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Original Article

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Sex-linked polygenic architecture in advertisement signals and mate preferences, and ecological compatibility, in two members of the *Enchenopa binotata* complex of treehoppers (Hemiptera: Membracidae)

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Abstract

We used a hybridization experiment to explore the genetic architecture of ecological adaptation and advertisement signals and mate preferences in two members of the *Enchenopa binotata* species complex of treehoppers, a clade of plant-feeding insects that offers a case study of speciation involving ecological and sexual selection. We found evidence of ecological compatibility (comparable survival to the adult stage on the same host plant in the parental species and the hybrids); and evidence for sex-linked polygenic architecture of male signals and female mate preferences. These results have intriguing implications for the course of speciation: selection to avoid maladaptive hybridization might not be crucial; the establishment of signal–preference linkage disequilibrium might often be facilitated by trait genetic architecture; and sexual divergence might suffice to keep species distinct.

Keywords genetic architecture, mate preference function, mating signals, speciation, substrate-borne signals, vibrational signalling

Introduction

The course of speciation depends, in part, on the genetic architecture of ecological and sexual traits (Kirkpatrick and Ravigné 2002, Coyne and Orr 2004, Kopp *et al.* 2018). The creation of distinct, reproductively isolated forms may be simplest through change in a single trait. For instance, divergence in one trait can produce reproductive isolation when the site of mating is dictated by the site of feeding and there is a change in that site; or when receivers assess signals by comparison with their own phenotype (i.e. by phenotype matching) and there is change in that phenotype (Servedio *et al.* 2011, Kopp *et al.* 2018). But how common are such scenarios?

Speciation often involves divergence in advertisement signals, mate preferences, and other sexual traits (West-Eberhard 1983, Eberhard 1985, Coyne and Orr 2004, Shaw *et al.* 2024). In such cases, the course of speciation may be less straightforward, because those suites of traits are often polygenic, i.e. they are influenced by many loci. Divergence then requires linkage disequilibrium, and factors like gene flow may interfere with the build-up of linkage disequilibrium and lower

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the likelihood of speciation (Kirkpatrick and Ravigné 2002, Kopp *et al.* 2018). But there is evidence that signal–preference physical linkage might not be rare (e.g. Kronforst *et al.* 2006, Shaw and Lesnick 2009, McNiven and Moehring 2013, Merrill *et al.* 2019, Xu and Shaw 2019, Dunn *et al.* 2024). Even without physical linkage, factors such as sex-linkage could facilitate the establishment of signal–preference linkage disequilibrium. Thus, broadening our understanding of the genetic architecture of ecological and sexual traits is key to assess which speciation scenarios might be common or rare.

Here, we use a hybridization experiment to explore the genetic architecture of ecological adaptation and advertisement signals and mate preferences in the *Enchenopa binotata* complex of treehoppers. This species complex of plant-feeding insects offers a case study of sympatric speciation involving both ecological selection, leading to host plant specialization, and sexual selection, leading to signal–preference co-evolution (Wood and Guttman 1982, 1983, Wood 1993, Rodríguez *et al.* 2004, 2006, 2021, 2025, Cocroft *et al.* 2008, 2010).

Like many host specialists (Drès and Mallet 2002, Cocroft *et al.* 2008), the members of the *E. binotata* complex develop well only on their own host plants (Wood and Guttman 1983, Cocroft *et al.* 2008). This would present a problem for rearing hybrids formed by crossing species that require different host plants to live. Fortunately, at some sites across the range of the complex, different *E. binotata* species occur on the same host plant species (Cocroft *et al.* 2010). We took advantage of one such case at our field sites in Wisconsin, where two members of the complex live on *Viburnum lentago* (Adoxaceae) plants (Rodríguez *et al.* 2018, Desjonquères *et al.* 2023). These two species diverged from each other within the last ~130 000–60 000 years, and their presence on *V. lentago* plants is likely to represent secondary colonization from another host for at least one of them (Cocroft *et al.* 2010, Hsu *et al.* 2018).

The ability to work with two *E. binotata* species on the same host plant representing separate colonizations allowed us to ask whether the species present mutually incompatible ecological adaptations, according to hypotheses of ecological or mutation-order speciation (Schluter 2009). Such mutual incompatibility predicts lowered hybrid survivorship.

Regarding the genetic architecture of advertisement signals and mate preferences, there is considerable evidence of continuous variation in signals and preferences within species in the *E. binotata* complex (Rodríguez *et al.* 2006, 2013, Cocroft *et al.* 2010). There is also evidence of broad-sense heritability in *E. binotata* signals and preferences (Rodríguez *et al.* 2008, 2013, Fowler-Finn *et al.* 2015, 2018). This suggests that physical linkage/super-gene scenarios are unlikely. We therefore tested the hypothesis that the inheritance of signals and preferences in *E. binotata* is polygenic. This hypothesis predicts continuous distributions for signal and preference phenotypes within the parental species and hybrid crosses, with the hybrid distributions shifted towards intermediate values relative to the parental species (e.g. Ritchie 1996). We also tested the non-exclusive hypothesis that the inheritance of signals and preferences in *E. binotata* shows sex-linkage. This hypothesis predicts that the distributions of hybrid signal–preference phenotypes will be closer to either the paternal or maternal species (cf. Ritchie 1996). If sex-linkage is present, we consider closer alignment with the maternal species more likely, given either XX/XO or XX/XY female/male chromosomes in *E. binotata* (Kornhauser 1914, Bhattacharya and Manna 1973, Palmer Droguett *et al.* 2024).

Materials and methods

Our study species, like most members of the *E. binotata* complex, have not been described formally (Hamilton and Cocroft 2009). However, species in the complex can be distinguished by their host plant, nymph coloration, and adult signals and preferences (Wood 1980, Rodríguez *et al.* 2004, 2006, 2025, Rodríguez and Cocroft 2006, Cocroft *et al.* 2008, 2010).

Here, we refer to our study species according to the distinctive dominant frequency of the male advertisement signals, as follows: 'LF' refers to the *E. binotata* species that lives on *V. lentago* in Wisconsin and has male signal frequencies of ~165 Hz; 'HF' refers to the species that lives on *V. lentago* in Wisconsin and has male signal frequencies of ~275 Hz (Rodríguez *et al.* 2013, 2018, Rebar and Rodríguez 2015, Desjonquères *et al.* 2023; Fig. 1). These species have correspondingly divergent female mate preferences for signal frequency (peak preferences at ~185 and 295 Hz, respectively; Rodríguez *et al.* 2013, Rebar and Rodríguez 2015, Desjonquères *et al.* 2023). These species also differ in nymph coloration: grey–green in LF, and dark brown with white stripes in HF (Rodríguez *et al.* 2018, Desjonquères *et al.* 2023; Fig. 1). We therefore also tracked nymph coloration in the crosses.

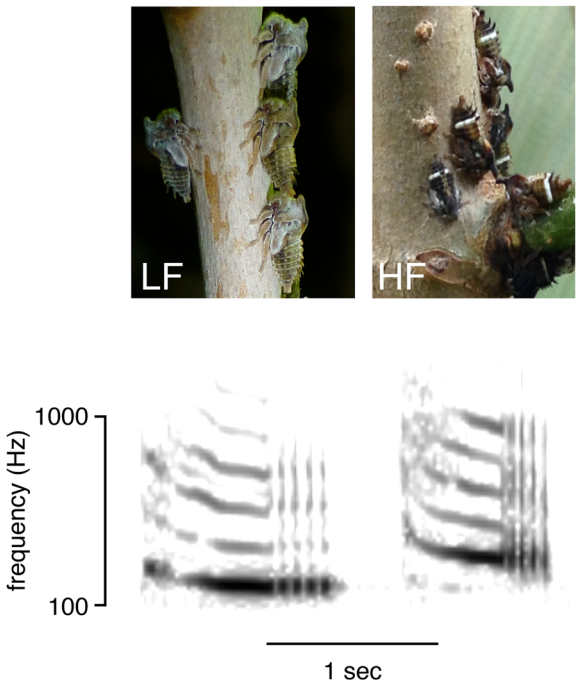

Figure 1 Distinctive nymph coloration and adult male advertisement signals of our study species, members of the *Enchenopa binotata* complex that live on *Viburnum lentago* host plants in Wisconsin, USA. Modified with permission from Rodríguez *et al.* (2018).

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We conducted the experiment over 2021–2023. We collected the treehoppers as nymphs at the University of Wisconsin-Milwaukee (UWM) Saukville Field Station (Saukville, WI, USA) and at nearby sites (Desjonquères *et al.* 2023). For each species, we consider the sites to constitute a single population. We kept voucher specimens in the Rodríguez Lab collection.

Establishing the crosses

We reared the nymphs on potted *V. lentago* plants at the UWM Greenhouse. We separated the treehoppers by sex when they reached the adult stage, then established the crosses. We established own-species (LF female × LF male or HF female × HF male) and hybrid crosses (LF female × HF male or HF female × LF male). Henceforth, we refer to the females and males in the crosses as f and m, respectively, thus: own-species crosses (LF f × LF m or HF f × HF m); hybrid crosses (LF f × HF m or HF f × LF m). To establish the crosses, we placed unmated males and females on replicate potted *V. lentago* plants for mating and oviposition. For each cross, we placed either 10 females and 10 males (2021) or 5 females and 5 males (2022 and 2023) per plant.

As is the case across the *E. binotata* complex (Wood 1980, Cocroft *et al.* 2008, Hamilton and Cocroft 2009), our study species cannot easily be told apart by eye as adults. We therefore took special care when establishing the crosses. We only used adults identified as HF or LF through nymph coloration when collected. When we separated adults by sex, each person sorted only a given species (e.g. D.W.L. sorted only HF treehoppers), and one of us (S.S.) kept oversight of the assignments. Likewise, for assembling the crosses, each person loaded adults of only one species to any given plant under the supervision of S.S.

Females laid eggs on the mating/oviposition plants over the summer and autumn. *Enchenopa binotata* females lay eggs under the bark of the host plant and cover them with a sculptured waxy secretion (Wood 1980, Fowler-Finn *et al.* 2018; Fig. 2A). We brought the plants outdoors in October in order that they would go into dormancy, then overwintered them in cold storage. The following spring, we brought the plants into the greenhouse to trigger nymph hatch as the sap began to flow in the plants. When the nymphs hatched, we moved them to fresh rearing plants, then we separated the sexes as above with additional rearing plants upon the adult moult.

Because we have background information about the signals and preferences of the two species (Rodríguez *et al.* 2013, 2018, Rebar and Rodríguez 2015, Desjonquères *et al.* 2023), we placed most of our effort into establishing hybrid crosses. We lost replicates owing to plants dying during the autumn (mainly from spider mite or aphid infestations) and during overwintering (i.e. some plants did not leaf out when we brought them to the greenhouse after overwintering). Furthermore, no nymphs hatched in many hybrid cross plants (see below). Thus, our final replication of rearing plants was limited (Table 1).

Assessing treehopper survivorship

Nymphs hatched from all plants for the own-species crosses, but in very few of the plants for the hybrid crosses (Table 1). In this experiment, no nymphs hatching for any one leafed-out plant with egg masses could represent hybrid embryo inviability. However, it could also represent little to no mating on that plant during the prior autumn, given the species differences in signals and preferences (Rodríguez *et al.* 2013, 2018, Rebar and Rodríguez 2015, Desjonquères *et al.* 2023; see below). The latter is because unmated *E. binotata* females sometimes deposit the wax they use to cover their egg masses on plant stems without eggs underneath or with seemingly unfertilized eggs (R.L.R. pers. obs.; see below).

We therefore checked stems with egg masses for a subset of the plants, by gently lifting the wax and the thin bark covering the eggs (Fig. 2). In most of the cases we examined (9 of 10 plants with 1–40+ egg masses), there were eggs with embryos under the wax (as indicated by their dark coloration;

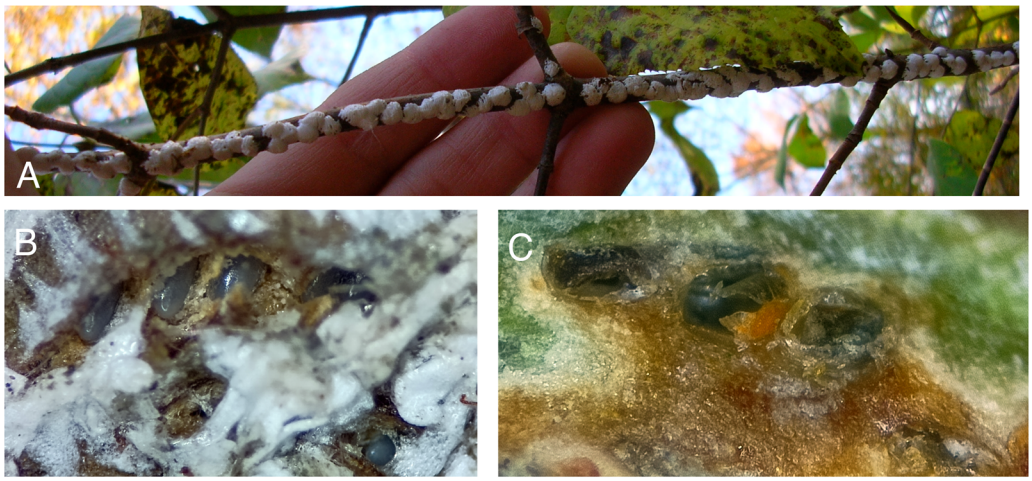

Figure 2 *Enchenopa binotata* females lay eggs in masses that they cover with a waxy secretion. A, example of a *Viburnum lentago* stem with egg masses in the field. (Picture by G.H.; Riveredge Nature Center, Saukville, WI, USA; October 2008). B, example of eggs under the wax on a *V. lentago* stem collected in the field (Picture by G.H. & Hossein Asgari; Downer Woods, Milwaukee, WI, USA; April 2026). C, example of eggs under the wax on a stem from a hybrid cross (LF f × HF m) (Picture by G.H. & Hossein Asgari; May 2026).

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Table 1 Replication by nymph rearing plant in the *Enchenopa binotata* hybridization experiment.

Cross	Replicate plants with:				
	adults for mating and egg laying	egg masses (autumn)	egg masses (into overwintering)	egg masses (leafed-out, spring)	hatched nymphs (spring)
LF	10	9	7	7	7
HF	10	9	5	4	4
LF f × HF m	26	18	16	8	1
HF f × LF m	26	18	17	10	2

Fig. 2B and C); this included one hybrid cross plant (LF f × HF m; Fig. 2C). However, another plant from that hybrid cross (LF f × HF m) had very few eggs per mass under the wax (approximately three eggs per mass, whereas the usual is ~10), and those eggs were transparent (i.e. seemed to lack an embryo). Yet another plant (LF) had one mass with no eggs under the wax among other masses with eggs. Thus, we cannot completely rule out embryo inviability as a cause of no nymphs hatching on any one plant. However, we consider that the more likely scenario is little to no mating on the hybrid cross plants during the prior autumn.

We therefore scored treehopper survivorship as the proportion of hatched nymphs that reached the adult stage per rearing plant. We also scored survivorship as the ratio of adults over the number of egg masses per plant, for plants that had at least some nymphs eclose.

Describing male advertisement signals and female mate preferences

Enchenopa binotata communicate with plant-borne vibrational signals (Cocroft and Rodríguez 2005, Cocroft *et al.* 2008, Hill 2008, Hill *et al.* 2019). Mate-searching males visit plants and produce bouts of advertisement signals, each mainly consisting of a frequency-modulated tone (a 'whine') and a series of pulses (Hunt 1994, Cocroft *et al.* 2008, 2010; Fig. 1). If a receptive female is on the plant and if she finds the male's signals attractive, she responds with a 'duetting' signal that alerts the male of her presence; the pair then duet as the male searches for the female (Rodríguez and Cocroft 2006, Cocroft *et al.* 2008). This selective duetting provides a natural assay of signal preferences, which are strongest for dominant signal frequency, the most species-specific signal trait across the complex (Rodríguez *et al.* 2004, 2006, Cocroft *et al.* 2008, 2010). We thus focused on male signal frequency and the female mate preference for it.

Male signals

We recorded male signals with a laser vibrometer (Polytec PDV-100, Polytec, Auburn, MA, USA). We focused the beam of the laser on a piece of adhesive reflective tape on the stem of a recording plant (a potted *V. lentago* plant). We band-pass filtered the laser vibrometer output (40–3000 Hz) with an electronic filter (Krohn-Hite 3202, Brockton, MA, USA) and sent the signal through a USB audio interface (Edirol USB Audio Capture UA-25, Roland, Hamamatsu, Japan) to an iMac computer (MacBook Pro, Apple, Cupertino, CA, USA). We recorded the signal on the iMac with the program AUDACITY (v.2.1.2; http://audacity.sourceforge.net/) at a sampling rate of 44.1 Hz. We monitored the temperature near the treehoppers on the recording plant with a digital thermometer (Fisher Scientific, Pittsburgh, PA, USA; Extech Instruments SDL500, Nashua, NH, USA).

To record a male, we placed him on the recording plant. Males generally signal spontaneously shortly after this. If the male did not signal within 1 min, we primed him with a playback of a recorded male–female duet (see below for playback method). To avoid assuming the signal phenotypes resulting from the experiment, we primed all males with playbacks of both parental species in random order, separated by 30 s of silence. If males did not signal within 10 min, we returned them to their rearing plant and tried again every 2–4 days or until they died. Male adult age at recording was 12–20 days. We measured dominant signal frequency from the recordings with the program AUDACITY.

Female mate preferences

We used an approach that recognizes that mate preferences are function-valued traits (Meyer and Kirkpatrick 2005, Stinchcombe *et al.* 2012). Mate preferences are expressed as a function of the features of the signals that females encounter, and describing them requires assessment over a range of signal trait values (Ritchie 1996, Rodríguez *et al.* 2013, Kilmer *et al.* 2017). We presented each female with vibrational playback stimuli. We imparted the stimuli onto the playback plant with a piezoelectric stack attached with soft wax to the stem of the plant. We drove the piezoelectric stack with a piezo controller (Thorlabs, Newton, NJ, USA). We recorded female duetting signals and the playback stimuli with the above laser vibrometer. We set the amplitude of the playbacks to 0.15 mm/s using an oscilloscope (HMO 1002; Rohde and Schwarz, Munich, Germany).

To test a female, we placed her on the playback plant, allowed her to settle for 30 s, and assessed whether she was receptive with playbacks of recorded male signals from both species, in random order. We returned unreceptive females to their rearing plant and tried again every 2–4 days, or until they died. Female adult age at testing was 25–42 days. We presented receptive females with synthetic playback stimuli that varied in frequency, with all other features set to the population mean of each species (Desjonquères *et al.* 2023). To capture the full shape of the preference functions, we used stimuli that exceeded the range of signal frequency values in the two species (Kilmer *et al.* 2017). Thus, we presented each female with a random sequence of 18 stimuli that varied from

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100 to 440 Hz in 20 Hz increments. Each stimulus consisted of a bout of four signals for any given frequency and was separated from the next by 15 s of silence.

We analysed female responses to playbacks from the recordings with the program AUDACITY. Our assay of response was the number of responses (ranging from zero to four) that females produced in response to each stimulus (Rodríguez *et al.* 2004, 2006, 2012). We visualized individual mate preference functions by fitting cubic spline regressions for each female with the program PFUNC (v.1.0.0; https://github.com/Joccalor/PFunc and https://hub.docker.com/r/joccalor/pfunc/; Kilmer *et al.* 2017). PFUNC uses the gam function in the MGCV R package (Wood and Wood 2015) to fit the functions without making any assumptions about shape, except that the curves will be somewhat smooth (Kilmer *et al.* 2017, cf. Schluter 1988). We also used PFUNC to extract the preferred signal frequency value (peak preference) of each female from her function.

Statistical analysis

We ran all analyses on JMP PRO v.19.0.3 (JMP Statistical Discovery 2025).

Likelihood of nymphs hatching

We fitted a logistic regression model with whether any nymphs hatched on leafed-out plants with egg masses (yes/no) as the dependent variable. The explanatory variables were cross and year. In this model, each plant contributes only one yes/no data point.

Survivorship

We fitted separate linear models for each survivorship score (adults/hatched nymphs, adults/egg masses; the former arcsine-transformed) as the dependent variable. Each model had cross and year as explanatory terms. In these models, each rearing plant contributes only one data point for each score.

Male signals

We fitted a linear mixed model with signal frequency as the dependent variable and with the following fixed explanatory terms: cross, recording temperature, and year. In this model, each rearing plant contributes one to several data points, according to how many males survived to recording on it. We therefore also included a random term for the rearing plant replicate of the males.

Female mate preferences

We fitted a linear mixed model with female responses to the stimuli (ranging from zero to four) as the dependent variable. We fitted a normal error distribution because, for *Enchenopa* females, there is a continuum of variation in the expression of mate preferences that underlies whether and how females respond that links the likelihood of responding with the number and length of responses (Rodríguez *et al.* 2004, 2012). The model had the following fixed explanatory terms: cross; stimulus frequency with linear and quadratic fits; the interactions between cross and the stimulus frequency terms; recording temperature; and year. In this model, each rearing plant contributes one to several data points, according to how many females survived to testing on it; and each females contributes 18 data points (her responses to the stimuli). We therefore also included random terms for the rearing plant replicate of the female, and for female identity. In this model, the linear and quadratic stimulus frequency terms model linear and curvilinear preference shapes, respectively. Thus, the key term is the cross × stimulus frequency^2, which tests for cross differences in preference shape.

We also analysed variation in the preferred signal frequencies of females (their peak preferences). We fitted a linear mixed model with the peak preference for each female as the dependent variable and with the following fixed explanatory terms: cross, recording temperature, and year. In this model, each rearing plant contributes one to several data points, according to how many females survived to testing on it. We therefore also included a random term for plant replicate.

Results

Likelihood of nymphs hatching

Nymphs were much more likely to hatch in the own-species crosses than in the hybrid crosses: cross term: $\chi^2_3 = 21.24$, $P < .0001$; year term: $\chi^2_2 = 9.76$, $P = .008$ (Fig. 3A). (Data underlying this and the below analyses are provided as supplementary material.)

Survivorship

We found no significant difference between crosses in survivorship to the adult moult with either of the survivorship scores (Table 2; Fig. 3B). In other words, when hybrid nymphs hatched, survivorship was comparable to survivorship in the own-species crosses.

Nymph coloration

As seen in prior work (Rodríguez *et al.* 2018, Desjonquères *et al.* 2023; Fig. 1), the nymphs of the two study species differed in coloration: LF nymphs were grey–green, and HF nymphs were brown with white stripes. The coloration of hybrid nymphs was fully aligned with the maternal species, with 100% of nymphs showing the corresponding colour pattern.

Male signals

Our sample of recorded males was low, owing, in part, to plant mortality and to low survivorship of males to sexual maturity (which starts ~2–3 weeks after the adult moult; Cocroft *et al.* 2008) regardless of cross. Nevertheless, our analysis detected clear and strong differences in signal frequency between the crosses (Table 3), with continuous variation in signal frequency within each species and cross and with the hybrid signal phenotypes closely aligned to the maternal species phenotype (Fig. 4). Note that for the analysis in Table 3, we included only the data for hybrid and own-species crosses indicated with 'this study' in Fig. 4.

For visual inspection, we supplemented our sample of male signals from our library of recordings of the two study species (Desjonquères *et al.* 2023). This confirmed continuous

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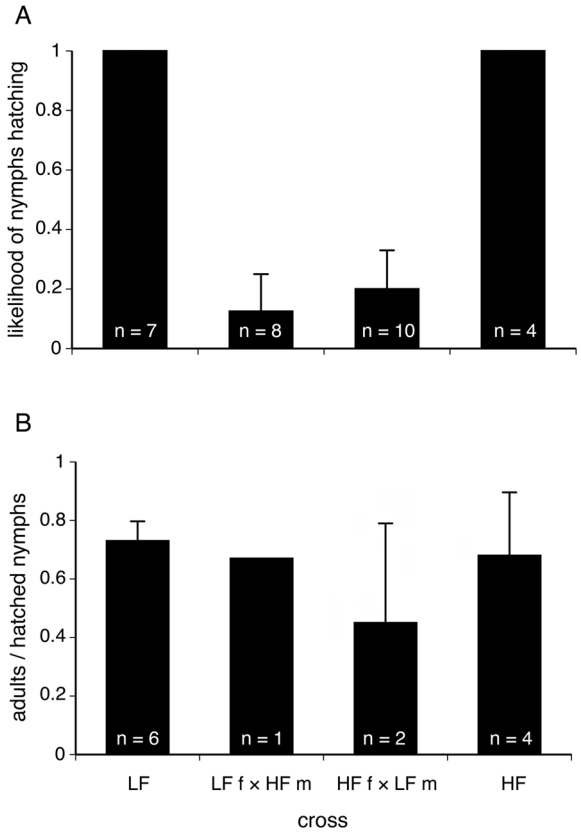

Figure 3 A, variation in the likelihood of *Enchenopa binotata* nymphs hatching in the parental species and hybrid crosses. B, variation in the survivorship of *E. binotata* treehoppers in the parental species and hybrid crosses. Sample sizes correspond to rearing plant replicates (Table 1). Note that we excluded one LF replicate from the analysis in B (Table 2) because an undercount of the nymphs that eclosed would have resulted in an estimate of >100% survivorship to the adult stage.

Table 2 Analysis of variation of the survivorship of *Enchenopa binotata* treehoppers in the own-species and hybrid crosses.

		d.f.	*F*	*P*-value
Adults/hatched nymphs	Cross	3, 7	0.53	.68[a]
	Year	2, 7	**8.22**	**.015**
Adults/egg masses	Cross	3, 7	0.10	.96[b]
	Year	2, 7	1.05	.40

Significant terms are highlighted in bold. [a]Results were the same using counts of males or females over hatched nymphs: $F \leq 0.36$, $P \geq .79$. [b]Results were the same using counts of males or females over egg masses: $F \leq 0.17$, $P \geq .91$.

variation in signal frequency within each species and also showed hybrid alignment with the maternal species phenotypes (Fig. 4).

Female mate preferences

We had better survivorship of females to sexual maturity, although our sample of HF females was also relatively low. Nevertheless, we detected clear and strong differences in

Table 3 Analysis of variation in male signal frequency of *Enchenopa binotata* treehoppers in the own-species and hybrid crosses.

Term	d.f.	*F*	*P*-value
Cross	3, 4.0	**146.8**	**.0001**
Temperature	1, 6.6	**13.1**	**.0094**
Year	3, 4.5	0.20	.68
Rearing plant replicate	95% CI = −32.7 to 50.3		.68

We report the 95% confidence interval and Wald *P*-value for the random term. Significant terms are highlighted in bold.

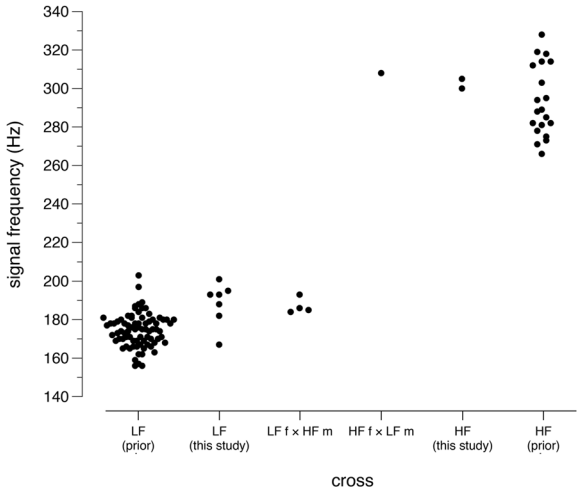

Figure 4 Variation in the dominant frequency of male signals of *Enchenopa binotata* treehoppers in the parental species and hybrid crosses. Because our sample of recorded male signals was low (see text), we supplemented the data for this figure from our library of recordings of the two study species (indicated in the figure with 'prior'; Desjonquères *et al.* 2023).

female mate preferences for signal frequency between the crosses (Tables 4 and 5). The mate preference functions varied continuously in peak preference (the highest point of the curves) and shape within each species and cross, and the hybrid phenotypes were closely aligned to the maternal species phenotype (Figs 5 and 6). Note that some females from the LF species and from the LF f × HF m hybrid cross had high peak preference values (Fig. 6); these were females with 'open' mate preferences that favoured extreme signal values, as opposed to the more common 'closed' shapes that favour intermediate signal values (Fig. 5). We have observed such individual variation in the shape of mate preferences in prior work (e.g. Rodríguez *et al.* 2013).

Discussion

We used a hybridization experiment to explore the genetic architecture of ecological adaptation and advertisement signals and mate preferences in two members of the *E. binotata* complex. We found evidence of ecological compatibility (comparable survival to the adult stage on the same host plant species in the parental species and the hybrids); and we found evidence for sex-linked polygenic architecture of male signals and female mate preferences.

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Table 4 Analysis of variation in female mate preference functions for male signal frequency of *Enchenopa* treehoppers in own-species and hybrid crosses.

Term	d.f.	*F*	*P*-value
Cross	3, 11.6	**4.97**	**.012**
Frequency	1, 1743	0.48	.49
Frequency^2	1, 1743	**77.12**	**<.0001**
Cross × frequency	3, 1743	**19.16**	**<.0001**
Cross × frequency^2	3, 1743	**11.71**	**<.0001**
Temperature	1, 67.2	0.14	.71
Year	1, 12.7	0.38	.55
Rearing plant	95% CI = −0.039 to 0.074		.54
Female identity	95% CI = 0.029–0.126		**.0018**

We report the 95% confidence interval and Wald *P*-value for the random term. Significant terms are highlighted in bold.

Table 5 Analysis of variation in the peak of female mate preference functions for male signal frequency of *Enchenopa* treehoppers in own-species and hybrid crosses.

Term	d.f.	*F*	*P*-value
Cross	3, 23.0	**8.60**	**.0005**
Temperature	1, 70.8	0.35	.56
Year	1, 43.1	0.74	.39
Rearing plant	95% CI = −256.4 to 305.9		.86

We report the 95% confidence interval and Wald *P*-value for the random term. Significant terms are highlighted in bold.

Our observation of ecological compatibility suggests that selection for assortative mating to avoid maladaptive hybridization (according to ecological or mutation-order speciation; Schluter 2001, 2009, Coyne and Orr 2004, Nosil 2012, Kopp *et al.* 2018) has not been a major factor in the sustained co-existence of these species. This conclusion must be tentative, for two main reasons. First, working with species that share the same host facilitated rearing of the hybrids, but most members of the *E. binotata* complex show strong host specialization (Wood and Guttman 1982, 1983, Wood 1993, Cocroft *et al.* 2008), as do many plant-feeding insects (Drès and Mallet 2002, Cocroft *et al.* 2008). Thus, our study addresses a real co-existence scenario (Cocroft *et al.* 2010), but not the main scenario of speciation involving colonization of novel hosts. Second, our results are based on a small sample of replicated rearing plants (Table 1). This is in part because some plants died before nymphs could hatch and because we did not count cases where no nymphs eclosed in rearing plants towards hybrid inviability (Table 1). We made this decision because we consider that hybrid matings were likely to be rare, given the widely divergent advertisement signals and mate preferences of the parental species (Rodríguez *et al.* 2018, Desjonquères *et al.* 2023, this study), and because in at least one case we observed fertilized eggs in a hybrid cross (Fig. 2C). Nevertheless, we cannot fully rule out hybrid embryo mortality, although when hybrid nymphs hatched they had similar survivorship to the own-species crosses.

We also found continuous variation in male advertisement signals and female mate preferences within the parental species and the hybrid crosses, with hybrid phenotypes closely aligned to the maternal species phenotypes. Interestingly, the degree of alignment with maternal species phenotypes that we observed is stronger than in other studies that have used a function-valued approach for describing mate preferences (e.g. Ritchie 1996). These results are also based on a relatively small sample of individuals, especially in the case of male signals. Nevertheless, there was close correspondence between the data from this study and prior observations with larger samples (Fig. 4; Rodríguez *et al.* 2013, 2018, Rebar and Rodríguez 2015, Desjonquères *et al.* 2023). Thus, we consider that our results support a hypothesis of sex-linked, polygenic architecture for signals and preferences. Such genetic architecture might facilitate the establishment of linkage disequilibrium and promote signal–preference co-divergence. It is intriguing, however, how this genetic architecture might evolve. It might not be so surprising for male signals, given likely XX/XO or XX/XY sex chromosome systems (Kornhauser 1914, Bhattacharya and Manna 1973, Palmer Droguett *et al.* 2024). However, the pattern was similar for female mate preferences, which is more puzzling. We speculate that the explanation might involve a process of X chromosomose–autosome fusion that has been found in other treehoppers, which could 'recruit' variation from the genome towards sex linkage (Palmer Droguett *et al.* 2024). Further work will be required to assess this intriguing possibility.

The coloration phenotypes of hybrid nymphs also showed close alignment with the maternal species phenotypes. Besides adult signals and preferences and host plant use, nymph coloration is a distinctive feature among species in the *E. binotata* complex (Wood 1980). Thus, this observation suggests that sex-linkage might extend to aspects of the ecology of these treehoppers besides their sexual traits.

Conclusion

In conclusion, our results highlight the role of sexual selection and divergence in signals and preferences in speciation (West-Eberhard 1983, Eberhard 1985, Higashi *et al.* 1999, Coyne and Orr 2004, Janicke *et al.* 2018, Shaw *et al.* 2024). Complete speciation can often involve divergence in multiple traits (Nosil *et al.* 2009, Riesch *et al.* 2017). Such is the case in the *E. binotata* complex (Wood and Guttman 1982, 1983, Wood 1993, Rodríguez *et al.* 2004, 2006, Cocroft *et al.* 2008, 2010). Nevertheless, there is also evidence that in this complex signal divergence might arise before ecological incompatibilities (Rodríguez *et al.* 2021, 2025) and perhaps even facilitate subsequent ecological specialization. Furthermore, should such incompatibility not arise, as is seemingly the case with our two treehoppers, species might be kept distinct purely by sexual divergence (M'Gonigle *et al.* 2012, cf. McEntee *et al.* 2016).

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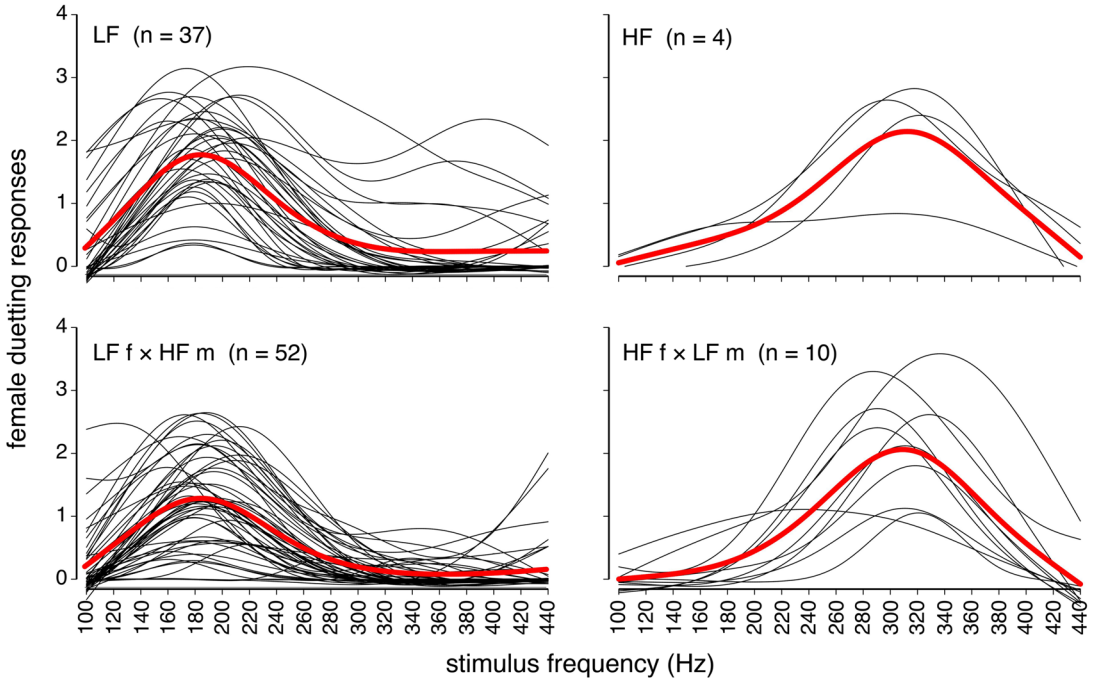

Figure 5 Variation in female mate preference functions for male signal frequency of *Enchenopa binotata* treehoppers in the parental species (top row) and hybrid crosses (bottom row). We show individual mate preference functions for all females (female sample sizes indicated in parentheses) with thin, black curves; and the mean preference function for each species/cross with thick red curves. The *x*-axis tick marks correspond to the playback stimuli we used.

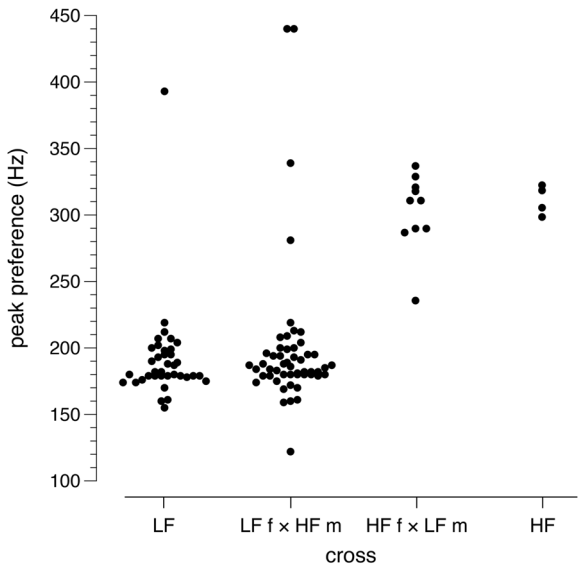

Figure 6 Variation in the female peak preferences for male signal frequency of *Enchenopa binotata* treehoppers in the parental species and hybrid crosses.

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Supplementary material

Supplementary material is available at *Biological Journal of the Linnean Society* online.

Conflicts of interest

None declared.

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Data availability

The data underlying the work are available in the Supplementary material.

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