Divergence in Female Duetting Signals in the *Enchenopa binotata* Species Complex of Treehoppers (Hemiptera: Membracidae)

Rafael L. Rodríguez & Reginald B. Cocroft

Biological Sciences, University of Missouri–Columbia, Columbia, MO, USA

Correspondence
Rafael L. Rodríguez, 223 Tucker Hall, Biological Sciences, University of Missouri–Columbia, Columbia, MO 65211–7400, USA.
E-mail: rafa@missouri.edu

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Abstract

Sexual communication often involves signal exchanges between the sexes, or duetting, in which mate choice is expressed through response signals. With both sexes acting as signalers and receivers, variation in the signals of males and females may be important for mate choice, reproductive isolation, and divergence. In the *Enchenopa binotata* species complex – a case study of sympatric speciation in which vibrational duetting may have an important role – male signals are species-specific, females choose among males on the basis of signal traits that reflect species and individual differences, and female preferences have exerted divergent selection on male signals. Here, we describe variation in female signals in the *E. binotata* species complex. We report substantial species differences in the spectral and temporal features of female signals, and in their timing relative to male signals. These differences were similar in range to differences in male signals in the *E. binotata* complex. We consider processes that might contribute to divergence in female signals, and suggest that signal evolution in the *E. binotata* complex may be influenced by mate choice in both sexes.

Introduction

Sexual communication often takes the form of duetting – signal interchanges between males and females – across a variety of taxonomic groups and signaling modalities (Claridge 1985; Henry 1994; Greenfield 2002; Bailey 2003; Hall 2004; Virant-Doberlet & Cokl 2004; Cocroft & Rodríguez 2005). In the mating systems of many duetting species, female signals express mate choice (Claridge 1985; Henry 1994; Bailey 2003; Virant-Doberlet & Cokl 2004; Cocroft & Rodríguez 2005). In these cases, males must signal attractively, and also detect and react appropriately to female responses; females, on the other hand, must choose among males, and effectively express their choice with their signals. Because each sex is a signaler and a receiver, variation in the signals of males and females may influence their reproductive success and contribute to patterns of reproductive isolation and sexual selection that may lead to population divergence.

Female signals are often simpler and shorter than male signals, with the timing relative to male signals being the feature that most varies between species (Lloyd 1966; Claridge 1985; Bailey & Hammond 2003; Bailey 2003). However, female signals can also resemble male signals in their spectral and temporal features, and may likewise be species- or population-specific (Henry 1994; Wells & Henry 1998; Virant-Doberlet & Cokl 2004). Variation in signal length and specificity may reflect the balance of natural and sexual selection stemming from mate choice and signaling costs (Greenfield 2002; Bailey 2003): very short female response signals may evolve when the risk of detection by predators is high, while longer responses may be favored when male mate choice has a relatively stronger role. Comparing the length and specificity of the signals of males and females

can thus generate hypotheses about the selection regimes under which they evolve, and about their contribution to variation in reproductive success and divergence.

Here, we describe variation in female signals and compare it with variation in male signals in a clade of plant-feeding insects where duetting sexual communication may be important in speciation. The *Enchenopa binotata* complex of treehoppers consists of 11 or more species that specialize on different host plants across eastern North America, and it is a case study of sympatric speciation through shifts to novel host plants (Wood & Guttman 1983; Lin & Wood 2002; Cocroft et al. 2007). Host shifts promote reproductive isolation between populations on ancestral and novel hosts, through a combination of changes in life history timing and high host fidelity; host shifts also result in divergent ecological selection among host plants (reviewed in Wood 1993). As with many insects that live on plants (Claridge 1985; Henry 1994; Virant-Doberlet & Cokl 2004; Cocroft & Rodríguez 2005), the members of the *E. binotata* complex communicate with plant-borne vibrational signals (Hunt 1994; Sattman & Cocroft 2003). Mate-searching males produce advertisement signals, and receptive females alternate their own signals with those of the male (Fig. 1), eliciting localized searching by the male. Male signals vary among species in the *E. binotata* complex, and females discriminate among males by selectively responding on the basis of species and individual signal differences (Rodríguez et al. 2004). Male signals closely match the preferred values of the stronger female preferences (Rodríguez et al. 2006), indicating that female preferences have been important in male signal divergence, and suggesting that sexual communication may be important in the diversification of this clade.

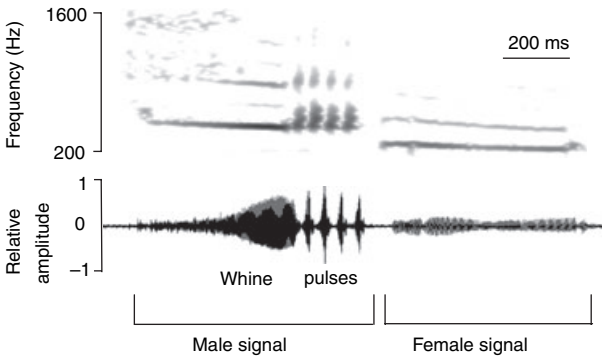

Fig. 1: Spectrogram (top) and waveform (bottom) of a portion of a duet of *Enchenopa binotata* 'Celastrus', showing one male signal and one female response. The male signal has two components: the whine (a tone with harmonics that sweeps downward in frequency) and a series of pulses. The female signal consists of a single, long component having a set of harmonically related frequencies

Here, we turn to the other side of sexual communication in the *E. binotata* complex, and assess variation in female signals. We studied three aspects of female signals: frequency, length, and timing relative to male signals. Male signal frequency and length are important for female choice in the *E. binotata* complex (Rodríguez et al. 2004, 2006), and differences in female signals may in turn influence male-mate choice. Since duetting is an interactive process, the relative timing of male and female signals can influence pair formation through their effects on detection by the receiver (Bailey & Hammond 2003; Bailey 2003). We focused on four species whose male signals span much of the range of variation in the complex.

Methods

Experiments were performed during April to August 2003–2005. We elicited female signals with playbacks of recorded and simulated male signals (see below). To ensure that females were sexually receptive and responsive, we tested virgin females 4–6 wk after their adult molt, at the peak of their sexual receptivity. We reared females from nymphs collected in the field, or from eggs laid by females collected in the field. Collecting sites were in Boone County, Missouri, near the University of Missouri–Columbia campus. We reared nymphs on their host plants in an outdoor facility at the University of Missouri–Columbia. We tested the *E. binotata* species that occur on *Celastrus scandens*, *Cercis canadensis*, *Ptelea trifoliata*, and *Viburnum rufidulum* plants. [We also used these experiments to describe female signal preferences (Rodríguez et al. 2006).] Species in this complex await description, so we refer to them by the names of their host plants (e.g. *E. binotata* 'Celastrus').

Stimulus Design

To describe female signal frequency and length, we elicited female responses with playbacks of recorded male signals having characteristics close to the mean values of each species in their Missouri populations (using one recording for each species), obtained from a library of *E. binotata* signals (R.B. Cocroft, unpublished data). Recordings were made using laser vibrometry (see below), by placing individual males on the stem of a potted plant, within a few cm of a

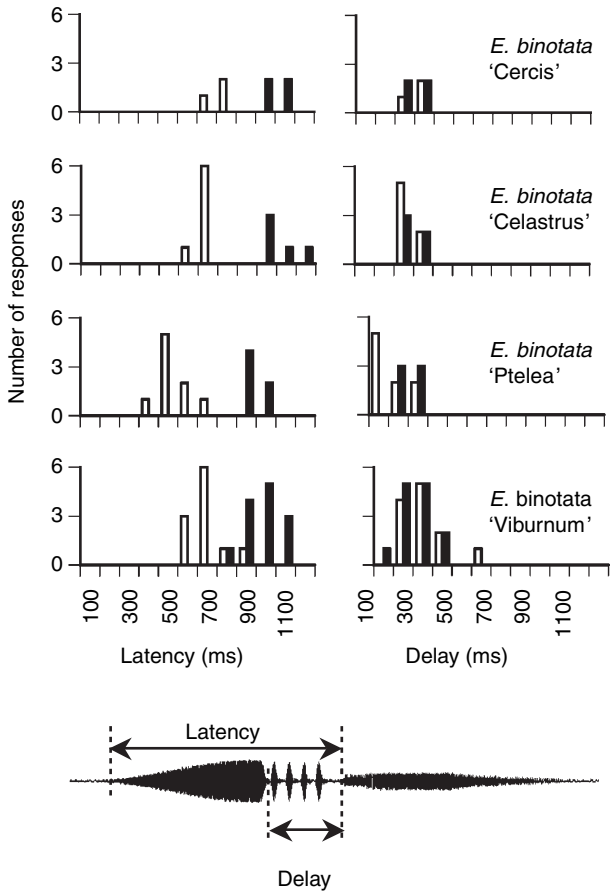

Fig. 2: Female response timing in four species in the *Enchenopa binotata* complex. Stimulus whine length influenced response latency, but not response delay, indicating that females trigger their signals using the end of the whine (Table 1). Open bars indicate responses elicited by stimuli with 400 ms long whines; filled bars, responses elicited by stimuli with 800 ms long whines. The inset shows a recording of a stimulus and the elicited female response, to illustrate our measures. Each female tested contributed two data points (one for her response to each whine length) for each measure

small piece of reflective tape on which the laser beam was focused.

To describe signal timing, we used synthetic playbacks generated with a custom-written program in Matlab Version 5.2.1 (Mathworks, Inc., Natick, MA, USA) that allowed us to manipulate stimulus features with precision. The main difference between synthetic signals and natural signals is that synthetic signals have a constant frequency. Preliminary trials showed these stimuli to be about as effective in eliciting female responses as playback of natural signals (unpublished data).

The whine or pulse components of male signals may influence female response timing. The pulse component probably does not have an influence: stimuli with many pulses are often overlapped by female responses, and stimuli with and without pulses result in similar or identical female response timings (unpublished data). Thus, females trigger their responses by the whine component, and we focused on whether the trigger is the beginning or end of the whine. We compared response timing between stimuli with 400 and 800 ms – long whines (Fig. 1). These values span the range of variation among the populations where our females were drawn. Other stimulus features were set to the population means. We used stimuli with whine and pulses, varying only the whine, because such stimuli elicit female responses more effectively than stimuli having only the whine component (unpublished data). We measured the time from the beginning and end of the whine to the beginning of the female response (latency and delay; Fig. 2, Table 1). If females trigger their responses by the beginning of male signals, latency should not be affected by whine length; if the trigger is the end of male signals, delay should not be affected. Once we determined the feature of the whine that influences female signal timing, we examined species differences by eliciting responses with stimuli having all features set to the mean values of each species.

Stimulation

We recorded females on potted plants, with all females of a given species being recorded on one to two individuals of their host plant species. We describe the procedure for introducing vibrational stimuli into plant stems and recording female response signals elsewhere (Rodríguez et al. 2006). Briefly, we attached a magnet to a plant stem, placed an electromagnet close to it, and used this system to send signals from a playback computer to the plant stem through an amplifier. We placed females on the stem close to the magnet, and recorded the play-

Table 1: Effect of variation in the length of stimulus whine length on the latency and delay of the elicited female response signals in the *Enchenopa binotata* complex

	E. binotata 'Cercis'	*E. binotata* 'Celastrus'	*E. binotata* 'Ptelea'	*E. binotata* 'Viburnum'
Latency	$F_{1,5} = 80.53$, $p = 0.0003$	$F_{1,10} = 101.03$, $p < 0.0001$	$F_{1,13} = 162.38$, $p < 0.0001$	$F_{1,23} = 58.27$, $p < 0.0001$
Delay	$F_{1,5} = 1.95$, $p = 0.22$	$F_{1,10} = 0.26$, $p = 0.67$	$F_{1,13} = 3.99$, $p < 0.067$	$F_{1,23} = 1.47$, $p < 0.24$

backs and elicited female response signals with a laser vibrometer. Air temperature in the recording room was maintained at 24–25°C.

Before playback we modified the recordings of male signals to compensate for frequency-dependent attenuation due to propagation along the plant stem (see Cocroft 1996; Cocroft & Rodríguez 2005 for details). This compensation was not necessary for the constant-frequency stimuli.

We adjusted stimulus peak amplitude at the point where females were placed on the stem. We used an amplitude (0.3 mm/s) corresponding to a male signaling *c.* 5 cm from the female on a stem whose thickness was typical of those used in the field, on the basis of the mean amplitude of over 10 males from three species in the *E. binotata* complex (from *Cercis*, *Ptelea* and *Viburnum* host plants), which showed similar amplitudes. Females usually walked a short distance after being placed on the stem, and thus received stimulation within a few centimeter of the point of amplitude calibration. Male *E. binotata* produce bouts of several signals of increasing amplitude (Sattman & Cocroft 2003). We matched this bout structure by presenting four signals that increased in amplitude in the pattern: 25%, 75%, 100% and 100% of maximum amplitude.

Analysis of Female Signals

We analyzed the spectral features of female signals with Canary 1.2.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA). We measured fundamental frequency, and the frequency of maximum amplitude (dominant frequency; Fig. 3a). Since dominant frequency was not always at the fundamental, we noted which harmonic had maximum amplitude. We analyzed the length and timing of female signals (Figs 3b and 2) to the nearest 2 ms using SoundEdit Version 2 (Macromedia, Inc., San Francisco, CA, USA). We described signal timing by measuring the interval between the end of stimulus whines and the beginning of female signals (the end of the whine is the feature females use to time their responses; see Results). We took these measures from the last two responses to each stimulus bout, which were the responses elicited by stimuli with 100% of maximum amplitude. We thus had two data points per stimulus (two signals) for each female, which we averaged to improve estimate accuracy.

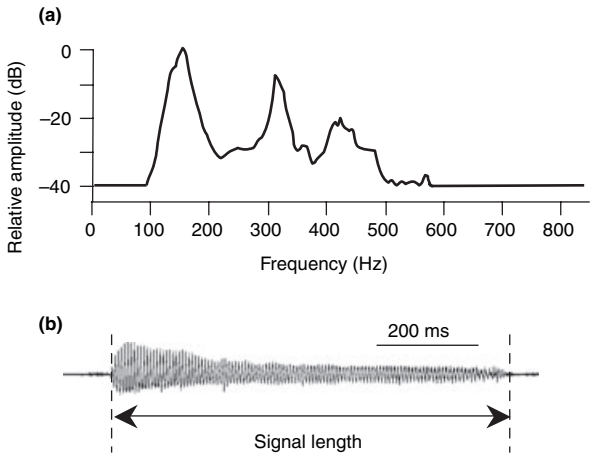

Fig. 3: Signal of an *Enchenopa binotata* 'Viburnum' female, showing the temporal and spectral features measured in the study. (a) Amplitude spectrum. (b) Waveform

An important question is whether our measures of female signals are influenced by filtering caused by the plants used to obtain the recordings (Michelsen et al. 1982; Cocroft & Rodríguez 2005). Fundamental frequency is not affected by recording individual insects on different plant substrates (Sattman & Cocroft 2003; Cocroft et al. 2006). However, dominant frequency may be influenced by plant substrates: the amplitude of different harmonics was often within a few dB (Fig. 3), and substrate filtering may influence the relative amplitude of these harmonics (Michelsen et al. 1982). We report results for females on their species-specific host plants, and further work will be necessary to test the substrate-dependence of this feature. Signal length is not likely to be influenced by substrate filtering (Cocroft et al. 2006); however, since *E. binotata* are highly host-specific, insects placed on foreign plant species are less prone to signal, and produce fewer and shorter signals than on their native plants (Sattman & Cocroft 2003). This effect should be absent from our data, since we recorded females on their species-specific host plants. Finally, we minimized variation due to plant filtering by focusing the beam of the laser vibrometer within a few cm of the females (see Cocroft et al. 2006).

Comparing Variation in Male and Female Signals

We compared male and female signals across species, drawing the data for the males from a library of recordings of the *E. binotata* complex (R.B. Cocroft, unpublished data). Since male signals consist of nearly a pure tone, we measured frequency in the time domain from the duration of 10 cycles at the end of the whine; this method yields substrate-independent frequency measurements (Sattman & Cocroft 2003). Because the end of the whine is

typically the point of highest amplitude in the signal (Fig. 1), our frequency measures for males will usually correspond to the dominant frequency of the signal. Male signals also have low-amplitude harmonics, which are evident in frequency-domain analyses. We inspected sonograms of male signals to determine whether peak amplitude was at the fundamental frequency or at a harmonic, and in the latter case we inferred the fundamental frequency based on the interval between harmonics. Male signals in *E. binotata* 'Cercis' have peak amplitude at the fundamental, but in the other three species peak amplitude is at the second harmonic (R.B. Cocroft, unpublished data). We thus compared both the fundamental and dominant frequencies of male and female signals. For females, the presence of strong harmonics precludes time-domain frequency measures, so we used FFT analysis (fft size 4096 points). Temperatures at which males and females were recorded differed by <1°C for each species, so temperature correction was not necessary.

Statistical Analysis

In comparisons across species there was homogeneity of variance for most traits (Levene test, $F_{3,57} \leq 2.19$, $p \geq 0.10$), and accordingly we used one-way ANOVAS. There was heterogeneity of variance for dominant frequency ($F_{3,57} = 33.53$, $p < 0.001$), so we used a Welch ANOVA allowing for unequal standard deviations. We compared the species-specificity of male and female signals qualitatively, by plotting the mean values of female signal traits in relation to the mean values of male signals traits for visual inspection. We obtained a preliminary indication of the relationship between male and female signal traits across species with Pearson correlation coefficients. We treated the four species as four closely related but independent lineages: studies in progress on variation in mitochondrial and nuclear genes between and within species in the *E. binotata* complex (R.L. Snyder, unpublished data) reveal that they are very similar for the genetic markers used (perhaps reflecting recent divergence), and their relationships are not yet resolved.

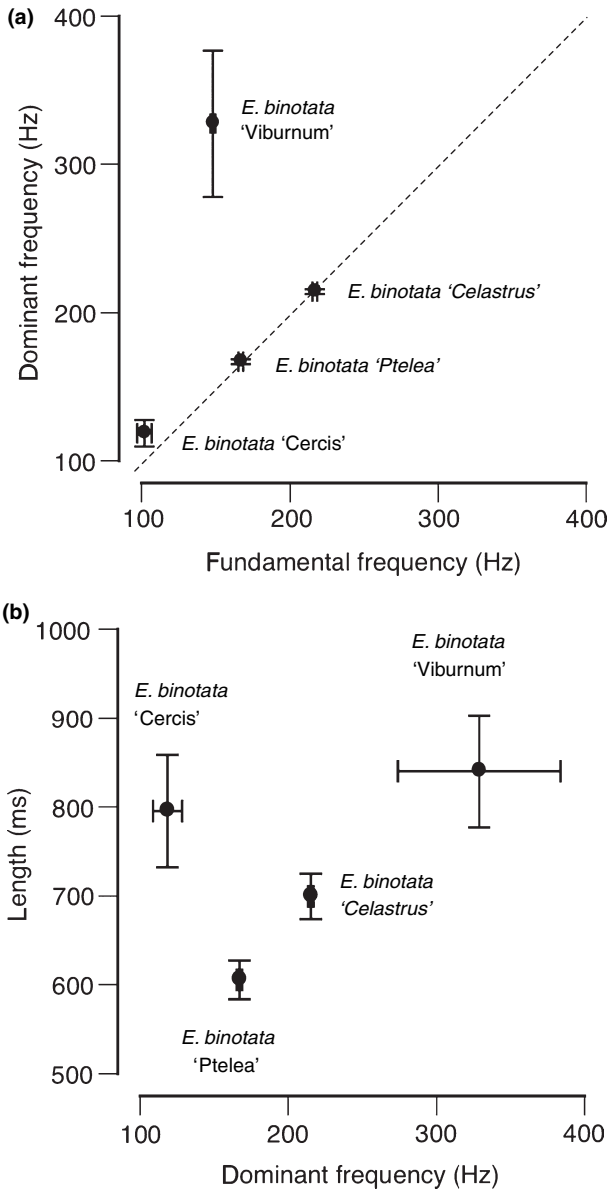

Fig. 4: Species-specificity in the spectral and temporal features (mean ± 1 SE) of female signals in the *Enchenopa binotata* complex. (a) Variation in fundamental frequency ($F_{3,57} = 216.19$, $p < 0.0001$) and dominant frequency ($F_{3,18.32} = 74.88$, $p < 0.0001$, Welch ANOVA allowing unequal standard deviations). Dashed line indicates an 1:1 relationship. Some variation in dominant frequency was not associated with variation in the fundamental: *E. binotata* 'Viburnum' females emphasized different harmonics and were thus higher and more variable in dominant frequency. (b) Variation in signal length ($F_{3,57} = 6.68$, $p = 0.0006$) in relation to variation in dominant frequency

Results

Species differed in all the features of female signals measured: fundamental and dominant frequency, length, and timing (Figs 4 and 5). Although females from all species most often had maximum amplitude at the fundamental, females from *E. binotata* 'Viburnum' sometimes had maximum amplitude at up to the fourth harmonic, and consequently their dominant frequency was higher and more variable

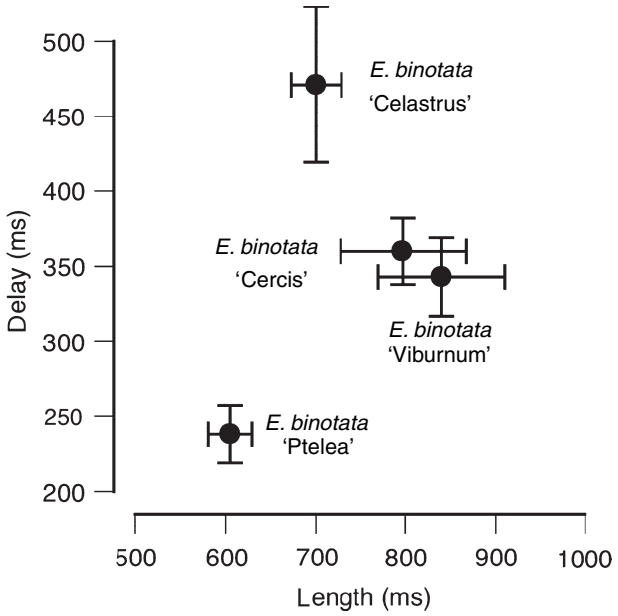

Fig. 5: Species-specificity in female response delay in the *Enchenopa binotata* complex ($F_{3,30} = 7.53$, $p = 0.0007$) relative to variation in female signal length. Data are means ± 1 SE

than for the other species (Fig. 4). In all four species, stimulus whine length influenced female response latency but not response delay (Fig. 2). From this result, we conclude that females use the end of the whine to trigger their responses. We thus compared response delay among species and found substantial differences, with only *E. binotata* 'Cercis' and *E. binotata* 'Viburnum' having similar delays (Fig. 5).

Comparing the traits of male and female signals revealed high species-specificity in both sexes (Fig. 6). Overall, female signals tended to be longer and to have lower dominant frequencies than male signals, but there was a positive relationship between males and females in these signal features (Fig. 6a–c). Furthermore, species with longer male signals also had longer female response delays (Fig. 6d). Significance tests for these correlations have low power: with n = 4 species, power is adequate ($1–\beta > 0.80$) only for $r \geq 0.98$ (Cohen 1988; Zar 1999), and the correlation was significant only for male and female signal length (Fig. 6c).

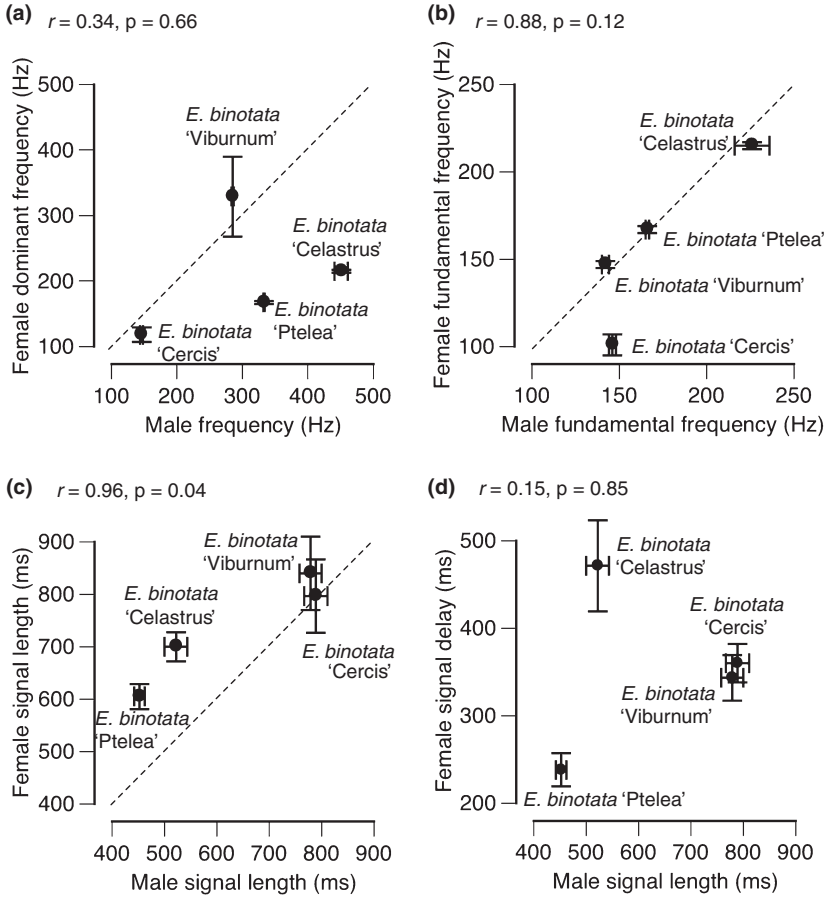

Fig. 6: Comparison of divergence in male and female signals (mean ± 1 SE) in the *Enchenopa binotata* complex. Dashed lines indicate an 1:1 relationship. (a) Dominant frequency. (b). Fundamental frequency. (c) Signal length. For male signals, we used the length of the whine component (see Fig. 1). (d) Delay of female response signals in relation to the length of male signals. We show Pearson correlation coefficients

Discussion

Hypotheses about the evolution of sexual communication in the *E. binotata* complex must account for two features: among-species divergence in male and female signals, and within-species differences between males and females. One hypothesis is that – since the members of the *E. binotata* complex specialize on different host plants – developing on different host species may generate signal differences. We have not evaluated plasticity in female signals, but experiments on male signals argue against this hypothesis. Rearing males on host vs. non-host plant species reveals substantial plasticity, but not exceeding the species-typical range (unpublished data). If plasticity in female signals has a similar range, host-plant effects are unlikely to account for species differences. We thus suggest that species differences in female signals in the *E. binotata* complex represent evolutionary divergence.

There are several potential sources of selection on female signals. Selection for efficient signal transmission may favor use of frequencies that experience less attenuation; if host species differ in their filtering properties, then use of different hosts may exert divergent selection on signal frequency (Cocroft & Rodríguez 2005; but see Henry & Wells 2004). However, selection on a given host plant would be the same for males and females, so this hypothesis would not account for within-species differences between males and females, unless the strength of selection on signal transmission also differed between the sexes. Alternatively, female signals may diverge as a correlated response to selection on male signals – which would be consistent with signal differences between the sexes; or divergence may be driven by mutual male–female mate choice on the basis of each others' signals. In the latter case, differences in the preferences of males and females could explain differences in the signals of each sex.

Female signal divergence in the *E. binotata* complex suggests the hypothesis that differences in female signals may have similar importance to differences in male signals, in terms of mate choice and its consequences for patterns of sexual selection, reproductive isolation, and population divergence. This hypothesis is supported by the observation that female signals in the *E. binotata* complex are long in comparison with many other duetting species, where female signals are often not more than a brief click (Gerhardt & Huber 2002; Greenfield 2002; Bailey & Hammond 2003; Bailey 2003). Long signals may evolve when mate choice has a prominent role relative to factors favoring less conspicuous signals, such as eavesdropping by predators (Greenfield 2002; Bailey & Hammond 2003; Bailey 2003). Thus, long female signals – such as in the *E. binotata* complex, other hemipterans, green lacewings, and some stoneflies and fireflies (Lloyd 1966; Stewart 1997; Wells & Henry 1998; Greenfield 2002; Bailey 2003; Virant-Doberlet & Cokl 2004) – may indicate that male mate choice on the basis of female signal variation is important in their mating systems. There have been few tests of the effect of variation in female signals on male mate choice, but differences among species and even populations influence male mate choice in planthoppers (Ichikawa et al. 1975; De Winter & Rollenhagen 1993; Claridge & de Vrijer 1994), green stink bugs (Miklas et al. 2003), and green lacewings (Henry et al. 2002).

Differences in male advertisement signals and female preferences contribute to behavioral isolation among species in the *E. binotata* complex (Rodríguez et al. 2004, 2006), and may have been important in the process of speciation following a host shift (Cocroft et al. 2007). If mutual mate choice based on signal differences does occur in these species, this may amplify the importance of the evolution of sexual signals in divergence and speciation.

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