Phenotypic Plasticity and Repeatability in the Mating Signals of *Enchenopa* Treehoppers, with Implications for Reduced Gene Flow among Host-Shifted Populations

Danielle A. Sattman & Reginald B. Cocroft

Division of Biological Sciences, University of Missouri-Columbia, Columbia, MO, USA

Abstract

Mate signaling systems, because of their role in assortative mating, have often been implicated in the origins of evolutionary independence between lineages. We investigated three sources of phenotypic plasticity in mating signals with potential relevance to assortative mating in a species in the *Enchenopa binotata* complex of treehoppers. This group has been a model for speciation in sympatry through shifts to novel host plants. Host shifts result in partial reproductive isolation in *Enchenopa binotata* because of their effects on life history timing, but interbreeding is still possible if there is dispersal and some overlap of mating periods. Courtship in these plant-feeding insects is mediated by plant-borne vibrational signals. We asked whether variation in male mate signaling behavior is influenced by plant substrate, age, or size, each of which may play a role in interactions among host-shifted populations. Males produced fewer, shorter signals when on non-hosts than when on hosts. However, there were no effects of age or size on signal variation. Significant repeatability of some signal features (carrier frequency and the number of signals produced in a signaling bout) is consistent with the presence of genetic variation and thus the potential to respond to selection. Our results suggest that plasticity in mate signaling systems, and in particular in male mate searching behavior on hosts and non-hosts, may have the potential to reduce interbreeding between populations that use different species of host plant.

Corresponding author: Reginald B. Cocroft, Division of Biological Sciences, University of Missouri-Columbia, Columbia, MO 65211, USA. E-mail: cocroftr@missouri.edu

Introduction

Ecological differences between populations can cause divergent selection (Schluter 1998), but gene flow and recombination may prevent evolutionary

differentiation unless there is also assortative mating (Mayr 1963; Maynard Smith 1966; Bush 1992; Johnson & Gullberg 1998; Kondrashov et al. 1998). Mate signaling systems, with their potential for rapid evolution, have often been implicated in assortative mating and ultimately in the origins of evolutionary independence between lineages (Fisher 1958; Lande 1981; West Eberhard 1983; Kaneshiro & Boake 1987; Turner & Burrows 1995; Seehausen et al. 1997; Higashi et al. 1999; Higgie et al. 2000). In addition to their potential for evolutionary divergence, signaling systems can also exhibit substantial phenotypic plasticity (e.g. Lesna & Sabelis 1999; Jia et al. 2000; Qvarnstrom et al. 2000; Rodriguez & Greenfield 2003), which might give rise to variation in signals and/or preferences and play a role in assortative mating between populations that occupy different habitats. Here we investigate the possibility that plasticity in male mate signaling behavior can contribute to assortative mating, in insects hypothesized to have speciated under conditions in which the possibility of gene flow between diverging populations is present from the outset.

Treehoppers in the *Enchenopa binotata* species complex provide a model for speciation in sympatry through shifts to novel host plants (Wood 1980, 1993; Wood & Guttman 1981, 1982, 1983; Guttman & Weigt 1989; Guttman et al. 1989; Wood & Keese 1990; Wood et al. 1990, 1999; Tilmon et al. 1998). The life history timing of these host-specific insects is closely tied to the phenology of their plant host. If there are differences in the phenologies of ancestral and novel hosts, then populations on those hosts will differ in the timing of mating (Wood & Keese 1990). Furthermore, individuals show a high degree of host fidelity, with limited dispersal and a marked tendency to mate and oviposit on their natal host (Wood et al. 1999). Both of these features of their life history will reduce gene flow after a host shift has occurred, but some interbreeding is still possible due to partial overlap of mating periods and occasional dispersal, especially by mate-searching males (Wood 1980). However, the potential for interbreeding is reduced by interactions during courtship (Wood 1980), which is mediated by vibrational signals (Hunt 1994). Vibrational mate attraction signals differ among species in the *E. binotata* complex (Rodriguez et al. in press; R.B. Cocroft, R. Hunt and R.L. Rodriguez, unpubl. data), and female responses to male signals contribute to reproductive isolation among currently differentiated species (Rodriguez et al. in press). If patterns of phenotypic plasticity in signals across rearing or signaling conditions contribute to assortative mating, then mate signaling systems might also play a role in the earliest stages of divergence between host-shifted populations.

We address three potential sources of phenotypic plasticity in mating signals with relevance to gene flow among host-shifted populations of *E. binotata* treehoppers. First, we ask whether male signaling behavior differs when on host and non-host plants. In other treehopper and leafhopper species, males increase the active space of their signaling effort by moving between sites on one plant or between plants (Hunt & Nault 1991; Cocroft 2003). Given the high host fidelity of female *E. binotata* in mating and oviposition (Wood 1993), males might be expected to show behavioral mechanisms that restrict their signaling to plants of

their host species, or otherwise to reduce their investment in signaling in contexts where encountering receptive conspecific females is unlikely. Such variation in signaling behavior between hosts and non-hosts could reduce the likelihood of mating between individuals living on different host plants.

Secondly, we ask whether male age influences signal variation. Age-dependent variation in secondary sexual traits, where it occurs, can influence female preferences, often leading females to prefer older males (Andersson 1994; Kokko & Lindstrom 1996). Alternatively, selection might favor females that mate with younger males (Beck & Powell 2000). In acoustically signaling insects, there is evidence for female preference for both older (Simmons 1995) and younger (Ritchie et al. 1995) males. Although mating periods differ between *Enchenopa* populations on different host plant species, there can be some temporal overlap (Wood & Guttman 1982), and females may encounter older or younger males from a population on a non-host, in addition to males from their natal population. If signal variation is age-dependent, and if this variation influences female preferences, then the signals of males from the non-host population may differ in attractiveness from those of males from a female's natal population. Such differences in signal attractiveness might influence the likelihood of mating between individuals from populations on different hosts, depending on their relative phenologies. For example, if older males produce less attractive signals, surviving males from a population with an earlier mating period would be less likely to mate with females from a population with a later mating period.

Examination of signal variation over time also allowed us to identify traits that differ consistently between individuals. Consistent differences between individuals suggest the possibility of selection on signal traits, and once host-associated populations begin to diverge, selection could lead to signal differences and further isolation. Although the relationship between a trait's repeatability and its heritability is not always predictable (Boake 1989; Dohm 2002), repeatabilities greater than zero suggest the presence of genetic variation (Lessells & Boag 1987; Lynch & Walsh 1998), which is important if sexual selection on male signals is to result in evolutionary change.

Thirdly, we ask whether variation in size influences the characteristics of male mate-attraction signals. In the *E. binotata* species on *Viburnum*, phenotypic plasticity in size can result in individuals that are poorly adapted to a new host plant being smaller at the time of adult eclosion, perhaps reflecting reduced condition or viability (K.J. Tilmon, pers. comm.). If size is correlated with signal features that influence attractiveness (Andersson 1994; Gerhardt & Huber 2002), then signals might function as indicators of viability. Offspring that result from mating between individuals adapted to different host plants would thus have less attractive signals, and this offspring disadvantage would limit the impact of gene flow when it does occur.

There is considerable evidence that the *E. binotata* complex of treehoppers speciated in sympatry, where gene flow was possible throughout the process of divergence (Wood 1980, 1993). Accordingly, it is important to understand how assortative mating may have arisen between populations using different resources.

We investigate potential sources of phenotypic plasticity in mating signals that could influence the likelihood of mating between host-shifted populations in the early stages of divergence, before genetic differentiation in signals or preferences has occurred.

General Methods

The *E. binotata* complex consists of nine species that use different host plants, and that are currently awaiting taxonomic description (Lin & Wood 2001). We studied the *E. binotata* species on wafer ash (Rutaceae: *Ptelea trifoliata*). Individuals were collected in May and June 2002 as nymphs or adults near the University of Missouri campus in Columbia, MO, USA. Males were maintained in a greenhouse on potted wafer ash plants.

Males began producing signals (Fig. 1) by approx. 1.5 wk after adult eclosion. Recorded males were first transferred individually to a plant stem and
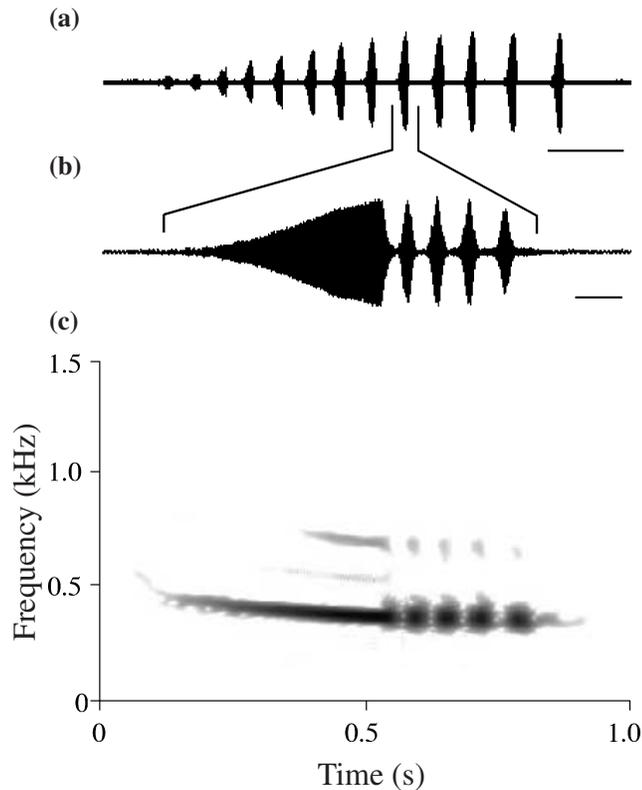

Fig. 1: Vibrational mate-attraction signals of male *Enchenopa binotata* from wafer ash (*Ptelea trifoliata*). (a) Waveform of one signal bout; (b) waveform of one signal; (c) spectrogram of one signal. Scale bar in (a) 4 s and (b) 80 msec

played a pre-recorded male–female duet to elicit signaling. As playback of airborne signals was sufficient to induce vibrations in the plant and evoke behavioral responses from males, the male–female duet was played from a MacIntosh G4 computer with an Optimus loudspeaker. Signals were recorded using a CLV 1000 laser vibrometer with a CLV M030 decoder module (Polytec PI, Inc., Auburn, MA, USA) at 5 mm/s/V sensitivity, mounted on a Vibraplane isolation table (Kinetic Systems Inc., Boston, MA, USA). To increase reflectance of the substrate, small pieces of reflective tape (approx. 1 mm^2) were placed on the plant stem. The laser was focused on a point ≤ 5 cm from the signaling insect. The laser output signal was high-pass filtered at 60 Hz using a Krohn-Hite model 3202 filter (Krohn-Hite Co., Brockton, MA, USA), digitized at 44.1 kHz, and stored as WAV files. Signals were analyzed using SoundEdit 16 v. 2 (Macromedia, Inc., San Francisco, CA, USA). Signals are produced in groups, here termed signal bouts (Fig. 1). Signal bout variables analyzed included the total number of signals and the inter-signal interval. Within each signal bout, signal variables measured included signal length; carrier frequency at the end of the initial, non-amplitude modulated portion of the signal (this provided a standard landmark for comparison among signals); and pulse rate during the second, amplitude-modulated portion of the signal. Carrier frequency, signal length, and pulse rate were measured from the last signal in the bout, which typically has the highest amplitude (see Fig. 1a).

The ambient temperature in the recording room was maintained at 24 ± 1.5°C. Signal variables that were influenced by temperature variation were corrected to a common temperature of 24°C. Statistical analyses were conducted using SPSS v.10 (SPSS Inc., Chicago, IL, USA) and SAS v. 6.12 (SAS Institute, Cary, NC, USA).

Experimental Design

Host plant effects on mating signals

To determine if there were plant effects on signal variation, we recorded males on two different plant species, one host and one non-host. We used bittersweet (Celastraceae: *Celastrus scandens*) as the non-host plant; this climbing vine is used by another member of the *E. binotata* complex and often grows in close proximity to wafer ash in the field. To test for the effects of plant species and individual on signals, we used three different wafer ash–bittersweet pairs (using potted plants < 0.5 m tall), recording 10 males on both plants in each pair for a total of 30 males (one recording of one of the males was not of sufficient quality for accurate measurement, and this male was excluded from the analysis). Each recording was of a single bout of signals. We altered treatment order between subjects to control for order effects, and analyzed the data using a repeated measures ANOVA with one between-subjects factor (individual plant) and one within-subjects factor (plant species). We analyzed the results in PROC GLM in SAS, testing for the effect of plant species and individual, nested within species.

Age effects on mating signals

The peak mating period in *E. binotata* from wafer ash differs from that of other species in the complex by 1–12 d (Wood & Guttman 1982, p. 239), and at a given time male *E. binotata* from different hosts are likely to differ in average age by a similar number of days. We therefore examined signal variation over a 2-wk period after the beginning of a male's reproductive maturity. We recorded each of 17 males (collected as nymphs) on three occasions, at approx. days 10, 17, and 24 after each male's adult eclosion date.

To control for substrate-induced differences in signals, all males were recorded on the same petiole of a single potted wafer ash plant. Each male was given a distinctive permanent mark by applying a dot of enamel paint to the pronotum; individuals differed in the color and position of the dot. Between recordings, each male was maintained individually in the greenhouse on a sleeve-caged wafer ash plant, accompanied by a female (males maintained without females are more likely to attempt to disperse).

To analyze age-related signal variation, we used a repeated measures analysis of variance of the temperature-corrected data. We used the Huynh–Feldt epsilon correction (Littell et al. 1996) for potential violations of the sphericity assumption. We also calculated the repeatability of signal features as in Lessells & Boag (1987).

Size effects on mating signals

A sample of recorded males (N = 26) was frozen and preserved in 95% ethanol. Size was measured from digital images (which included a scale) generated using a LEICA MZ7.5 microscope (Leica Microsystems Inc., Bannockburn, IL, USA) with an attached Sony SSC-CD50A digital video camera (Sony Corp., New York, USA). Images were captured on Macintosh G4 computer using File Cut Pro (Apple Computer, Inc., Cupertino, CA, USA). Body length (front of head to tip of hind wing) was measured using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://www.rsb.info.nih.gov/nih-image/). Pearson product-moment correlation coefficients were then calculated between size and signal variables.

Temperature corrections

Three signal features were significantly affected by temperature: carrier frequency (N = 42 males, $p < 0.01$, slope = 9.73 Hz/°C), pulse rate (N = 42 males, $p < 0.01$, slope = 1.28 Hz/°C) and inter-signal interval (N = 42 males, $p < 0.01$, slope = −0.558 s/°C). The other features measured (signal length, number of signals per bout) were not significantly correlated with temperature (N = 42 males; all $p > 0.14$). For all subsequent analyses, the measured values of carrier frequency, pulse rate and inter-signal interval were corrected to a common temperature of 24°C.

Results

Males signaled differently on hosts and non-hosts, producing fewer and shorter signals on non-hosts. We found no evidence that any signal traits were influenced by male age or size, but some traits showed consistent differences between individuals.

Plant Effects on Mating Signals

Males signaled differently when on host and non-host plants (Table 1, Fig. 2). On a non-host, males produced 28% fewer signals (6.8 vs. 4.9 signals per bout), and the signals they did produce were 15% shorter (580 vs. 490 ms). The difference in signal length was due to a decrease in the length of the initial, non-amplitude-modulated portion of the signal, rather than a decrease in the number of pulses at the end of the signal. There were no significant effects on either variable of within-species variation in plant substrate, and thus variation in male signaling behavior is due to the differences in plant species rather than to differences among individual plants of the same species. Other signal traits did not differ between signals produced on wafer ash and bittersweet.

Age Effects on Mating Signals

There were no significant effects of age on any signal feature (Table 2; results did not differ qualitatively with outliers removed). Carrier frequency varied the

Table 1: The results of repeated measures analyses of variance, testing for the effect of variation between and within plant species (wafer ash vs. bittersweet) on signal variables. Also shown is the percentage of change in the signal features that differed when males were recorded on the two plant species (N = 29 males)

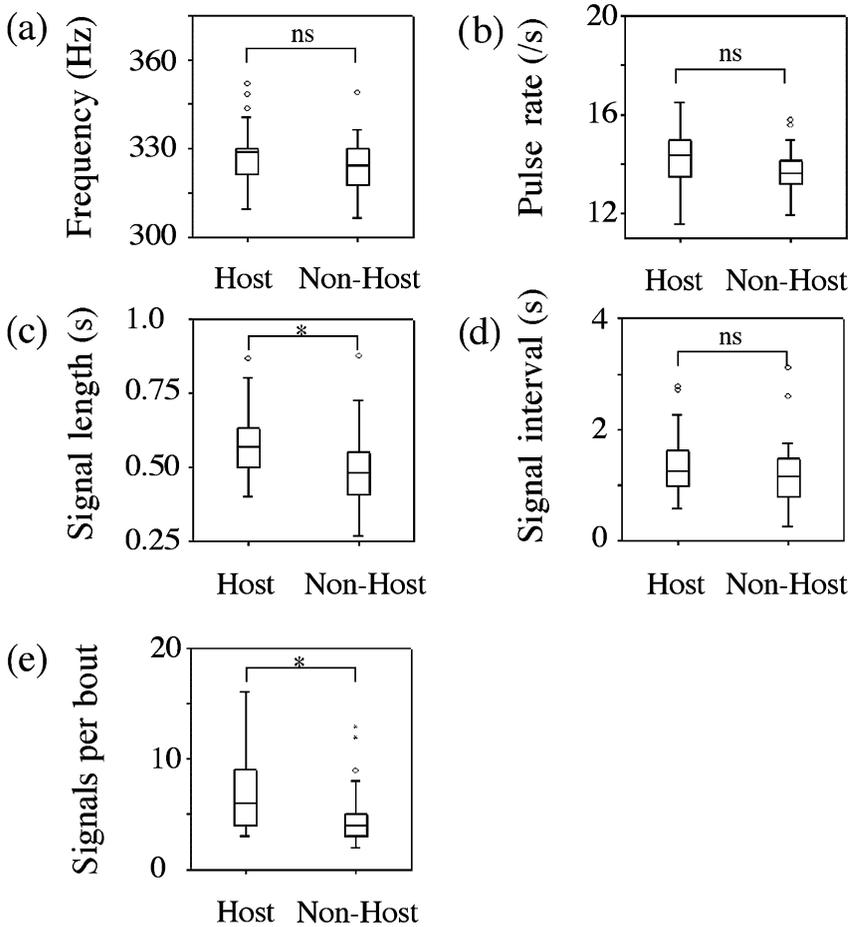| Variable	F	p	Change
Carrier frequency			
Species	2.88	ns	
Within species	2.47	ns	
Pulse rate			
Species	2.91	ns	
Within species	1.37	ns	
Signal length			
Species	7.35	< 0.01	−15%
Within species	1.61	ns	
Signal interval			
Species	0.01	ns	
Within species	0.87	ns	
Signals per bout			
Species	5.18	< 0.05	−28%
Within species	1.4	ns	

Fig. 2: (a–e) Signal characteristics of male *E. binotata* recorded on host and non-host plants. There were significant differences in signal length and the number of signals per bout

least, with a maximum difference of 1% between mean values of the three age classes. For signal length, pulse rate, and signal interval the maximum difference between age class mean values was ≤5%. The number of signals per bout varied the most, with age class mean values differing by up to 47%. Although there was no significant effect of age on number of signals per bout, males appeared to produce fewer signals when they were younger. A power analysis (Sokal & Rohlf 1995) suggests that we would have had a high chance of detecting a difference $(1-\beta = 0.95)$ only if the differences had been very large (Table 2), and thus our failure to reject the null hypothesis for this variable does not provide strong evidence that it does not change with age. Although signal amplitude might be expected to change with age, the data acquisition system used in the study did not allow us to evaluate changes in absolute signal amplitude between recordings.

Table 2: The results of repeated measures analysis of variance, testing for the effect of age on signal variables; and repeatabilities (with standard errors) from one-way ANOVA obtained as in Lessells & Boag (1987) and Becker (1984). N = 17 males. The power analysis shows the minimum difference between mean values that could be detected with high probability. The total number of observations for repeatability estimates is shown; some variables were not measurable for some recordings. *p < 0.05

Variable	Age effect (p)	x̄ ± SD	Max. diff. observed	Detectable diff. at 1−β = 0.95	No. of observation	Repeatability
Carrier frequency (Hz)	ns	323 ± 11	2.9 (1%)	18.0	51	0.32 ± 0.16*
Pulse rate (Hz)	0.09	14.4 ± 0.9	0.81 (5%)	1.7	50	0.22 ± 0.16
Signal length (ms)	ns	667 ± 86	24 (4%)	200	51	0.06 ± 0.15
Signal interval (s)	ns	2.82 ± 0.53	0.08 (3%)	1.1	47	0.10 ± 0.17
Signals per bout	ns	7.4 ± 3.6	2.6 (47%)	6.0	51	0.27 ± 0.16*

Table 3: Pearson product-moment correlation coefficients for the relationship between male size and signal variation

Variable	N	r	p
Carrier frequency	24	−0.215	> 0.3
Pulse rate	24	0.117	> 0.5
Signal length	26	0.365	0.067
Signal interval	24	0.014	> 0.5
Signals per bout	26	0.136	> 0.5

Carrier frequency had the highest within-individual repeatability (Table 2). The number of signals per bout also had a relatively high repeatability, in spite of the possible trend for an increase in the number of signals per bout in older males.

Size Effects on Mating Signals

Size was not significantly correlated with variation in any signal feature (Table 3). There was a trend for signal length to be correlated with size (p = 0.067); however, neither component of signal length – i.e. length of the first, non-amplitude modulated section or the number of pulses – was correlated with size (p > 0.3), nor were the two variables correlated with each other (p > 0.2), suggesting that this apparent trend does not represent a meaningful correlation.

Discussion

We investigated three sources of phenotypic plasticity in signals that could be important in the early stages of sympatric differentiation in *E. binotata* treehoppers. Our focus was on potential mechanisms of signal variation that

could influence attractiveness and thus the likelihood of interbreeding between populations adapted to different resources, but which do not require genetic differentiation in the mate signaling system. The first proposed mechanism was that males would bias their mate searching behavior toward hosts over non-hosts. Our results supported this hypothesis: when signaling on a non-host species, males produced shorter signals, and fewer signals in a bout, than when signaling on a host plant. Observations also suggested that males took longer to signal on a non-host, and were more likely to fly off the host before signaling (D.A. Sattman, pers. obs.).

How likely is this difference in signaling behavior to translate into assortative mating? Answering this question required additional evidence, particularly with respect to its effect on female choice. The effect of variation in signal length on female choice in *E. binotata* has not yet been evaluated. However, preferences for greater numbers of signals per unit time are widespread (e.g. Ryan & Keddy-Hector 1992), and in *E. binotata* from *Viburnum* (the only species in the complex for which the relevant experimental data are currently available), the number of signals per bout is positively correlated with the probability of evoking a response from receptive females (R.L. Rodriguez and R.B. Cocroft, unpubl. data). Therefore, even in the absence of changes in mating signals between host-shifted populations, the differences in the number of signals produced on hosts and non-hosts should have the effect of reducing the likelihood of mating between host-shifted populations.

There were no obvious effects of host plant transmission properties on the frequency or fine-temporal features of signals (as opposed to host plant effects on the number and length of signals produced). This is perhaps not surprising in the case of *E. binotata* signals. Transmission through a plant stem can change the relative amplitude of different frequencies in a signal (Michelsen et al. 1982), but most features of the relatively pure-tone signals of *E. binotata* males should not be greatly affected. Because these signals are frequency modulated, differential frequency filtering is likely to alter the shape of the overall amplitude envelope of the signal, but this aspect of signal variation was not quantified in this study. It is worth noting that the signals of the *Enchenopa* species that lives on bittersweet are distinct, especially in frequency, from signals of wafer ash *Enchenopa* males recorded on bittersweet (wafer ash males: 323 Hz; bittersweet males: 409 Hz, in sympatric Missouri populations of the two species; R.B. Cocroft, R. Hunt and R.L. Rodriguez, unpubl. data). In light of our results, such differences among the signals of different species in the *E. binotata* complex (Rodriguez et al. in press) represent inherent differences, and not the result of variation in the signal-transmitting properties of their host plants.

The observation that the signaling behavior of male *E. binotata* differed between host and non-host plants has implications for studies of vibrational communication in insects. Given the heterogeneous nature of natural plant substrates, individuals from different species or populations are sometimes recorded on a single common substrate such as a dowel or other platform. Our results suggest that, for highly host-specific taxa, use of a substrate other than

their host plant can yield recordings that are not representative of signaling behavior in the field. In contrast, for at least one species that uses multiple species of hosts (the treehopper *Umbonia crassicornis*), male signals differ only slightly, if at all, when produced on stems of plant species (R.B. Cocroft, unpubl. data), and in such cases a common recording substrate might be appropriate.

The second proposed mechanism was that mating signals would change with male age. Age-related signal variation often influences female choice (Andersson 1994; Kokko & Lindstrom 1996; Beck & Powell 2000) and thus could have consequences for interactions between populations that differ in life history timing. However, we found no effects of age on signals of male *E. binotata* over an approx. 2-wk period after the onset of reproductive maturity. It is possible that effects would be revealed if males were measured over longer time spans. However, because allochronic shifts in development between the species on wafer ash and other species in the complex are generally < 12 d, the period examined is probably the most relevant one for evaluating age-related signal variation as it relates to gene flow among host-shifted populations.

There were consistent differences between males in some signal features, as reflected in their repeatability. Although the relationship of repeatability and heritability is not straightforward (Boake 1989; Aragaki & Meffert 1998; Dohm 2002), the presence of reliable differences between individuals is consistent with the presence of genetic variation (Boake 1989; Martins 1991) and thus the potential to respond to sexual selection. In this study, carrier frequency was the signal trait with the highest repeatability, and is subject to stabilizing selection by female choice in the population used in this study (R.L. Rodriguez and R.B. Cocroft, unpubl. data) and in the *E. binotata* species on *Viburnum* (Rodriguez et al. in press). In accord with this potential for evolutionary change, carrier frequency is among the signal traits that differ most among species in the complex (Rodriguez et al. in press; R.B. Cocroft, R. Hunt and R.L. Rodriguez, unpubl. data). Although the repeatabilities reported here are lower than many of those reported for signal traits in the literature (e.g. Boake 1989; Rivero et al. 2000), this may be due in part to the relatively long period (>2 wk) over which they were estimated, which is likely to increase the contribution of environmental variation (see Martins 1991) when compared with the repeatability of traits measured over shorter time periods.

Our third hypothesis was that variation in size would correlate with signal features. In the context of a host shift, differences in size may reflect the viability of different individuals growing up on the same host (K.J. Tilmon, pers. comm.), and size-related signal variation might thus provide an indicator this viability, a relationship that could be important for female choice in the context of a shift to a novel host plant. However, we detected no size-related variation in the signals of these male *E. binotata*. These results may seem surprising, because in communication systems using airborne sound, size is often correlated with signal features (reviewed in Andersson 1994; Gerhardt & Huber 2002). In particular, the carrier frequency is often negatively correlated with size, ultimately because of the constraints inherent in coupling an acoustic signal

to the atmosphere, i.e. for small objects radiating sound frequencies with long wavelengths, there is an acoustic 'short-circuit' that reduces the amplitude of the broadcast signal (Ewing 1989). The physics of substrate vibration are very different from those of airborne sound (Michelsen et al. 1982; Markl 1983) and do not impose the same size constraint on signal frequency. Accordingly, unless constraints are imposed by the mechanics of signal production, we might not expect the same size–frequency relationship. However, this question has not been well studied in vibrationally communicating animals. In wolf spiders, there is no relationship between spider size and the dominant frequency of the signal (Rivero et al. 2000), but for these drumming signals the spectral features of the signals are a function of the substrate, not the signaler. De Luca & Morris (1998) found that larger male katydids produced vibrational signals at a higher rate than did smaller males.

There is considerable evidence that treehoppers in the *E. binotata* complex speciated in sympatry as a result of shifts to novel host plants. Although a host shift has immediate consequences for assortative mating (Wood & Guttman 1982, 1983; Wood & Keese 1990; Wood et al. 1999), there still exists the possibility for interbreeding between populations specializing on different resources. Our results with one species in the *E. binotata* complex suggest that differences in male signaling behavior on hosts and non-hosts may further contribute to assortative mating between populations using different species of host plant. The reduction in male signaling on a non-host species is essentially an aspect of host fidelity, and may occur in host-shifted populations of other specialized insect herbivores. The importance of this behavioral mechanism for population differentiation will need to be tested with recently host-shifted populations.

Acknowledgements

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