#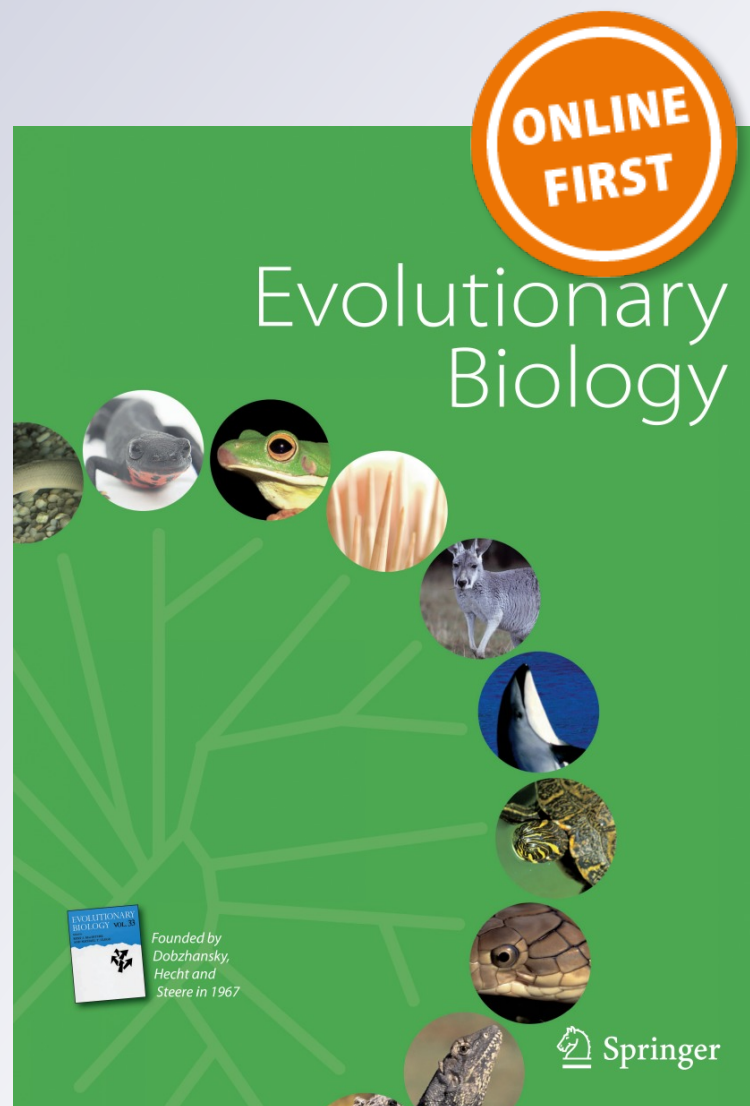 Allometric Convergence, Acoustic Character Displacement, and Species Recognition in the Syntopic Cricket Frogs *Acris* crepitans *and* A. gryllus

Jonathan P. Micancin & R. Haven Wiley

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Allometric Convergence, Acoustic Character Displacement, and Species Recognition in the Syntopic Cricket Frogs *Acris crepitans* and *A. gryllus*

Jonathan P. Micancin · R. Haven Wiley

Abstract Evidence for reproductive character displacement (RCD) has accumulated more slowly than for ecological character displacement, perhaps because sampling scales and environmental covariates can obscure the role of RCD in speciation. We examined an early example of RCD in an anuran species group, the vocalizations of the sympatric cricket frogs *Acris crepitans* and *A. gryllus*. With a relatively fine spatial scale, we compared mixed-species choruses (syntopy), nearby locations where *A. gryllus* is recently extirpated (historic sympatry), and surrounding areas without secondary contact (allopatry). In each of these areas, we evaluated variation in dominant frequency, click rate, and mass of males. In addition, we determined the acoustic preferences of syntopic females. Temperature influenced dominant frequency of vocalizations in *A. crepitans*, but not in *A. gryllus*. Body size varied more and had a stronger influence on dominant frequency in *A. crepitans* than in *A. gryllus*. Consequently, the decrease in mass of *A. crepitans* in syntopy resulted in convergence of body size and divergence of dominant frequencies of the two species. In contrast, dominant frequency of *A. crepitans* did not differ between historic sympatry and allopatry. Females of both species used fine temporal structure to discriminate between conspecific and heterospecific vocalizations and showed no preferences for dominant frequency. Chorus noise limited the ability of *A. gryllus* females to detect and discriminate vocalizations, so convergence in mass might have resulted from RCD in dominant frequency to reduce heterospecific acoustic interference. However, influences other than RCD might have caused syntopic convergence in body size.

Keywords Amphibian decline · Body size · Conservation behavior · Reproductive isolation · Vocal communication · Sympatry

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J. P. Micancin (✉) · R. H. Wiley
Department of Biology, University of North Carolina at Chapel Hill, CB# 3280 Coker Hall, Chapel Hill, NC 27599-3280, USA
e-mail: micancin@gmail.com

R. H. Wiley
e-mail: rhwiley@email.unc.edu

Introduction

Closely related species sometimes diverge phenotypically in sympatry to reduce competitive or reproductive interactions, a phenomenon known as character displacement (CD) (Brown and Wilson 1956; Blair 1958; Butlin 1987; Schluter 2000; Pfennig and Pfennig 2010). Ecological character displacement (ECD) in traits such as bill size and body size can reduce heterospecific competition for resources such as food (Brown and Wilson 1956; Grant and Grant 2006; Kirschel et al. 2009; Pfennig and Pfennig 2009). Reproductive character displacement (RCD; Fouquette 1975), usually in signals for mate attraction or preferences for those signals, evolves as a result of reproductive interference (Gröning and Hochkirch 2008) when hybrids are inviable, sterile, or otherwise maladapted (reinforcement; Dobzhansky 1940; Blair 1974; Noor 1999; Servedio and Noor 2003; Pfennig and Pfennig 2009) or competition for reproductive resources such as position in a breeding aggregation (Höbel and Gerhardt 2003) or signal space (Amezquita et al. 2006; Crampton et al. 2011). Evidence for RCD has accumulated more slowly than for ECD (Schluter 2000; Dayan and Simberloff 2005; Gröning

and Hochkirch 2008). Integrated studies of ECD and RCD can help to clarify the relative importance of these two processes in speciation (Rundle and Schluter 2004; Goldberg and Lande 2006; Grant and Grant 2006; Pfennig and Pfennig 2009).

In general, CD can be difficult to detect in wild populations. Clinal variation along ecological gradients can produce phenotypic differences that resemble or obscure CD, especially RCD (Goldberg and Lande 2006; Adams and Collyer 2007; Meiri et al. 2011). Selection for CD should be highest where encounters between species are most common (Pfennig and Pfennig 2009). Nevertheless, studies of CD sometimes treat sympatric (in the same geographic area) and syntopic populations (in the same microhabitat; Rivas 1964) as equivalent by pooling data from allotopic and syntopic populations within sympatry or by comparing allopatric populations with allotopic populations within sympatry. Even with diffusion of phenotypes from syntopy to allotopy within a region of sympatry, comparisons of allopatry and sympatry might fail to detect CD by sampling allotopic populations with mean phenotypes that are an intermediate of allopatric and syntopic populations. A focus on strictly syntopic populations might also be necessary to detect RCD in mating signals or responses, which are instantaneous and socially variable behaviors (Gerhardt 1994). For these reasons, many recent studies of RCD have compared syntopic and allotopic populations rather than sympatric and allopatric populations (Höbel and Gerhardt 2003; Amezquita et al. 2006; Johanet et al. 2009; Kirschel et al. 2009; Richards-Zawacki and Cummings 2011).

Examples of CD in the communication systems of anuran species groups were described when the concept was first introduced (Brown and Wilson 1956; Blair 1958). In many anurans, females use temporal or spectral features of advertisement calls to discriminate conspecific and heterospecific males (Gerhardt and Huber 2002; Wells 2007). Most early anuran examples of RCD were subsequently examined at a regional scale (in sympatry and nearby allopatry) and produced evidence of RCD in timing, frequency, or amplitude (Littlejohn 1959, 1965; Littlejohn and Fouquette 1960; Fouquette 1975; Loftus-Hills and Littlejohn 1992; Smith et al. 2003; Lemmon 2009). RCD was not subsequently tested in one of the originally proposed examples, the hylid *Acris* (cricket frogs) of the eastern and central United States.

To determine the origin of acoustic variation and reproductive isolation in *Acris* species, Nevo and Capranica (1985) sampled *A. c. crepitans*, *A. c. blanchardi*, and *A. gryllus* at 21 sites throughout the range of the genus in eastern North America, including one site with syntopic *A. crepitans* and *A. gryllus*. They concluded that interspecific differences in spectral and temporal components of

vocalizations arose from allopatric divergence in body size along continental gradients in temperature and precipitation or from selection for transmission in forests and grasslands, not reinforcement in sympatry. They thus identified clinal variation and habitat differences that can obscure CD at smaller scales or within habitats (Goldberg and Lande 2006). In the narrow forest-grassland ecotone in eastern Texas, Ryan and Wilczynski (1991) found that acoustic differences between parapatric *A. c. crepitans* and *A. c. blanchardi* were the result of environmental selection for efficient sound transmission in different habitats, rather than body size differences along a climate gradient or RCD. Recent phylogenetic evidence elevated *A. c. blanchardi* to *A. blanchardi* and suggested that Texas populations were entirely *A. blanchardi* (Gamble et al. 2008; Collins and Taggart 2009; but see Brown et al. 2010). Regardless of whether the populations studied by Ryan and Wilczynski (1991) were parapatric subspecies of *A. crepitans* or *A. blanchardi*, the system was unsuitable for examining the role of RCD in sympatry. Thus, RCD has yet to be evaluated in sympatric or syntopic *Acris* species.

The sympatric cryptic sibling species *A. crepitans* and *A. gryllus,* an early proposed example of RCD, warrant examination at a small spatial scale. The two species are sympatric on the upper Atlantic Coastal Plain in the southeastern United States (Conant and Collins 1991; Mount 1996; Jensen et al. 2008; Micancin and Mette 2009; Beane et al. 2010). Morphological and acoustic similarities of *A. crepitans* and *A. gryllus* obscure the extent of syntopy of the two species (Micancin and Mette 2009). Although Nevo and Capranica (1985) concluded that RCD was unlikely because *A. crepitans* and *A. gryllus* were broadly sympatric with no evidence of hybridization, new molecular evidence suggests that hybridization has occurred in the Coastal Plain (Haenel et al. 2012). Furthermore, a recent assessment indicates that *A. gryllus* is widely extirpated across three river basins in North Carolina where it was formerly sympatric and syntopic with *A. crepitans* (Micancin and Mette 2009). Both species appear stable in sympatry and syntopy in the adjacent basin to the northeast, but *A. gryllus* has declined in the next basin at the northern limit of its range (Micancin et al. 2012). Therefore, the stability of secondary contact of the two species varies at a small geographic scale. In such conditions, RCD might arise to reduce interference between the vocalizations of syntopic *A. crepitans* and *A. gryllus* and then subsequently decrease in *A. crepitans* once *A. gryllus* disappears.

In this study, we investigated the possibility that RCD in the communication systems of *A. crepitans* and *A. gryllus* contributes to reproductive isolation. We examined acoustic variation with body size and temperature across a small region encompassing both species in allopatry, the
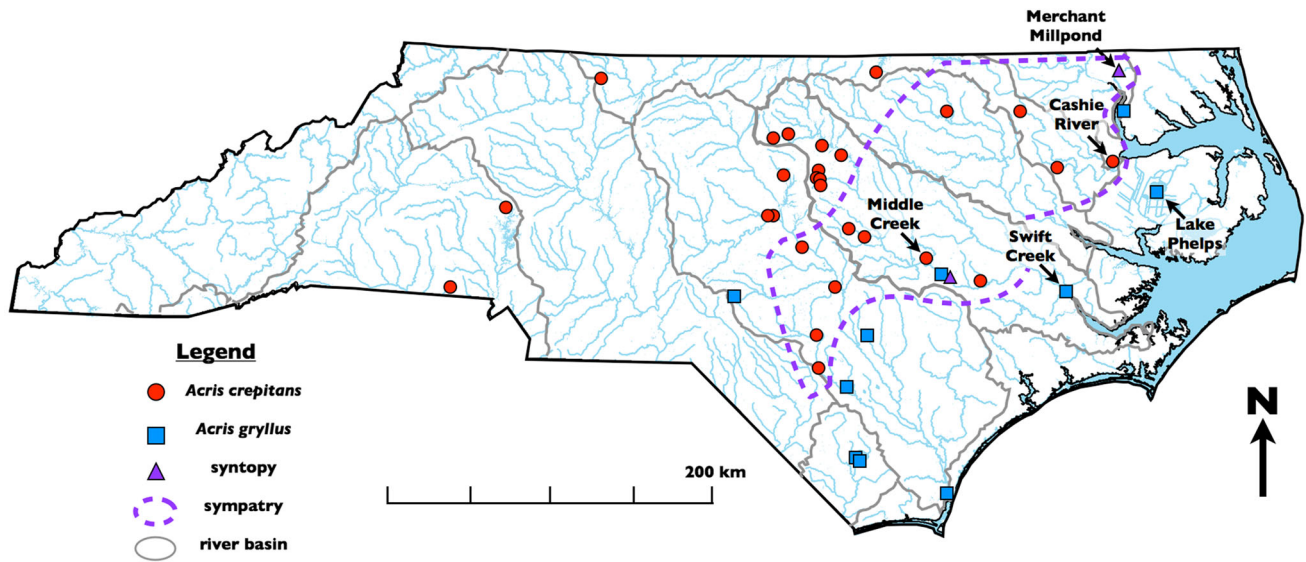
Fig. 1 Populations of *A. crepitans* and *A. gryllus* studied in North Carolina. Sympatry was defined by range overlap and microhabitat co-occurrence of museum specimens in the mid-20th Century. (Micancin and Mette 2009). Recordings from Merchants Millpond (36.4318° N, 76.6967° W; Garmin GPS, NAD83 datum), Cashie River (35.9242° N, 76.7345° W), Lake Phelps (35.7340° N, 76.4408° W), Middle Creek (35.5714° N, 78.5837° W), and Swift Creek (35.1922° N, 77.0836° W) were used in Experiment 1 at Merchants Millpond
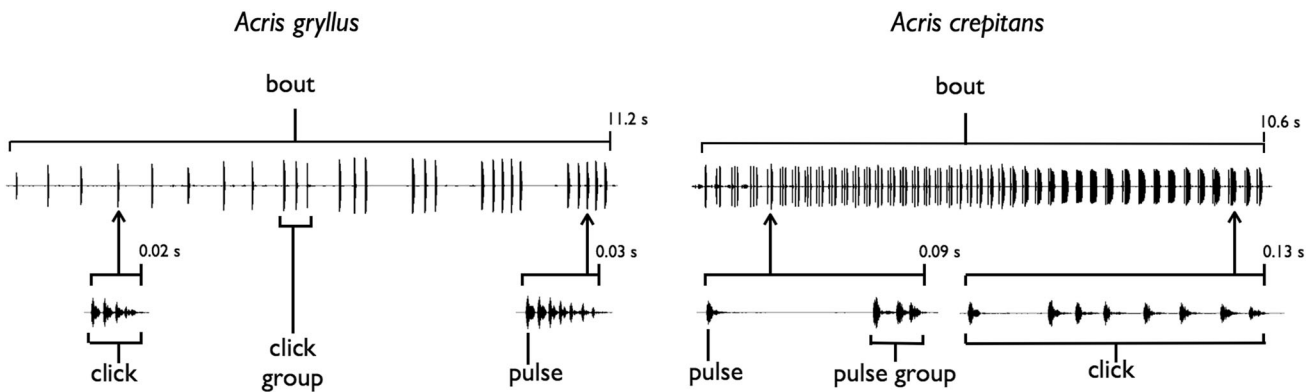

Fig. 2 Oscillograms of representative vocalizations of *Acris crepitans* and *A. gryllus* from the syntopic chorus at Merchants Millpond State Park. See OR 2 for .wav files of these vocalizations

area of historic sympatry where *A. gryllus* has recently disappeared, and syntopy. We also evaluated species recognition by syntopic females in playback experiments of signals and noise.

Methods

Acoustic Variation of Males

Prior to fieldwork, we examined museum specimens and identified sympatry and syntopy in the upper Coastal Plain of North Carolina (Micancin and Mette 2009). We then recorded 258 *A. crepitans* and 107 *A. gryllus* males at 39 choruses in North Carolina from May through July in 2004, 2005, and 2007 (Fig. 1 and Online Resource OR 1.1). Choruses were separated by at least 500 m. We recorded from 2100 hours until a chorus waned (between 0100 and 0300 hours). We identified males by acoustic (Fig. 2) and morphological features (Micancin and Mette 2009). We recorded vocalizations of each male using a Marantz PMD-221 or PMD-421 cassette recorder or a Marantz PMD-670 digital recorder (at 22.05 kHz) and an Audio-Technica 815a microphone. We captured and placed each male in a damp plastic jar and measured the surface temperature at its calling site (Miller and Weber T-6000 thermometer). We then weighed, euthanized, and preserved each male for a study of identification and

range shifts in sympatric *Acris* (Micancin and Mette 2009).

We used WildSpectra (version 060125; http://www.unc.edu/~rhwiley/wildspectra/) to digitize recordings at a sampling rate of 22.05 kHz and for all acoustic analyses. *Acris* vocalizations have complex temporal structures in which pulses are repeated to produce larger units of increasing duration: pulse groups, clicks, click groups, bouts, and episodes (Fig. 2 and OR 1.2; see OR 2 for audio files for Fig. 2). We randomly selected 5 bouts from each male (http://www.random.org/) unless <5 bouts were available for analysis (1,808, 4.95 bouts per male). We counted the number of clicks in each bout and used the SongSignatures function in WildSpectra (OR 1.3) to measure the duration of each bout, the dominant frequency of each click, and the interval between each click. We used a sampling rate of 11.025 kHz and a transform size of 1,024 (21 Hz frequency resolution; 46.43 ms temporal resolution). We calculated the click rate and mean dominant frequency of each bout and then the mean click rate and mean dominant frequency of each individual. Data are archived in OR 3.

To investigate the contributions of temperature, mass, and contact between species to variation in vocalizations, we used linear regressions, nested analyses of covariance (ANCOVA) and variance (ANOVA), and least-squares-means Student's *t* tests separately for each species in JMP (v. 9.0; SAS Institute 2009; http://www.jmp.com/). We used linear regressions to describe variation in dominant frequency and click rate with temperature and mass (Nevo and Capranica 1985; Wagner 1989b; Ryan and Wilczynski 1991; Gerhardt and Huber 2002). To investigate differences between allopatry, historic sympatry, and syntopy, we conducted ANCOVA of dominant frequency and click rate (with temperature as the covariate) and ANOVA of mass with individuals and choruses nested within biogeographic regions. Using these analyses, we calculated adjusted means for regions with control for effects of temperature and chorus (least squares means; SAS Institute 2009). We then compared adjusted mean dominant frequency, click rate, and mass between regions with *t* tests. Each *t* test used degrees of freedom for the error variance from the ANCOVA or ANOVA and assumed unequal variances within regions.

The sympatric distribution of *A. crepitans* suggests that it colonized the upper Coastal Plain by moving down rivers from the Piedmont Plateau (Jensen et al. 2008; Micancin and Mette 2009; Beane et al. 2010). We compared dominant frequency, click rate, and mass of each species between adjacent sympatric and syntopic choruses in a river basin by adjusting click rates and dominant frequencies to 22.5 °C with linear regressions and then comparing means of adjacent choruses with *t* tests.

Female Preference Tests

We examined the acoustic preferences of *A. crepitans* and *A. gryllus* females at Merchants Millpond, North Carolina (Fig. 1), a blackwater impoundment with extensive mixed-species choruses containing many more vocalizing males and gravid females of each species than other wetlands in the study. Heterospecific males intermingled in close proximity, often vocalized within 50 cm of each other, and engaged in heterospecific agonism (Micancin and Mette 2010). Both species had mean vocal amplitudes of 80 dB at 1 m and mixed chorus amplitude fluctuated between 78 and 82 dB (mode, 80 dB) (RadioShack Sound Level Meter 33-2055, C weighting, fast response). Gravid females potentially had many opportunities to mate with conspecific or heterospecific males.

Experiment 1: Discrimination of Natural Vocalizations by A. gryllus

In June 2006, we tested the ability of female *A. gryllus* to discriminate between vocalizations of both species recorded at five locations (Fig. 1). We sorted bouts of the two species that differed by no more than four clicks and 2 s and then paired heterospecific bouts to present antiphonal signals with similar gross temporal properties. Otherwise, paired bouts differed in dominant frequency, the species-specific fine temporal structure of pulses and clicks, and the intervals between clicks by the normal amounts for males in mixed or separate choruses. From these paired bouts, we randomly (http://www.random.org/) selected four pairs from syntopic Merchants Millpond, two pairs from Cashie River (sympatric *A. crepitans*) and Lake Phelps (allopatric *A. gryllus*), and two pairs from Middle Creek (sympatric *A. crepitans*) and Swift Creek (allopatric *A. gryllus*; OR 1.4). We used Sound Studio (v. 2.2.4; http://felttip.com/ss/) to concatenate each pair into a two-channel AIFF file with 1.0 s of silence separating antiphonal bouts which repeated for 15 min. Half of these files began with an *A. crepitans* bout. Each file contained an equal number of bouts of the two species and ended after approximately 15 min (after the bout nearest the 15 min point was complete). We removed background vocalizations and equalized the amplitudes of the two channels.

Amplexed *A. gryllus* females were collected between 2100 and 2400 hours. We photographed and captured each pair, and confirmed the species of the female and male based on morphological features (Micancin and Mette 2009), placed the female in a damp plastic jar, and released the male at the point of capture. These females were tested within 5 h in a playback arena under a semi-enclosed structure (17–25 °C ambient temperature, within the range of source choruses) and released them on the same night at

a different chorus. The playback arena (dimensions: 240 × 120 × 71 cm high) had internal walls covered with acoustic tile and dim illumination from a centrally suspended red light. We placed an amplified speaker (Radio-Shack 277-1008) 20 cm from each end of the arena at 180° and connected the speakers to a third-generation iPod (Apple Inc.). We alternated the speaker presenting each species from one night to the next. Before each trial, we measured the amplitude of a test vocalization from each speaker with a sound pressure level meter (RadioShack 33-2050, C weighting, fast response) and adjusted the speaker until each click produced a maximal reading of 80 dB SPL at 1 m. For each trial, we randomly selected a playback file and a female using a 16-sided die, placed the female at the center of the arena under a perforated plastic cone, and released it remotely after one set of antiphonal bouts. We continued each playback for 15 min or until the female made a choice by phonotaxis to within 20 cm of a speaker (or until the female left the arena). We conducted trials until 2 females responded to each of the 8 pairs of bouts (N = 16).

Experiments 2–4: Discrimination of Synthetic Vocalizations Differing in Frequency or Timing

In May–July 2007, we examined the preferences of female *A. crepitans* and *A. gryllus* for signals synthesized on a PowerPC with customized software (SoundSynth2, www.unc.edu/~rhwiley/soundsynthesis/SoundSynth2_070416B.zip) and modeled on the vocalizations of 5 *A. crepitans* and 4 *A. gryllus* males recorded on 12 May 2005 at Merchants Millpond (OR 1.5). See OR 1.6 for details of the modeling and synthesis of stimuli. The resulting signals represented a range of bouts of males a female might encounter in a mixed chorus rather than the average vocalizations for each species or population.

Experiment 2 checked the sufficiency of synthesized vocalizations by testing females' abilities to discriminate between vocalizations with species-typical parameters. We synthesized four *A. crepitans* bouts with a dominant frequency of 3,962 Hz and four *A. gryllus* bouts with a dominant frequency of 3,502 Hz, each with the number and intervals of clicks and total duration of a recorded natural bout. We randomly paired a bout from each species to create four pairs of exemplars. The two signals in each exemplar pair differed in dominant frequency, click structure, bout duration, and the number and pattern of clicks. Experiment 3 tested the ability of females to discriminate between synthesized vocalizations differing only in species-specific click structure. We paired each bout used in Experiment 2 with a bout in which we replaced the clicks of one species with the clicks of the other species. This procedure resulted in four pairs of exemplars for females of each species. The two

signals differed in the fine temporal structure of pulses within clicks but had the same species-typical mean dominant frequency and gross temporal structure. Experiment 4 tested the preference of females for synthesized conspecific vocalizations with dominant frequencies at the population mean or displaced from the heterospecific species. Female *A. crepitans* received bouts with the mean conspecific dominant frequency (3,962 Hz) and with a dominant frequency one standard deviation above the mean (4,150 Hz). Female *A. gryllus* received bouts with the mean conspecific dominant frequency (3,502 Hz) and with a dominant frequency one standard deviation below the mean (3,353 Hz). We paired each bout from Experiment 2 with a bout resynthesized at the other dominant frequency to produce four pairs of exemplars for females of each species. We used the same procedures from Experiment 1 except we conducted the trials in an enclosed structure to maintain ambient temperature above 22 °C (22–29 °C for all trials). We also connected the speakers to a compact two-channel mixer (RadioShack 32-2056) with input from the iPod. We recorded the time and direction of the female's first hop and each hop thereafter. We continued each playback until the female demonstrated a phonotactic response and approach toward one of the signals as in Experiment 1 (choice) or for a maximum of 15 min (no choice). We conducted each experiment until we observed 16 choices. See OR 1.7 for details on the execution of trials.

Experiments 5–6: Detection and Discrimination in Noise

In June–July 2007, we examined the ability of female *A. gryllus* to detect and discriminate signals in chorus noise. From a recording of a mixed-species chorus at Merchants Millpond, we mixed two 10 s samples using Sound Studio 2.2.4 to eliminate major amplitude shifts and discriminable individual vocalizations. The mixed sample had a larger frequency peak at 3,789 Hz produced by *A. crepitans* and *A. gryllus* and a smaller peak at 818 Hz produced by *H. cinerea*. We looped the mixed sample to produce 15 min of continuous noise, applied a fade-in filter from 0 to 5 s, and inserted 3 min of silence at the beginning of the file for acclimation of each female.

Experiment 5 tested the ability of *A. gryllus* females to detect a conspecific signal in chorus noise. We constructed playback files by removing the antiphonal *A. crepitans* signal from each stereo file used in Experiment 2 (leaving the *A. gryllus* signal in the left or right channel) and adding the two-channel noise file. We mixed the signal and noise files so that during each trial, the chorus noise from both speakers was −3, −1.5, or −0 dB relative to the 80 dB *A. gryllus* signal from one speaker (measured at 1 m in the center of the arena). This procedure resulted in 4 exemplars of *A. gryllus* signals at each level of noise. Experiment 6 tested the ability of female *A. gryllus* to discriminate

Table 1 Regressions of dominant frequency and click rate on temperature and body mass

Species	Acoustic feature	Effect	Summary of fit		
			Slope	R²	P
A. crepitans	Dominant frequency	Temperature	34.387	0.37	<0.0001*
		Mass	−762.275	0.37	<0.0001*
	Click rate	Temperature	0.185	0.45	<0.0001*
		Mass	−1.524	0.06	<0.0001*
A. gryllus	Dominant frequency	Temperature	−7.614	0.01	0.2419
		Mass	−479.437	0.26	<0.0001*
	Click rate	Temperature	0.129	0.21	<0.0001*
		Mass	−0.016	0.00	0.9662

between conspecific and heterospecific calls in chorus noise. We repeated the preparation for Experiment 5 except we kept the antiphonal *A. crepitans* signals in the files from Experiment 2. We conducted each set of trials until 12 choices were made. Otherwise, the procedure for Experiment 5–6 was identical to the procedure for Experiment 2–4. Because *Acris* vocalizations likely have spherical far-field propagation (Gerhardt 1975; Gerhardt and Klump 1988; Gerhardt and Huber 2002), we assumed −6 dB attenuation of vocalizations per doubling of distance to estimate the range at which *A. gryllus* females might be able detect conspecific signals within typical intensities of chorus noise (Wollerman 1999; Wollerman and Wiley 2002).

Statistical Analysis

For each experiment, we used exact tests of goodness-of-fit (McDonald 2009) to assess female preference and JMP to conduct Wilcoxon rank-sum tests of the latency to the first hop and latency to choice.

Results

Acoustic Variation of Males

Variation with Temperature and Mass

The two species differed in the relationships of dominant frequency and click rate to temperature and mass (Table 1). There was a significant positive relationship between temperature and dominant frequency in *A. crepitans* and a non-significant relationship in *A. gryllus*. Click rate increased with increasing temperature in both species, but the relationship was weaker in *A. gryllus* than in *A. crepitans*. Dominant frequency decreased with increasing mass in both species, but again the relationship was weaker in *A. gryllus*. The negative relationship between mass and click rate in *A. crepitans* was significant, but had a low R².

There was no significant relationship between mass and click rate in *A. gryllus*. Acoustic differences among choruses of both species were likely influenced by seasonal variation in temperature and mass (OR 1.8–1.10). However, for both species, body mass contributed substantially to the total variances in dominant frequency and click rate while chorus, month of recording, and temperature at the calling site had comparatively little or no influence.

Variation in Allopatry, Historic Sympatry, and Syntopy

The two species diverged in dominant frequency in syntopy and converged in click rate in historic sympatry, despite substantial variance within species and overlap between species (Table 2). Overall, *A. crepitans* had higher mean dominant frequency and click rate than *A. gryllus*. Both species varied significantly between regions in dominant frequency and click rate (Table 3). Adjusted mean dominant frequency of *A. crepitans* was significantly higher in syntopy than in allopatry or sympatry but did not differ between sympatry and allopatry (Table 4). Adjusted mean dominant frequency of *A. gryllus* was significantly higher in syntopy than in allopatry but did not differ between syntopy and sympatry or between sympatry and allopatry (Table 4). The differences between dominant frequencies of the two species were larger in syntopy (415.12 Hz) than in sympatry (390.20 Hz) or allopatry (386.90 Hz). Adjusted click rate of *A. crepitans* was significantly lower in syntopy than in sympatry and sympatry than in allopatry but did not differ between sympatry and syntopy (Table 4). Adjusted click rate of *A. gryllus* was significantly higher in sympatry than in allopatry or syntopy but did not differ between syntopy and allopatry (Table 4). The differences in adjusted click rates of the two species were larger in allopatry (2.14/s) than in sympatry (0.76/s) or syntopy (1.53/s). Therefore, the click rates of *A. crepitans* and *A. gryllus* were more similar among allotopic choruses in historic sympatry than in allopatry or syntopy.

Masses of the two species converged in syntopy because of significant reductions in mass of *A. crepitans* from

Table 2 Mean dominant frequency, click rate, body mass, and temperature of *A. crepitans* and *A. gryllus* males in allopatry, allotopy, syntopy, and throughout North Carolina

Species	Region	N		Dom. freq. (Hz)		Click rate (N/s)		Mass (g)		Temperature (°C)	
		Chorus	Male	Mean	SD	Mean	SD	Mean	SD	Mean	SD
A. crepitans	Allopatry	15	123	3,817.48	184.43	4.00	0.95	1.102	0.160	21.39	3.61
	Allotopy	12	104	3,892.29	235.12	4.21	1.08	1.024	0.176	23.77	3.67
	Syntopy	2	31	3,940.83	178.55	3.44	1.01	0.980	0.113	21.75	2.99
	Total	29	258	3,862.46	209.99	4.02	1.03	1.056	0.168	22.39	3.73
A. gryllus	Allopatry	9	62	3,475.73	161.96	2.06	0.43	1.013	0.176	24.86	1.71
	Allotopy	1	13	3,473.00	155.14	3.21	0.82	1.048	0.145	26.17	1.35
	Syntopy	2	32	3,526.24	142.84	2.00	0.60	0.993	0.158	22.92	2.86
	Total	12	107	3,490.50	156.01	2.18	0.66	1.011	0.167	24.44	2.34

Table 3 Nested analyses of variance of dominant frequency, click rate, and mass of *A. crepitans* and *A. gryllus* by region and chorus

Species	Feature	Summary of fit		Source	df	F	p
A. crepitans	Dominant frequency (Hz)	R^2	0.61	Model	29	12.15	<0.0001*
		Adj. R^2	0.56	Region	2	8.37	0.0003*
		RMSE	139.73	Chorus	26	4.51	<0.0001*
		Mean	3,862.46	Temperature	1	12.52	0.0005*
		N	258	Error	228		
	Click rate (/s)	R^2	0.64	Model	29	14.13	<0.0001*
		Adj. R^2	0.60	Region	2	8.44	0.0003*
		RMSE	0.66	Chorus	26	3.84	<0.0001*
		Mean	4.02	Temperature	1	76.69	<0.0001*
		N	258	Error	228		
	Mass (g)	R^2	0.45	Model	28	6.59	<0.0001*
		Adj. R^2	0.38	Region	2	12.65	<0.0001*
		RMSE	0.13	Chorus	26	5.91	<0.0001*
		Mean	1.06	Error	229		
		N	258				
A. gryllus	Dominant frequency (Hz)	R^2	0.27	Model	12	2.87	0.0021*
		adj. R^2	0.17	Region	2	3.50	0.0342*
		RMSE	141.73	Chorus	9	3.46	0.0010*
		Mean	3,490.51	Temperature	1	1.52	0.2201
		N	107	Error	94		
	Click rate (/s)	R^2	0.48	Model	12	7.37	<0.0001*
		adj. R^2	0.42	Region	2	19.96	<0.0001*
		RMSE	0.50	Chorus	9	0.83	0.5873
		Mean	2.18	Temperature	1	14.03	0.0003*
		N	107	Error	94		
	Mass (g)	R^2	0.25	Model	11	2.81	0.0032*
		adj. R^2	0.16	Region	2	1.32	0.2731
		RMSE	0.15	Chorus	9	3.31	0.0015*
		Mean	1.01	Error	95		
		N	107				

allopatry through historic sympatry to syntopy. Overall, *A. crepitans* had higher mean mass than *A. gryllus* (Table 2). Mass varied significantly between regions in *A. crepitans* but not in *A. gryllus* (Table 3). Adjusted mass of *A. crepitans* was higher in allopatry than sympatry or syntopy and lower in syntopy than sympatry (Table 4). In contrast,

Table 4 Comparisons of adjusted mean dominant frequencies, click rates, and masses of *A. crepitans* and *A. gryllus* males in allopatry, historic sympatry, and syntopy (*t* tests based on least-squares-means from ANCOVA and ANOVA)

Species	Feature	Syntopy		Sympatry		Allopatry		*t* test			
		Mean	SD	Mean	SD	Mean	SD	Diff.	df	t	p
A. crepitans	Dominant frequency (Hz)	3,958.24	27.52	3,846.69	16.69			111.54	228	3.30	0.0011*
				3,846.69	16.69	3,835.16	15.13	11.54	228	0.47	0.6386
		3,958.24	27.52			3,835.16	15.13	123.08	228	4.05	<0.0001*
	Click rate (/s)	3.70	0.13	3.80	0.08			−0.10	228	−0.62	0.5358
				3.80	0.08	4.17	0.07	−0.37	228	−3.22	0.0015*
		3.70	0.13			4.17	0.07	−0.47	228	−3.29	0.0012*
	Mass (g)	0.98	0.03	1.03	0.01			−0.06	229	−1.98	0.0491*
				1.03	0.01	1.10	0.01	−0.07	229	−3.67	0.0003*
		0.98	0.03			1.10	0.01	−0.13	229	−4.45	<0.0001*
A. gryllus	Dominant frequency (Hz)	3,543.12	28.05	3,456.49	41.52			86.63	94	1.63	0.1064
				3,456.49	41.52	3,448.26	20.13	8.23	94	0.18	0.8548
		3,543.12	28.05			3,448.26	20.13	94.86	94	2.64	0.0098*
	Click rate (/s)	2.17	0.10	3.03	0.15			−0.87	94	−4.59	<0.0001*
				3.03	0.15	2.03	0.07	1.01	94	6.32	<0.0001*
		2.17	0.10			2.03	0.07	0.14	94	1.10	0.2729
	Mass (g)	0.98	0.03	0.98	0.03			−0.06	95	−1.25	0.2139
				1.05	0.04	1.04	0.02	0.01	95	0.26	0.7982
		0.98	0.03			1.04	0.02	−0.05	95	−1.47	0.1446

adjusted mass of *A. gryllus* did not differ between any two regions (Table 4). Because of the strong effects of body size on acoustic features, the higher dominant frequency and lower click rate of *A. crepitans* in syntopy probably resulted from the smaller masses of individuals in these choruses. In *A. gryllus*, the weak effects of mass on dominant frequency and click rate precluded an allometric basis for biogeographic variation.

We located allopatric, historically sympatric, and syntopic choruses in the Neuse River basin (13 *A. crepitans* and 3 *A. gryllus* choruses, including 1 syntopic chorus, Fig. 3). The two species overlapped in click rate and dominant frequency between sympatric choruses but not at the syntopic chorus. Click rates of *A. crepitans* did not differ significantly between the syntopic chorus (#13; $\bar{\chi} = 3.92$/s) and the adjacent sympatric choruses upriver (#11, $\bar{\chi} = 4.07$/s; *t* test, $df = 17$, $t = 0.5685$, $p = 0.5771$) or downriver (#14, $\bar{\chi} = /$ s; *t* test, $df = 18$, $t = 1.4407$, $p = 0.1668$). Click rate of *A. gryllus* differed between the syntopic chorus (#13, $\bar{\chi} = 2.01/$ s) and the sympatric chorus 1 km away (#12, $\bar{\chi} = 2.74$/s; *t* test, $df = 24$, $t = 2.6927$, $p = 0.0127$) but not between the syntopic chorus and the allopatric chorus 74 km downriver (#15, $\bar{\chi} = 1.76$/s; *t* test, $df = 19$, $t = 1.3196$, $p = 0.2026$). Dominant frequency of *A. crepitans* was higher at the syntopic chorus (#13, $\bar{\chi} = 4,007.08$ Hz) than at the adjacent sympatric choruses upriver (#11, $\bar{\chi} = 3,749.21$ Hz; *t* test, $df = 17$, $t = 6.0748$, $p < 0.0001$) or downriver (#14,

$\bar{\chi} = 3,754.23$ Hz; *t* test, $df = 18$, $t = 5.7306$, $p < 0.0001$). Mean adjusted dominant frequency of *A. crepitans* was at least 252.86 Hz higher at the syntopic chorus than at adjacent sympatric choruses and at least 50.69 Hz higher than any other chorus in the basin. Dominant frequency of *A. gryllus* did not differ between the syntopic chorus (#13, $\bar{\chi} = 3,545.67$) and the adjacent sympatric chorus (#12, $\bar{\chi} = 3,500.93$; *t* test, $df = 24$, $t = 0.6814$, $p = 0.5021$) or allopatric chorus (#15, $\bar{\chi} = 3,639.14$ Hz; *t* test, $df = 19$, $t = 1.3393$, $p = 0.1963$).

The high dominant frequency of *A. crepitans* in syntopy probably resulted from the significantly lower masses of frogs at that location (#13, $\bar{\chi} = 0.965$ g) than at the adjacent sympatric choruses upriver (#11, $\bar{\chi} = 1.169$ g; *t* test, $df = 17$, $t = 5.8466$, $p < 0.0001$) or downriver (#14, $\bar{\chi} = 1.146$ g; *t* test, $df = 18$, $t = 5.4659$, $p < 0.0001$). Mean mass of *A. crepitans* was at least 0.181 g lower at the syntopic chorus than at adjacent sympatric choruses and at least 0.143 g lower than any other chorus in sympatry (#9–14). The mean mass of *A. gryllus* at the syntopic chorus (#13, $\bar{\chi} = 0.936$ g) was not significantly lower than at the sympatric chorus (#12, $\bar{\chi} = 1.048$ g; *t* test, $df = 24$, $t = 1.7736$, $p = 0.0888$) or allopatric chorus (#15, $\bar{\chi} = 0.975$ g; *t* test, $df = 19$, $t = 0.5553$, $p = 0.5852$). Therefore, divergence of dominant frequency in syntopic *A. crepitans* and *A. gryllus* resulted from the significantly reduced size of *A. crepitans* and the stronger relationship
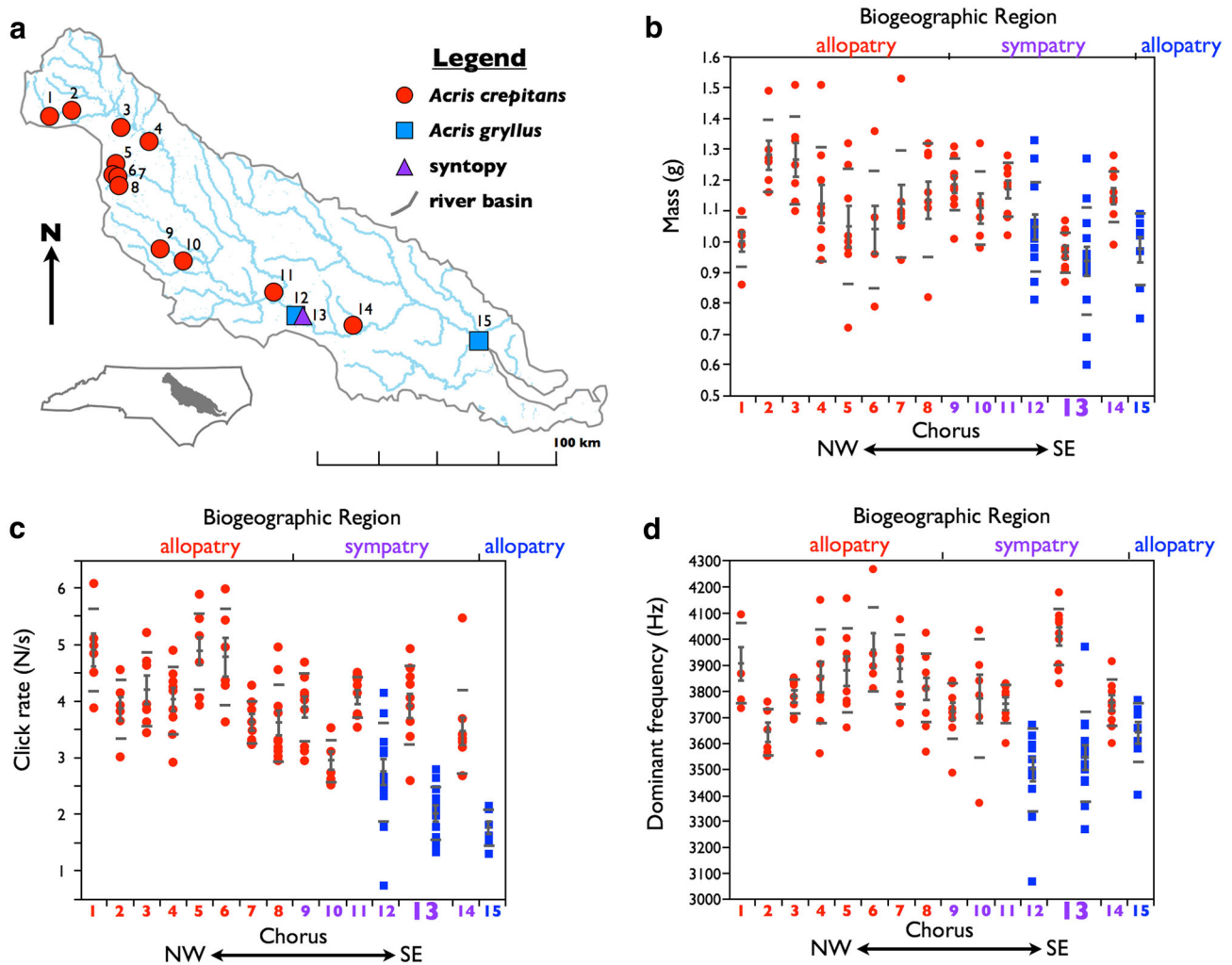
Fig. 3 Variation in the Neuse River Basin among **a** allopatric, sympatric, and syntopic populations of *Acris crepitans* and *A. gryllus* in **b** mass, **c** click rate, and **d** dominant frequency. Sympatry was determined by overlap in distribution of museum specimens in the mid-20th century (Micancin and Mette 2009). Each point in **b**, **c**, and **d** indicates an individual male's mass or mean temperature-adjusted click rate and dominant frequency. *Central* and *inner bars* indicate population means and standard errors. *Outer bars* indicate standard deviations

between body size and dominant frequency in *A. crepitans* than in *A. gryllus*.

Female Preference Tests

In Experiment 1, female *A. gryllus* preferred the natural vocalizations of conspecific males (exact test; odds = 15:1, N = 16, p < 0.001). The latency to choice for the female that chose a heterospecific signal did not differ significantly from the 15 choices for *A. gryllus* (Wilcoxon test; S = 11, N = 16, p = 0.66). In Experiment 2, both species preferred conspecific vocalizations (Exact test; *A. crepitans*, odds = 15:1, N = 16, p < 0.001; *A. gryllus*, odds = 13:3, N = 16, p = 0.021). The synthesized signals were therefore sufficient for species

discrimination. There were no significant differences between females that chose conspecific signals and those that chose heterospecific signals in latency to the first hop (Wilcoxon test; *A. crepitans*, S = 14, N = 16, p = 0.28; *A. gryllus*, S = 28, N = 16, p = 0.79) or latency to choice (Wilcoxon test; *A. crepitans*, S = 9, N = 16, p = 1.0; *A. gryllus*, S = 35, N = 16, p = 0.23). The species did not differ overall in latency to first hop (Wilcoxon test; S = 275, N = 32, p = 0.69) or latency to choice (Wilcoxon test; S = 230, N = 32, p = 0.21).

During Experiment 3 and 4, *A. crepitans* females stopped amplexing at the collection site but enough choices were available for statistical analysis. In Experiment 3, which tested preference for pulse structure of clicks, all *A. crepitans* females preferred the conspecific signal (exact

test; odds = 8:0, N = 8, $p < 0.001$) as did all *A. gryllus* females (exact test; odds = 16:0, N = 16, $p < 0.001$). The species did not differ in latency to first hop (Wilcoxon test; $S = 115$, N = 24, $p = 0.37$) or latency to choice (Wilcoxon test; $S = 117$, N = 24, $p = 0.31$). In Experiment 4, there was no preference by *A. crepitans* females for conspecific signals with the mean dominant frequency over those with a higher dominant frequency (exact test; odds = 8:6, N = 14, $p = 0.791$) and no difference in latency to choice (Wilcoxon test; $S = 49$, N = 14, $p = 0.65$). There was, however, a difference in latency to first hop (Wilcoxon test; $S = 69$, N = 14, $p = 0.002$) between *A. crepitans* females that chose the high-frequency bout (mean = 44 s) and those that chose the low-frequency bout (mean = 204 s). In Experiment 4, *A. gryllus* females had no preference for conspecific signals at the mean dominant frequency over those with a lower dominant frequency, as half the females chose each signal (exact test; odds = 8:8, N = 16, $p = 1.0$). In *A. gryllus*, there was no difference in latency to first hop (Wilcoxon test; $S = 65.5$, N = 16, $p = 0.83$) or latency to choice (Wilcoxon test; $S = 73$, N = 16, $p = 0.64$). The two species did not differ overall in latency to first hop (Wilcoxon test; $S = 244$, N = 30, $p = 0.27$) or latency to choice (Wilcoxon test; $S = 251$, N = 30, $p = 0.16$).

In Experiment 5, female *A. gryllus* demonstrated a tendency for phonotaxis toward a speaker broadcasting a conspecific signal (at 80 dB) in the presence of chorus noise at -3 dB (exact test; odds = 10:2, N = 12, $p = 0.039$) or -1.5 dB (exact test; odds = 12:0, N = 12, $p < 0.001$) but showed no preference for the speaker when the noise was -0 dB (exact test; odds = 5:7, N = 12, $p = 0.774$). In Experiment 6, female *A. gryllus* tended to prefer the speaker broadcasting a conspecific signal over the speaker broadcasting a heterospecific signal when the noise was -3 dB (exact test; odds = 10:2, N = 12, $p = 0.039$) or -1.5 dB (exact test; odds = 11:1, N = 12, $p < 0.001$) but showed no preference when the noise was -0 dB (exact test; odds = 8:4, N = 12, $p = 0.388$). With spherical spreading of vocalizations and a detection threshold of -1.5 dB noise-to-signal, females could detect conspecific males less than 1 m away at chorus levels of 80 dB or more. When chorus noise occasionally decreases to 78 dB, detection distance rises to over 1 m.

Discussion

Acoustic Variation of Males

Mass negatively influenced dominant frequency and had little or no influence on click rate in both species. Temperature had a positive influence on dominant frequency in *A. crepitans* and on click rate in both species, but had no significant effect on dominant frequency in *A. gryllus*. Temperature had less influence on dominant frequency in each species than did mass. Like *A. blanchardi* (McClelland et al. 1996), larger *A. crepitans* and *A. gryllus* males had lower dominant frequencies. Because the size-frequency relationship was steeper and stronger in *A. crepitans*, variation in mass produced larger differences in dominant frequency than in *A. gryllus*. In allopatry, *A. crepitans* was larger than *A. gryllus*. In syntopy, reduction of the size of *A. crepitans* resulted in displacement of dominant frequency away from *A. gryllus*.

Differences in the influence of mass and temperature suggest that sibling *A. crepitans* and *A. gryllus* differ in the proximate limitations on sound production. In *A. blanchardi* as in most anurans (McClelland et al. 1996; Gerhardt and Huber 2002), body size affects the size of vibratory and muscular structures of the larynx; larger males produce lower dominant frequencies and fewer and slower pulses. Temperature generally has more influence on physiologically constrained temporal components and less influence on morphologically constrained dominant frequency in anurans (Gerhardt and Mudry 1980; Ryan and Wilczynski 1991; Gerhardt and Huber 2002). The acoustic responses of *A. crepitans* to mass and temperature are typical of anurans, but *A. gryllus* does not exhibit an influence of temperature on dominant frequency that occurs in *A. crepitans* and many other anurans. Perhaps *A. gryllus* is closer to the physiological limits of nonmuscular vocal control than *A. crepitans*, as suggested by its faster and less variable pulse rate. Ryan and Wilczynski (1991) report a similar pattern of lower variation in dominant frequency than in temporal components in parapatric populations of *A. blanchardi* and likewise propose higher evolutionary lability in physiological constraints than in size constraints on vocalizations.

Difference in the regulation of vocalizations might permit convergence in the click structure of *A. crepitans* and *A. gryllus*. The relationship between larynx or body size and dominant frequency is usually fixed in anurans (Gerhardt and Huber 2002), but male *A. blanchardi* can facultatively decrease dominant frequency (Wagner 1989a, 1992; Burmeister et al. 1999, 2002) as well as click rate and structure (Wagner 1989a, b; Burmeister et al. 2002) to signal aggressiveness to neighboring males. While click structure of *A. gryllus* is static, the variable click structure of sympatric *A. crepitans* might arise from facultative shifts as in *A. blanchardi* (Wagner 1989a, b). Smaller *A. blanchardi* had shorter clicks containing more pulses at a faster pulse rate, while warmer males had shorter clicks containing more pulse groups at a faster pulse rate (Nevo and Capranica 1985; Wagner 1989c; McClelland et al. 1996). If the mechanisms of sound production are similar in *A. crepitans* and *A. blanchardi*, then smaller, warmer *A.*

crepitans might produce more clicks and pulse groups containing more pulses in faster succession. Higher calling temperatures among smaller *A. crepitans* males might explain the resemblance of some pulse groups of *A. crepitans* with the clicks of *A. gryllus* (Fig. 2). Such vocalizations might increase errors of mate recognition or even be an intermediate hybrid phenotype.

Female Preference Tests

Female *A. crepitans* and *A. gryllus* from a syntopic chorus used the consistent differences in temporal structure of clicks for species discrimination. In choices between natural vocalizations, female *A. gryllus* recognized conspecific signals, despite varying differences in dominant frequency and gross temporal structure. In choices between synthesized vocalizations, females of both species preferred conspecific clicks. Several differences separate the clicks of the two species, including duration, pulse number, variation in the interpulse interval, pulse shape, pulse rate, and amplitude profile (Nevo and Capranica 1985). One or more of these differences could have been used by females for selection of conspecific males. Furthermore, because the clicks of *A. crepitans* vary more than those of *A. gryllus*, females might have used variation among clicks, rather than any specific component, for species recognition.

In choices between conspecific signals, syntopic females of both species showed no preferences for dominant frequency. As in many anurans, dominant frequencies of syntopic *A. crepitans* and *A. gryllus* correlate with body size and potentially indicates male quality. However, dominant frequencies of conspecific vocalizations overlap broadly with those of heterospecific vocalizations; *A. crepitans* females using dominant frequency to identify vocalizations of larger conspecific males would encounter acoustic interference from *A. gryllus*. Studies of *A. crepitans* and *A. blanchardi* suggest that spectral and temporal preferences and the tuning of the female ear are population-specific (Capranica et al. 1973; Ryan and Wilczynski 1988; Ryan and Keddy-Hector 1992; Wilczynski et al. 1992; Perrill and Lower 1994; Kime et al. 2004). Smaller females prefer higher dominant frequencies than those preferred by larger females presumably because tuning of their basilar papilla is size-dependent (Keddy-Hector et al. 1992; Ryan and Keddy-Hector 1992), so selection by females is not necessarily directional even within populations. In this study, *A. crepitans* females that chose the higher dominant frequency hopped sooner than those that chose the lower dominant frequency. This result suggests that females searching for a conspecific mate in a syntopic chorus might respond more quickly to smaller males with dominant frequencies displaced from heterospecific males or move more quickly on the edge of a chorus where smaller males

predominate. Based on these results, the range of conspecific detection and discrimination in the middle of choruses (with sustained amplitude of 80 dB or more) at Merchants Millpond is less than 1 m. Distances between vocalizing males were often less than 50 cm, so a female would often be able to detect more than 10 calling males of the two species combined. In these conditions, errors of species recognition might occur as females move through a chorus. We occasionally observed heterospecific amplexus in syntopy. Future work could indicate whether allotopic (but sympatric) females express preferences for dominant frequency, unlike the syntopic females in this study.

Conclusions

Our investigation revealed that *A. crepitans* and *A. gryllus* diverged significantly in dominant frequency at two widely separated syntopic sites in North Carolina. Nevertheless, three factors prevent us from concluding that RCD has occurred in this case. First, because of the widespread decline of *A. gryllus* discovered during this study (Micancin and Mette 2009), our sample of syntopic choruses is small. We examined syntopic vocalizations and body size at only two choruses: one in the region of contracting sympatry in central North Carolina and the other in an adjacent area of northeastern North Carolina with stable sympatry and frequent syntopy (Micancin et al. 2012). Second, we did not determine whether reduction in the size of *A. crepitans* and CD in dominant frequency resulted from the presence of *A. gryllus*. Influences other than interspecific competition for signal space might have caused syntopic *A. crepitans* to be smaller. Genetic drift along the edge of the range of *A. crepitans* might result in lower mean mass in syntopy. Natural selection for smaller body size might occur at wetlands with low desiccation pressure (Nevo 1973; Blem et al. 1978; Nevo and Capranica 1985) or abundant small prey. Climate change is suspected of causing declines in body size, particularly in ectotherms and at range limits (Gardner et al. 2011; Bickford et al. 2011; McCauley and Mabry 2011). Third, we obtained evidence for a pattern but not a process of RCD (Pfennig and Pfennig 2009). The process of RCD might be studied in *Acris* species by observing differences in body size and acoustic interference before and after secondary contact. Because *Acris* species appear to have short generation times and low survivorship (Gray and Brown 2005; McCallum et al. 2011), it might be possible to detect rapid evolution of body size of *A. crepitans* along a shifting front of contact with *A. gryllus*. The widespread extirpation of *A. gryllus* and long distances between choruses in most of the area of sympatry in North Carolina precluded such an analysis. The study site at Merchants

Millpond, in contrast, is within a region with many choruses of both species in allopatry, sympatry, and syntopy (Micancin et al. 2012). Future study of RCD in sympatric *Acris* can focus on this area.

CD might explain high regional diversity (Pfennig and Pfennig 2009) such as the exceptional richness of amphibians in the southeastern United States (Duellman and Sweet 1999; Rissler and Smith 2010) especially in the family *Hylidae* (Smith et al. 2005). Our study and others in the southeastern United States show the importance of evaluating phenotypic variation in syntopy and allotopy in studies of RCD in anurans. In *P. feriarum*, pooled data from sympatric allotopy and syntopy confirmed RCD in the pulse rate and pulse number of vocalizations in sympatry with *P. nigrita* and *P. brimleyi* (Lemmon 2009). In *H. chrysoscelis,* females in sympatry, but not necessarily syntopy, with *H. versicolor* still preferred conspecific pulse rate more strongly than distantly allopatric females (Gerhardt 1994). In *H. cinerea*, females in sympatry with *H. gratiosa* preferred conspecific dominant frequency more strongly than distantly allopatric females (Höbel and Gerhardt 2003). However, in other components of vocalizations, RCD was only detected through comparisons of allotopic and syntopic populations within sympatry. Despite the preference of sympatric *H. cinerea* females, differences in dominant frequency and calling height of males were only detected in syntopy and allotopy (Höbel and Gerhardt 2003). In this study, the displacement of dominant frequency in syntopic *A. crepitans* was not detectable in allotopic populations in sympatry. *Pseudacris* and *Acris* are sibling genera (Faivovich et al. 2005) in which indications of declines of the Coastal Plain species (*P. nigrita* and *A. gryllus*) in parapatry or sympatry with the Piedmont species (*P. feriarum* and *A. crepitans*) have been found during studies of RCD (Lemmon 2009; Micancin and Mette 2009). The disappearance of *A. gryllus* has substantially reduced the area of sympatry with *A. crepitans* and the number of syntopic sites where RCD most likely occurs. The disappearance of *A. gryllus* from syntopy might also eliminate selection for smaller body size, thus contributing to differences between syntopic and allotopic *A. crepitans*. Worldwide declines of amphibians (IUCN et al. 2008) potentially result in other reductions in species interactions that could obscure the mechanisms of amphibian diversity. As a result of these potentially rapid and cryptic changes in the interactions of sibling species, studies of RCD in syntopy and allotopy potentially reveal more about the behavioral interactions of sibling species than can studies in allopatry and sympatry.

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