F = 55.1$, $P < .0001$).

To assess this scenario, we tested for differences between ancestral-line treehoppers on the ancestral egg and nymph hosts (leftmost black symbol in each panel of fig. 5 in sympatry and in figs. 6 and 7) and novel-line treehoppers on the novel egg and nymph hosts (rightmost red symbol in each panel of fig. 5 in sympatry and in figs. 6 and 7), including recording temperature and enclosure in the model. In most cases these differences were small and nonsignificant ($P \geq .15$). However, some differences were larger and significant: for shift 1 (*V. lentago*–*V. lantana*), pulse rate ($F_{1,8} = 6.10$, $P = .039$; fig. 6) and frequency sweep ($F_{1,6.949} = 8.76$, $P = .021$; fig. 7); for shift 2 (*V. lentago*–*V. prunifolium*), whine length ($F_{1,15} = 9.70$, $P = .0071$; fig. 6); and for shift 3 (*V. lentago*–*V. utile*), signals/bout ($F_{1,24.9} = 17.68$, $P = .0003$; fig. 6) and frequency sweep ($F_{1,22} = 4.71$, $P = .041$; fig. 7).

Another question of interest is whether evolution in the form of plasticity would result in signal divergence between ancestral- and novel-line treehoppers were they to meet on the ancestral host—that is, if novel-line treehoppers were to return to the ancestral host. To investigate this question, we tested for differences between ancestral- and novel-line treehoppers on the ancestral egg and nymph hosts (i.e., between the red and black symbols on the leftmost column on each panel in fig. 5 in sympatry and figs. 6 and 7), including recording temperature and enclosure in the models. Most of these differences were small and nonsignificant ($P \geq .15$; figs. 5–7). But for shift 3 (*V. lentago*–*V. utile*), there were significant differences for signals/bout ($F_{1,32.71} = 12.12$, $P = .0014$), whine length ($F_{1,33} = 3.66$, $P = .065$), and dominant frequency ($F_{1,31.31} = 5.64$, $P = .024$; figs. 5–7).

We also asked whether evolution in the form of plasticity would result in signal divergence between ancestral- and novel-line treehoppers were they to meet on a novel host—that is, if ancestral-line treehoppers initiated a second colonization of a novel host. To investigate this question, we tested for differences between ancestral- and novel-line treehoppers on the novel egg and nymph hosts (i.e., between the red and black symbols in the rightmost

Table 6: Test for divergence in signal number of pulses (fig. 6) in the *Enchenopa* sympatric host shifts

	Shift 1 (*V. lentago*–*V. lantana*)		Shift 2 (*V. lentago*–*V. prunifolium*)[a]		Shift 3 (*V. lentago*–*V. utile*)	
Term	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*
Line	1, 42.8	.46, .50	1, 76	.01, .93	1, 78	2.33, .13
Egg host	1, 42.12	2.02, .16	1, 76	.34, .56	1, 78	.23, .63
Nymph host	1, 42.57	**4.99, .031**	1, 76	**14.4, .0003**	1, 78	.39, .53
Line × egg host	…	…	1, 76	1.22, .27	1, 78	.18, .68
Line × nymph host	1, 42.24	.09, .76	1, 76	1.31, .26	1, 78	1.39, .24
Egg × nymph host	…	…	1, 76	**3.22, .076**	1, 78	1.65, .20
Line × egg × nymph host	…	…	1, 76	.97, .33	1, 78	**4.94, .029**
Temperature	1, 42.93	.02, .90	1, 76	.03, .86	1, 78	.42, .52
Enclosure	95% CI = −.22 to .40		95% CI = −.011 to −.006		95% CI = −.011 to −.006	

Note: Table composition is as for table 2. CI = confidence interval.

[a] Excluding the nonsignificant three-way interaction yielded similar results: terms that were nonsignificant remained so ($P \geq .23$), nymph host became nonsignificant ($F = 14.4$, $P = .16$), and egg host × nymph host became nonsignificant ($F = 2.43$, $P = .36$).

Table 7: Test for divergence in signal pulse rate (fig. 6) in the *Enchenopa* sympatric host shifts

	Shift 1 (*V. lentago*–*V. lantana*)		Shift 2 (*V. lentago*–*V. prunifolium*)[a]		Shift 3 (*V. lentago*–*V. utile*)[b]	
Term	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*
Line	1, 42.43	**8.89, .005**	1, 76	.00, 1.00	1, 74.57	1.19, .28
Egg host	1, 42.05	**4.44, .04**	1, 76	1.68, .20	1, 76.51	.22, .64
Nymph host	1, 42.28	.02, .90	1, 76	**4.85, .031**	1, 77.18	.0002, .99
Line × egg host	…	…	1, 76	.02, .88	1, 77.3	.92, .34
Line × nymph host	1, 42.11	**2.94, .094**	1, 76	.83, .37	1, 42.87	2.48, .12
Egg × nymph host	…	…	1, 76	**3.65, .06**	1, 77.48	**3.73, .057**
Line × egg × nymph host	…	…	1, 76	.02, .89	1, 76.63	.16, .69
Temperature	1, 42.54	**118.9, <.0001**	1, 76	**143.3, <.0001**	1, 43.38	**56.7, <.0001**
Enclosure	95% CI = −.83 to 1.64		95% CI = −.03 to −.02		95% CI = −.13 to .16	

Note: Table composition as for table 2. CI = confidence interval.

[a] Excluding the nonsignificant three-way interaction yielded similar results: terms that were nonsignificant remained so ($P \geq .20$), nymph host remained significant ($F = 5.82$, $P = .018$), egg host × nymph host remained marginally significant ($F = 3.93$, $P = .051$), and temperature remained significant ($F = 145.8$, $P < .0001$).

[b] Excluding the nonsignificant three-way interaction yielded similar results: most terms that were nonsignificant remained so ($P \geq .28$), female line × nymph host became marginally significant ($F = 3.16$, $P = .08$), egg host × nymph host remained marginally significant ($F = 3.97$, $P = .05$), and temperature remained significant ($F = 59.7$, $P < .0001$).

column on each panel in fig. 5 in sympatry and figs. 6 and 7), including recording temperature and enclosure in the models. For shift 1 (*V. lentago*–*V. lantana*), no comparison was possible because there were no data from treehoppers from the ancestral host on the novel egg host (figs. 5–7). For shift 2 (*V. lentago*–*V. prunifolium*), most differences were small and nonsignificant ($P \geq .21$), with the exception of whine length ($F_{1, 8.457} = 4.70$, $P = .06$; figs. 5, 6). For shift 3 (*V. lentago*–*V. utile*), most differences were small and nonsignificant ($P \geq .19$), but there were large and significant differences for signal PC1 ($F_{1, 6.666} = 11.67$, $P = .012$), pulse rate ($F_{1, 9.23} = 7.09$, $P = .025$), and dominant frequency ($F_{1, 10} = 121.00$, $P < .0001$; figs. 5–7).

No Signal Divergence in Allopatry

The analysis with signal PC1 revealed no signal divergence among treehoppers on the different host shifts (nonsignificant line term; table 11, pt. A; fig. 5). There was also no signal divergence among treehoppers on the ancestral host in the different host shifts (nonsignificant host shift term in table 11, pt. B; fig. 5).

Discussion

We assessed how signal divergence may arise in the early stages of a host shift using a quasi-natural host shift selection experiment (Fry 2003) followed by a reciprocal

Table 8: Test for divergence in signal dominant frequency (fig. 6) in the *Enchenopa* sympatric host shifts

	Shift 1 (*V. lentago*–*V. lantana*)		Shift 2 (*V. lentago*–*V. prunifolium*)[a]		Shift 3 (*V. lentago*–*V. utile*)[b]	
Term	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*
Line	1, 41.27	.79, .38	1, 76	.13, .72	1, 78	.06, .81
Egg host	1, 41.31	.08, .78	1, 76	.001, .97	1, 78	1.17, .28
Nymph host	1, 41.94	**5.55, .023**	1, 76	.10, .75	1, 78	.86, .36
Line × egg host	…	…	1, 76	.99, .32	1, 78	.12, .73
Line × nymph host	1, 41.77	1.27, .27	1, 76	.06, .80	1, 78	**6.77, .011**
Egg × nymph host	…	…	1, 76	.16, .69	1, 78	.48, .49
Line × egg × nymph host	…	…	1, 76	1.04, .31	1, 78	.02, .89
Temperature	1, 41.03	**52.09, <.0001**	1, 76	**56.1, <.0001**	1, 78	**59.4, <.0001**
Enclosure	95% CI = −20.7 to 28.3		95% CI = −3.32 to −1.72		95% CI = −2.31 to −1.21	

Note: Table composition is as for table 2. CI = confidence interval.

[a] Excluding the nonsignificant three-way interaction yielded similar results: all terms that were nonsignificant remained so ($P \geq .16$), and temperature remained significant ($F = 57.2$, $P < .0001$).

[b] Excluding the nonsignificant three-way interaction yielded similar results: terms that were nonsignificant remained so ($P \geq .28$), female line × nymph host remained significant ($F = 12.3$, $P = .0008$), and temperature remained significant ($F = 65.2$, $P < .0001$).

Table 9: Test for divergence in signal frequency sweep (fig. 6) in the *Enchenopa* sympatric host shifts

	Shift 1 (*V. lentago*–*V. lantana*)		Shift 2 (*V. lentago*–*V. prunifolium*)[a]		Shift 3 (*V. lentago*–*V. utile*)[b]	
Term	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*	ndf, ddf	*F*, *P*
Line	1, 18.89	.50, .49	1, 59.04	.74, .39	1, 65.96	**6.31, .014**
Egg host	1, 36.86	.31, .58	1, 67.74	.14, .71	1, 63.77	1.56, .22
Nymph host	1, 6.936	1.28, .30	1, 22.34	1.54, .23	1, 64.37	1.26, .27
Line × egg host	…	…	1, 66.52	.04, .84	1, 65.16	2.28, .14
Line × nymph host	1, 28.63	.56, .46	1, 65.99	.17, .69	1, 64.65	2.54, .12
Egg × nymph host	…	…	1, 49.59	.08, .77	1, 65.01	.81, .37
Line × egg × nymph host	…	…	1, 67.99	.48, .49	1, 65.03	.19, .67
Temperature	1, 5.10	1.27, .31	1, 67.21	.09, .77	1, 61.09	**5.25, .025**
Enclosure	95% CI = −264.8 to 179.9		95% CI = −252.3 to 333.2		95% CI = −379.1 to 826.1	

Note: Table composition is as for table 2. CI = confidence interval.

[a] Excluding the nonsignificant three-way interaction yielded similar results: all terms that were nonsignificant remained so ($P \geq .21$).

[b] Excluding the nonsignificant three-way interaction yielded similar results: most terms that were nonsignificant remained so ($P \geq .13$), female line remained significant ($F = 6.34$, $P = .014$), female line × nymph host became marginally significant ($F = 2.88$, $P = .094$), and temperature remained significant ($F = 5.49$, $P = .002$).

transplant experiment. We worked with the *Enchenopa binotata* complex of treehoppers, a clade where speciation begins with host plant shifts (Wood and Guttman 1983; Wood 1993) and involves remarkable signal-preference codivergence (Rodríguez et al. 2006; Cocroft et al. 2008, 2010). We found evidence that all potential means of signal divergence—standing genetic variation, plasticity, and evolution in the form of plasticity—played a role. This included signal differences when treehoppers that were shifted to a novel host went back to the ancestral host and vice versa.

The likelihood of a role for overall plasticity and for evolution in the form of plasticity differed across the experimental host shifts, being higher with the two most phylogenetically and phenotypically distinct novel hosts (*Viburnum lantana*, *V. utile*) from the ancestral host (*V. lentago*; Clement et al. 2014; Rodríguez et al. 2021). Interestingly, one of these novel hosts (*V. utile*) was also the only one in which adaptation occurred in the prior study (Rodríguez et al. 2021). This suggests that more distinct novel hosts induced higher levels of early plasticity and perhaps also generated stronger selection on the form of plasticity, while evolution involving standing genetic variation was similar among hosts. As we shifted the treehoppers to hosts that were novel to them, any initial form of plasticity was likely previously unselected (although the source population on *V. lentago* may have had some history one of those hosts, as it was surrounded by areas with mainly *V. prunifolium*).

Some signal traits were also more prone to exhibit overall plasticity (including the potential for maternal effects; see Little et al. 2024) and evolution in the form of plasticity. These tended to be those associated with relatively strong female mate preferences in the *E. binotata* complex, although seldom in the signal trait (dominant frequency) subject to the strongest preferences.

All instances of signal divergence occurred in conditions of sympatry, with no divergence of any form arising in allopatry. We thus interpret the observed signal divergence as involving a response to selection on novel versus ancestral hosts with gene flow. Gene flow from the ancestral host likely had a genetic rescue effect (Holt and Barfield 2011; Eriksson et al. 2014; Tomasini and Peischl 2020), facilitating the colonization of the novel hosts (Rodríguez et al. 2021; e.g., note that the *V. utile* allopatric populations became extinct) and promoting a response to selection (current article). An alternative possibility is that the treehoppers may have become adapted to encountering both ancestral and novel hosts in their adult lives, with signal differences between hosts representing adaptive plasticity. We favor the selection–gene flow interpretation, however, for three reasons. First, although there was some dispersal in sympatry, the treehoppers were strongly philopatric as adults and completely philopatric as juveniles (see above; Stearns et al. 2013). Thus, encounters with both ancestral and novel hosts were possible but probably relatively rare. Second, when adaptation occurred in sympatry, performance was improved not only on the novel host but also on the ancestral host when individuals adapted to the novel host were shifted back (Rodríguez et al. 2021). Third, any adaptation to encountering both ancestral and novel hosts should be mirrored in the corresponding allopatric conditions—for example, what might look like the scenario in figure 2*C* in sympatry should look like the scenario in figure 2*A* in allopatry. But there was no signal divergence at all in allopatry. We note that these results do not correspond to the situation for *E. binotata* in the wild, where strongly host-specific species with strongly divergent signals

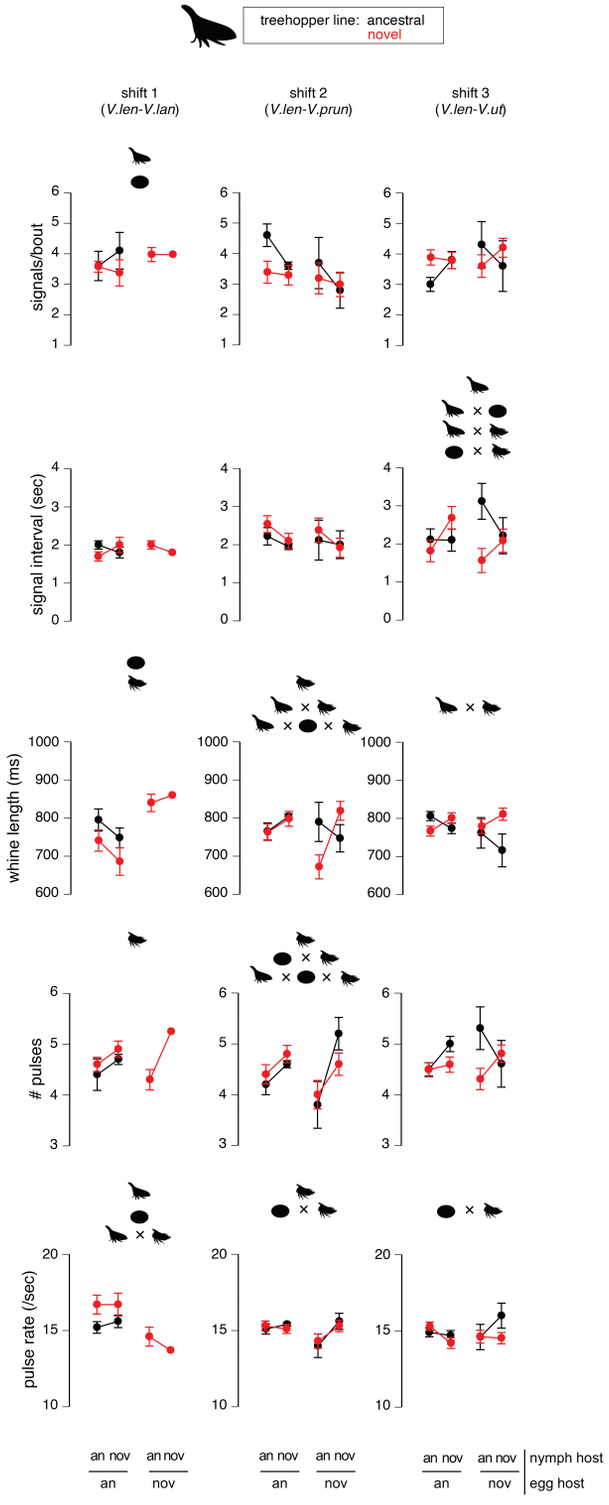

Figure 6: Variation in male advertisement signals in *Enchenopa* across host shifts and reciprocal transplant experiments. Here, we show the signal temporal features that we measured. Figure structure is as in figure 5. Symbols show least square mean ± SE values obtained from the linear mixed models (see text)—except for sympatric shift 1 (*V. lentago*–*V. lantana*), for which we show raw means because the statistical model for this shift was different from the other shifts (see text). Silhouette significance key and enclosure and male sample sizes are as in figure 5.

and preferences often occur in sympatry and syntopy—that is, not merely sharing the same geographic area but occurring in very close physical proximity (Wood and Guttman 1983; Wood 1993; Cocroft et al. 2008). Instead, we consider them to represent likely conditions at the beginning of speciation in sympatry.

These findings point to the presence of both standing genetic variation in signals and standing genetic variation in signal plasticity in the source population. We did not obtain estimates for these from the source population. However, work with another member of the *E. binotata* complex (the species that lives on *Ptelea trifoliata* host plants in Missouri) has detected both broad-sense heritability and genetic variation in plasticity (i.e., genotype × environment interaction [G × E]; Lynch and Walsh 1998), with treehoppers reared on different host species (Rodríguez et al. 2008). This included heritability and G × E in dominant signal frequency as well as other signal traits associated with relatively strong female mate preferences in the complex. Note that the evolution in the form of plasticity that we detected across ancestral and novel hosts could also be interpreted as G × E. However, we consider it more straightforward to view treehoppers shifted to different hosts as different selected lines rather than different genotypes per se. We therefore view our results as involving evolution in the form of plasticity.

The above patterns of change in signals could have important consequences for assortative mating. In the analysis with signal PC1, the largest differences between ancestral- and novel-line treehoppers occurred on the novel host (fig. 5). For several signal traits, there were also considerable differences between ancestral- and novel-line treehoppers on the ancestral host, including traits for which females have strong mate preferences, particularly dominant frequency, whine length, pulse number, and pulse rate (fig. 6; table 10; Rodríguez et al. 2006).

Whether these changes would result in assortative mating depends on how they relate to potential changes in female mate preferences. We did not obtain data for mate preferences in this experiment. However, we have information about the mate preferences of *E. binotata* from *Viburnum* for other localities (on *V. rufidulum* in Missouri, on *V. lentago* in Wisconsin; Rodríguez et al. 2006, 2013*b*). The differences of up to 10 Hz in dominant signal frequency and up to 100 ms in whine length that we observed between ancestral- and novel-line treehoppers on novel and ancestral hosts (figs. 6, 7) are sufficient to displace the signal

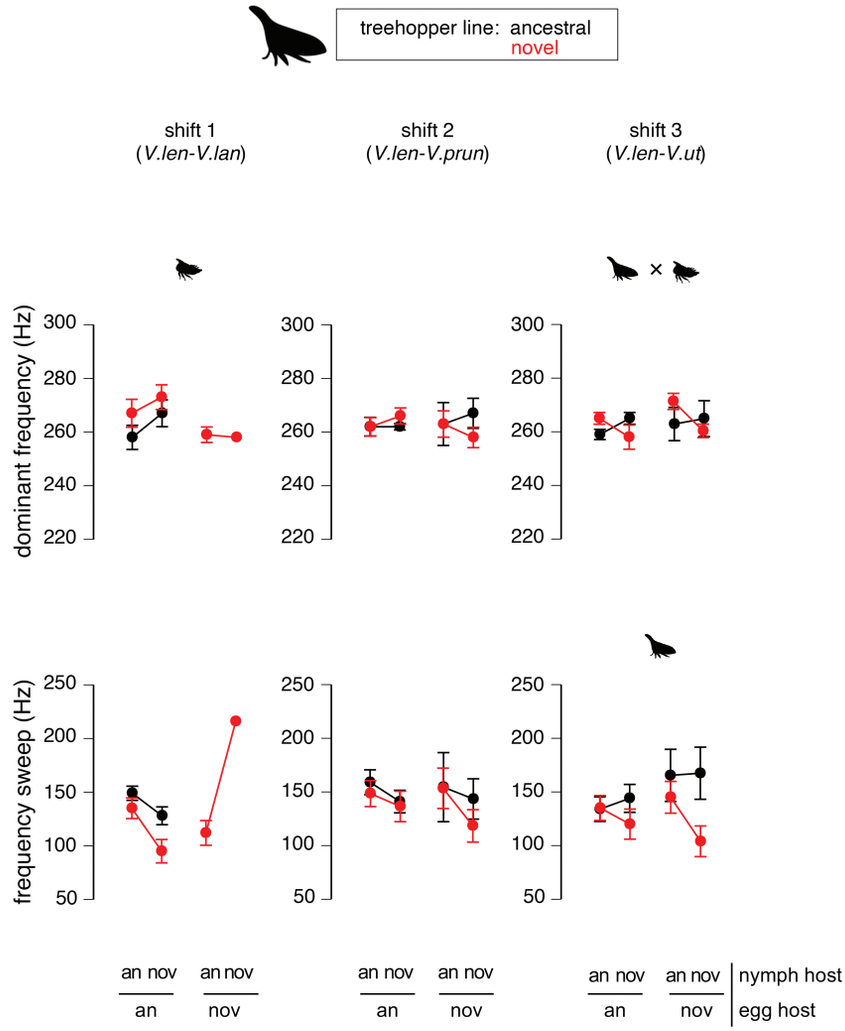

Figure 7: Variation in male advertisement signals in *Enchenopa* across host shifts and reciprocal transplant experiments. Here, we show the signal spectral features that we measured. Figure structure is as in figure 5. Symbols show least square mean ± SE values obtained from the linear mixed models (see text)—except for sympatric shift 1 (*V. lentago*–*V. lantana*), for which we show raw means because the statistical model for this shift was different from the other shifts (see text). Silhouette significance key and enclosure and male sample sizes are as in figures 5 and 6.

away from the peak of the female mate preference and reduce the likelihood of female response by approximately 50% and 40%, respectively (Rodríguez et al. 2006). Note, however, that even comparably slight changes in peak preferences in the other direction from the male signals would achieve much stronger decreases in female response and hence assortative mating. We have evidence of multiple causes of plasticity in mate preferences, with some factors generating signal-preference mismatches (such as experience of signaling before and during adult sexual maturation or developing in social groups of varying density; Fowler-Finn and Rodríguez 2012*a*, 2012*b*; Rebar and Rodríguez 2016; Fowler-Finn et al. 2017; Desjonquères et al. 2019*a*, 2019*b*, 2021; Desjonquères and Rodríguez 2023) and other factors generating signal-preference covariance (such as developing on different host plant genotypes and/or social groupings; Rebar and Rodríguez 2013, 2014*a*, 2014*b*, 2015; Desjonquères et al. 2023). In short, it seems likely that the signal changes we observed would alter the form of sexual selection due to mate choice and also result in assortative mating between ancestral and host-shifted treehopper populations within a few generations of the shifts.

Our analyses involved a large number of comparisons. The signal traits we measured were mostly weakly correlated with each other, so that the problem of testing multiple correlated traits was unlikely. Nevertheless, we had

Table 10: Summary of tests for *Enchenopa* signal divergence in the sympatric host shifts, for the seven signal traits (from the results in tables 3–9 and figs. 6 and 7)

Means of divergence	Shift 1 (*V. lentago*–*V. lantana*)	Shift 2 (*V. lentago*–*V. prunifolium*)	Shift 3 (*V. lentago*–*V. utile*)	Comparison of likelihood of detecting cause of divergence across the different host shifts and signal traits	
				Term	χ^2, *P*
Standing genetic variation (line term)	2 (signals/bout, pulse rate)	0	2 (signal interval, frequency sweep)	Host shift Signal trait	4.19, .12 5.66, .46
Overall plasticity (egg host, nymph host, and their interaction)	5 (signals/bout, whine length, no. pulses, pulse rate, dominant frequency)	3 (whine length, no. pulses, pulse rate)	0	Host shift Signal trait	**19.1, <.0001** **17.94, .0064**
Evolution in the form of plasticity (interactions between line and egg or nymph host)	1 (pulse rate)	1 (whine length)	4 (signal interval, whine length, no. pulses, dominant frequency)	Host shift Signal trait	**4.618, .099** 9.19, .16
Potential maternal effects (egg host, any interaction with egg host)	3 (signals/bout, whine length, pulse rate)	3 (whine length, no. pulses, pulse rate)	3 (signal interval, no. pulses, pulse rate)	Host shift Signal trait	0, 1.0 **13.41, .037**

Note: Means of divergence are as follows: divergence fueled by standing genetic variation (line term), plasticity (egg host, nymph host, and their interaction), evolution in the form of plasticity (interactions between line and egg host or nymph host, or both), and potential maternal effects (egg host, any interaction with that term). For each host shift, we show the number of signal traits out of the seven we measured for which the corresponding terms were significant or marginally significant. We then show the comparison across host shifts and signal traits of the likelihood that the cause of divergence was detected (rightmost column; significant terms or marginally significant terms from the logistic regression models are in boldface).

Table 11: Tests for *Enchenopa* signal divergence in allopatry

Term	ndf, ddf	*F*, *P*
	A. Across novel hosts in allopatry[a]	
Line	2, 105	.86, .43
Temperature	1, 105	**210.3, <.0001**
Enclosure	In this test, there was only one enclosure for each host shift	
	B. On the ancestral host across shifts in allopatry and sympatry[b]	
Host shift	3, 2.536	.26, .85
Temperature	1, 121.1	**207.61, <.0001**
Enclosure	95% CI = −.13 to .22	

Note: In both tests, the dependent variable is the first axis generated by the principal component analysis of the seven signal traits we measured. Part A tests for differences between the shifts in allopatry (line term), and part B tests for differences on the ancestral host between shifts in sympatry and allopatry. We report *F* ratios and *P* values for the fixed terms and 95% confidence intervals (CIs) for the variance component of the random term. Significant terms are in boldface.

[a] We confirmed that the line term was nonsignificant for each of the individual signal tests. In all cases, $F \leq 1.92$, $P \geq .15$.

[b] We confirmed that the host shift term was nonsignificant for each of the individual signal tests. In all cases, $F \leq 1.23$, $P \geq .45$.

some risk of spurious significance (Rice 1989). The table-wide criterion (Moran 2003) suggests that such a risk may apply for the means of divergence involving standing genetic variation (for at most two out of seven signal traits in any one shift; table 10), but less so for the other means of divergence.

In the context of host shifts (i.e., of populations colonizing of novel environments), bottlenecks may influence the signal-preference relationship and reproductive isolation. Bottlenecks may restrict the variety of displays in the novel populations and lower the availability of the mate types preferred in the ancestral population, which may in turn favor lower selectivity in mate choice (Kaneshiro 1980, 1983; Fraser and Boake 1997). We consider this factor to probably not be in play in the selection experiment. The starting population was very large (28,000 females), and the number of breeding adults on each replicate was likely always high enough to minimize the impact of drift (>50 breeding pairs/replicate; Fry 2003; Rodríguez et al. 2021). Furthermore, random variation should be evident as differences between replicates in our study, which were negligible (tables 2–9; Rodríguez et al. 2021). We note, however, that plasticity in mate preferences may generate a Kaneshiro-like effect (see Kaneshiro 1980, 1983; Fraser and Boake 1997) in at least some members of the *E. binotata* complex: females that experience conditions indicative of low availability of preferred mate types (created with playback experiments) as young adults show lower selectivity in their mate preferences than females that experienced conditions indicative of the presence of preferred mate types, whether alone or mixed with nonpreferred types (Fowler-Finn and Rodríguez 2012*a*, 2012*b*; Rodríguez et al. 2013; Desjonquères and Rodríguez 2023). Our conclusions regarding the consequences of the patterns of signal divergence we observed in the host shifts must be tempered until a fuller understanding of the corresponding patterns in mate preferences is achieved.

We now integrate the results we report here on signal divergence with the prior analysis of the adaptive consequences of the host shifts for female fecundity (Rodríguez et al. 2021). First, signal divergence was more common than adaptation in sympatry, and both were completely absent in allopatry. This suggests that gene flow from populations on the ancestral host may have provided variants that fueled the response to divergent selection (Holt and Barfield 2011; Eriksson et al. 2014; Tomasini and Peischl 2020). Second, of the two host shifts in which signal divergence was more likely and stronger, one was also the host shift in which adaptation occurred, which involved one of the more distinct novel hosts. This suggests some common features between selection on signals and performance on the host plants. However, the relationship between signal divergence and adaptation was not straightforward: it could not have involved selection against hybridization, given that the observed adaptation did not involve ecological specialization or performance trade-offs across ancestral and novel hosts, instead yielding even better performance for shifted treehoppers back on the ancestral hosts (Rodríguez et al. 2021). We note that specialization does arise in the *E. binotata* complex (Wood and Guttman 1983; Wood 1993; Lin and Wood 2002; Cocroft et al. 2008, 2010; Hsu et al. 2018). However, this does not seem to occur in the early stages of speciation.

Thus, our results suggest that assortative mating may arise early on from signal divergence. Importantly, this signal divergence can be due not only to overall genetic divergence but also to overall plasticity and evolution in the form of plasticity. Such early assortative mating could in turn help further adaptation and eventual specialization, but with adaptation/specialization and reproductive isolation originating independently and in parallel or with sexual divergence leading. If ecological and sexual divergence do originate independently and occur in parallel or if sexual divergence does arise first, it may be that ecological divergence is facilitated by plasticity and sexual divergence—rather than ecological divergence being the initiating factor. Furthermore, early sexual divergence may be especially important for speciation in cases of nonadaptive (nonecological) radiation (West-Eberhard 1983; Czekanski-Moir and Rundell 2019; see Anderson and Weir 2022; Anderson et al. 2023).

To the extent that our results are typical of the early stages of speciation, they suggest that plasticity and divergence in sexual traits may often facilitate seemingly problematic scenarios of speciation involving linkage disequilibrium between broad suites of polygenic traits (Kopp et al. 2018). Two features of our experiment make it especially able to address these questions. The transplant experiment after a brief interval of selection (five generations) offers an unusual level of resolution regarding the processes at play early in speciation. Examining later stages may add confounding factors, as more traits and processes are likely to be involved (Nosil et al. 2009; Sobel et al. 2010; Kulmuni et al. 2020). And working with a clade such as the *E. binotata* complex, where prior work has established a causal link between shifts to novel host plants and signal-preference divergence, offers an ideal context to test how signal divergence may arise from host shifts. We suggest that more experiments like ours will be necessary to determine the sequence of these events during the early stages of speciation and clarify the roles of divergence in ecological adaptation and sexual traits (Mendelson and Safran 2021; Anderson et al. 2023). It will also be interesting to broaden the scope of these questions for different kinds of sexual traits. For instance, divergence in chemical signals may have a more intimate connection to plant-derived compounds (Jarrett

and Miller 2024). Furthermore, focusing on traits involved in direct competition between males, such as weapons, or on traits involved in sperm competition and cryptic female choice may reveal yet more complex interactions (Jarrett and Miller 2024).

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Statement of Authorship

T.K.W. conceived the study. T.K.W., R.E.H., F.W.S., R.L.S., K.J.T., and M.S.C. conducted the experiment. R.B.C. and R.E.H. recorded male signals, and R.L.R. and R.B.C. analyzed the data and wrote the manuscript. F.W.S., R.L.S., K.J.T., M.S.C., R.E.H., and R.B.C. read the manuscript and contributed to revisions.

Data and Code Availability

Data for the analyses in this study are available as supplementary information submitted with the manuscript and are available from Harvard Dataverse (https://doi.org/10.7910/DVN/DYHRRJ; Rodríguez et al. 2025). We did not use any special code.

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