REPRODUCTIVE CHARACTER DISPLACEMENT IN THE ACOUSTIC COMMUNICATION SYSTEM OF GREEN TREE FROGS (*HYLA CINEREA*)

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Abstract.—Interactions between species can affect the evolution of their sexual signals, receiver selectivity, or both. One commonly expected outcome is reproductive character displacement, whereby adverse consequences of mismating select for greater differentiation of communication systems in areas of sympatry than in areas of allopatry. We found evidence of reproductive character displacement in the acoustic communication system of green tree frogs (*Hyla cinerea*). The strength of female preferences for the spectral properties of calls that distinguish conspecific calls from those of a closely related congener, *H. gratiosa*, was greater in areas of sympatry with *H. gratiosa* than in areas of allopatry. We also found subtle differences in advertisement calls and in the heights of male calling perches when we restricted our comparisons to localities in which *H. gratiosa* was also breeding (syntopy) with localities where this species was absent. *Hyla cinerea* and *H. gratiosa* show only weak genetic incompatibility, but the calls representative of interspecific hybrids were unattractive to females of both parental species. Hybrids might also be at an ecological disadvantage because of different habitat preferences of the two taxa. Thus, selection against production of less fit or less attractive hybrid or backcross offspring are probably the main causes responsible for the differences documented in this paper.

Key words.—Advertisement-call structure, call perches, female choice, preference function, preference strength, reinforcement, reproductive character displacement.

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Communication plays a major role in the lives of most organisms, and is especially important for mate choice. The selective consequences of such choice is thought to be a major contributor to the evolution of both signals and receiver selectivity. Although the mutual selective pressures exerted by senders and receivers of the same species is a constant factor, reproductive interactions between different species can also play a role in the evolution of communication. Such interspecific effects are usually couched in terms of reproductive character displacement, that is, divergence in the signals, receiver selectivity, or other aspects of reproductive ecology (time and place of breeding) of closely related species in areas of sympatry compared to areas of allopatry (Howard 1993). In all examples of which we are aware, such a pattern of divergence is probably best explained by selection to avoid mismatings that have adverse consequences, such as the production of inviable, sterile, ecologically unfit, or sexually unattractive offspring.

Reproductive character displacement has long been considered a rare phenomenon, in part because numerous studies have failed to find evidence for it (e.g., Asquith et al. 1988; Doherty and Howard 1996; for reviews, see Walker 1974; Butlin 1987), and also because only few examples demonstrating it have been generally accepted (e.g., Littlejohn 1965; Fouquette 1975; Waage 1979). However, cases of reproductive character displacement may often go undetected due to gene flow or a bias towards focusing solely on sexual signals. As pointed out by Waage (1979), there may be little selective pressure to increase signal differences in sympatry if the signals of the two taxa can be discrim-

inated by at least some receivers at the time sympatry is established. Rather, selection may act primarily on variation in receiver selectivity (Waage 1979), especially when receivers are females, which generally have more to lose from mating mistakes than do males (Gerhardt 1994). In some systems, shifts in female preferences may subsequently select for additional divergence in male secondary sexual traits that facilitate species recognition (e.g., Sætre et al. 1997). In fact, geographic variation in receiver selectivity has been assessed in a growing number of recent studies that have provided various degrees of empirical support for reproductive character displacement (Gerhardt 1994, 1999; Noor 1995; Marquez and Bosch 1997; Sætre et al. 1997; Rundle and Schluter 1998; Pfennig 2000; Marshall and Cooley 2000; Gabor and Ryan 2001).

One of the potential processes leading to the pattern of reproductive character displacement, namely reinforcement, has also been a subject of controversy, and even its definition has become contentious (Butlin 1987; Howard 1993; Gerhardt and Huber 2002). Here we follow Howard (1993) and view reinforcement as the evolution of prezygotic isolation barriers in response to selection against hybridization without regard to the degree of genetic compatibility of the interacting taxa. Although earlier theoretical work suggested that reinforcement is unlikely to occur (e.g., Paterson 1982; Butlin 1987), more recent studies showed that reinforcement can theoretically occur in a much wider range of conditions than previously supposed (e.g., Liou and Price 1994; Kelly and Noor 1995; Servedio and Kirkpatrick 1997; Kirkpatrick and Servedio 1999; Cain et al. 1999; Kirkpatrick 2000, 2001).

Frogs have long been a favorite subject for the study of acoustic communication. The potential for interspecific interaction is high because mixed-species breeding aggrega-

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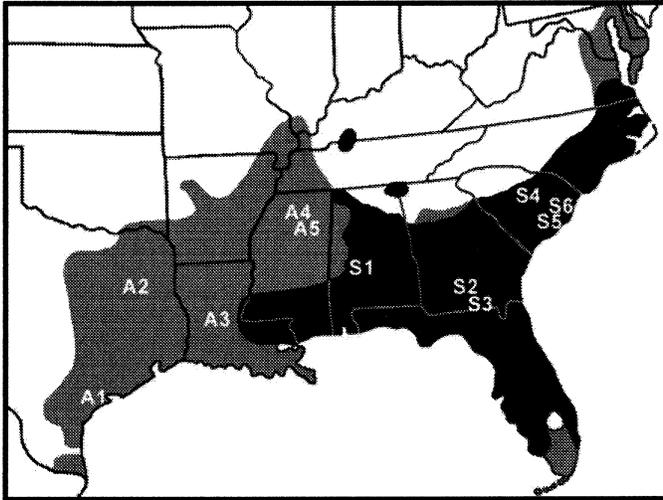
FIG. 1. Geographic distributions of *Hyla cinerea* (shaded area) and its congener *H. gratiosa* (black area), and the locations of the 11 study sites. The study ponds were situated at: Rob and Bessie Welder Wildlife Refuge (A1), San Patricio Co., Texas; Richland Creek Wildlife Management Area (A2), Freestone Co., Texas; Beechwood State Fish Hatchery (A3), Rapides Co., Louisiana; Private John Allen National Fish Hatchery (A4) and Tombigbee State Park (A5), both Lee Co., Mississippi; Marion State Fish Hatchery (S1), Perry Co., Alabama; Paradise Public Fishing Area (S2), Berrien Co., Georgia; Grand Bay Wildlife Management Area (S3), Loudes Co., Georgia; Southland Fisheries (S4), Calhoun Co., South Carolina; Francis Marion National Forest (S5) and Hobcaw Barony (S6), both Georgetown Co., South Carolina. Sites S2 and S4 were syntopic sites that had both species calling at the time the study was conducted. Ranges based on Conant and Collins (1998) maps.

tions are common (review, by Duellman and Trueb 1986). Moreover, mate choice is strongly dependent on selective phonotaxis of the female to the advertisement call of the male, which is typically indiscriminate in attempting to clasp other frogs of comparable size that touch or move nearby (Gerhardt 2001). The strong reliance on acoustic signals also has experimental advantages. Advertisement calls are easily recorded and analyzed, and playback experiments that can identify the acoustic properties used by females to make subtle mate-choice decisions have successfully been conducted with many species (for review, see Gerhardt 2001). We studied geographic variation in the reproductive behavior of green tree frogs (*Hyla cinerea*) to assess the hypothesis that these traits have been affected by sympatry with barking tree frogs (*Hyla gratiosa*). These two species, which are highly genetically compatible (Mecham 1960), are considered sister species (Cocroft and Ryan 1995). They also have all the necessary features of a suitable system to study reproductive character displacement as laid out by Howard (1993): (1) the traits under consideration (advertisement call, female call preferences) serve as reproductive barriers (Oldham and Gerhardt 1975); (2) the members of the species pair occur in sympatry and one (*H. cinerea*) or both also occur in allopatry (Fig. 1); (3) heterospecific matings, interspecific hybrids, and backcrosses occur frequently in mixed-species choruses, especially in human-disturbed breeding sites (Gerhardt 1974a; Gerhardt

et al. 1980; Mecham 1960; Lamb and Avise 1986; Schlefer et al. 1986); and (4) there is evidence of selection against hybrids (Gerhardt 1974a; Schlefer et al. 1986).

We studied three reproductive traits and behaviors (acoustic properties of the advertisement call; male calling perches, and female phonotactic selectivity), and demonstrate that the geographic pattern of variation in *H. cinerea* shows greater divergence in some of those isolating traits in areas of sympatry than areas of allopatry. Thus, that there is reproductive character displacement. Our results suggest that episodes of selection within sympatry may have caused present-day differences between frogs in sympatric and allopatric areas, and that selection against production of less attractive hybrid offspring is probably the main factor driving the process.

MATERIAL AND METHODS

Study Populations

We studied *Hyla cinerea* at eleven localities. Based on range maps in Conant and Collins (1998), five localities were in areas of allopatry (Texas, Louisiana, and Mississippi) and six localities were in areas of sympatry (Alabama, Georgia, and South Carolina) (Fig. 1). Ponds at Paradise Public Fishing Area (S2) and Southland Fisheries (S4) were syntopic sites with mixed breeding choruses of *H. cinerea* and *H. gratiosa*; we did not find *H. gratiosa* at the other localities in the area of sympatry.

Advertisement Calls

The advertisement calls of *H. cinerea* are relatively short (100–200 ms), and repeated about 80 times per minute. The spectral composition of the calls is characterized by two frequency peaks: one peak consists of a single component in the low-frequency range (LFP: 0.65–1.5 kHz); and the other peak usually consists of two components of about the same relative amplitude in the high-frequency range (HFP: 2.5–4.0 kHz) (Oldham and Gerhardt 1975). The advertisement calls of *H. gratiosa* also have a bimodal spectrum, but the frequencies of the two peaks are significantly lower than in the calls of *H. cinerea* (for sonograms of both species, see Oldham and Gerhardt 1975).

To investigate whether the presence of *H. gratiosa* has affected the sexual signals of *H. cinerea*, we recorded the calls of 378 *H. cinerea* males from four allopatric and six sympatric populations during the 1999–2001 breeding seasons. We located calling males at night with a headlamp and recorded their calls using a Sony WM-D6C (1999) or a Sony TC-D5M tape recorder (2000 and 2001) with a Sennheiser K3-ME88 microphone. After each recording we measured the air temperature at the location of the frog using a Miller and Weber Cloacal Thermometer. With the exception of frogs from the Grand Bay (Loudes Co., GA) Wildlife Management Area, we measured the snout-vent length (SVL) of each male to the nearest 0.1 mm with a caliper and weighed each male to the nearest 0.1g with a 30-g Pesola spring balance.

We digitized (22 kHz sampling rate) and analyzed the recorded calls using CoolEdit96 (Syntrillium Software Corporation, Phoenix, AZ) on a portable computer. We measured the duration of the call, the call rate (number of calls per

minute), the waveform periodicity of the call (estimated by measuring the time of 10 cycles of the repeating waveform), and the frequencies of each of the two spectral peaks. For each male we then calculated mean values based on the analysis of 10 calls. To comply with requirements of normality we square-root transformed values of all call properties before performing statistical tests.

Ambient temperature and body size can affect call parameters in frogs. We therefore performed linear regression analyses of call properties on temperature, and adjusted the datasets for which a significant effect of temperature was found (call rate and call duration) to a common temperature of 25°C. This analysis also revealed that body size (represented by SVL) was significantly and negatively correlated with call dominant frequency (represented by the LFP measurements) in seven of nine test populations (r^2-values ranged from 0.23 to 0.57; P-values were all less than 0.003 in A1, A3, S1–2, S4–6; r^2-values were less than 0.09 in A2 and A4). However, because we were interested in the call-frequency differences that would be experienced by females when choosing a mate in their respective population, we did not correct for size effects on call frequency when comparing populations. Because of the influence of body size on call parameters and the geographic division of our allopatric study sites in the west and the sympatric study sites in the east, we did test for clinal variation in body size (represented by SVL) that could confound possible selection on calling song with selection on body size. This analysis revealed no effects of geographic location on body size (regression of SVL on longitude: $r^2 = 0.00$, F = 0.56, $P = 0.45$).

Because call dominant frequency plays an important role in mate choice and species isolation in *H. cinerea* (Gerhardt 1982), we first focused on that character. We compared the frequencies of advertisement calls between sympatric and allopatric populations by calculating a nested ANOVA where study sites were nested within sympatry and allopatry. We also examined the possibility that a further enhancement of the species differences only occurs during episodes of direct behavioral interaction between the two species, by performing another ANOVA in which we nested study sites within syntopic sites and sites in which *H. gratiosa* was absent (allopatric and sympatric).

Because other call characteristic besides dominant frequency may be influenced by interspecific interactions, we also performed a principal-component analysis where we included all five measured call parameters. We visualize the results of the principal component analysis by plotting the population means of PC 1 and PC 2 against each other. We performed nested ANOVAs with PC 1 and PC 2 in the way described above for call dominant frequency. We adopt the traditional $P < 0.05$ criterion for statistical significance. Some authorities (e.g., Rice 1989) recommend adjusting for multiple comparisons by lowering this criterion to 0.025; thus some of our results in which P-values were between 0.025 and 0.05 could be interpreted as marginally significant.

Playback Experiments: Tests of Selective Phonotaxis in Females

Playback system.—Acoustic stimuli were generated from 16-bit digital files created by custom-designed software. We used commercial D/A hardware and interface software (CoolEdit96, Syntrillium Software Corp. Phoenix, AZ) and an IBM-compatible computer (266 MHz Pentium) to output the sound from these files at a sampling rate of 44 kHz. Signals were amplified and played back with a pair of loudspeakers (RadioShack Optimus XTS 40) situated just outside a rectangular playback arena (2 m × 1 m). The floor of the arena was a plywood board, and the sides consisted of 50-cm high wooden frames that supported a light black cloth to shield the movements of the researcher and the position of the loudspeakers from the frogs. The speakers were placed 2 m apart, in the middle of each of the two short sides of the arena, facing each other. We used measurements of the frequency response of the playback system to adjust (during software generation of the digital files) the relative amplitude of the frequency components to within ±2 dB at the release point of the females. The sound pressure level (dB SPL: re 20 μPa, fast root-mean-square [RMS]) of each stimulus was adjusted using a Lutron SL-4001 (Lutron Electronic Enterprise, Taipei, Taiwan) sound level meter prior to each test.

Test procedure.—We tested females from four allopatric and four sympatric localities. Each female was found in amplexus and kept with the amplexing male until shortly before testing. Some females were tested within a few hours of capture; others were held in a refrigerator at 6°C and tested the following night after acclimatizing them to ambient temperature (23–27°C). All females were tested in a two-speaker (choice) playback design. We placed females individually in an acoustically transparent container (small round hardware cloth cage, 10-cm diameter) midway between the loudspeakers. Once the alternative stimuli had been played back for at least five repetitions, we remotely removed the lid of the release box so that the female could move freely and observed her behavioral response under dim red light. We scored a positive response once a female touched or moved within 10 cm of the speaker after having showed phonotactic orientation movements, such as head and body scanning (Rheinlaender et al. 1979). Females were tested only once with a given stimulus pair at the same SPL, and there was a time-out period of at least 5 min between different tests. Previous research with *H. cinerea* found no evidence of carryover effects of one test to another when the two tests had a stimulus in common (Gerhardt 1981a). Within a series of choice tests, the stimuli were alternated between loudspeakers to guard against any side biases. Most females were released at the site of capture within one day of being tested.

We tried to test at least 10 females with each combination of stimuli, and tested an additional 5–10 females whenever time and availability of females permitted us to do so. Since two-tailed binomial tests are not strictly valid because we did not fix sample size ahead of time, we compute lower than 95%-confidence limits (or more accurately, Bayesian credible intervals, assuming a uniform prior probability distribution; see Gerhardt 1992) from standard tables (Burstein 1971), and judge a preference to be significant if the lower confidence limit (P) is above 0.6.

Preference strength

Playback experiments (Oldham and Gerhardt 1975; Gerhardt 1974a,b, 1981b) have shown that females of both spe-

cies prefer conspecific signals to heterospecific or hybrid signals. However, females respond to heterospecific and hybrid calls in ''no-choice'' situations (Gerhardt 1974a; Oldham and Gerhardt 1975), and by varying call intensity female preferences for calls of conspecific males to those of sympatric congeners can be reversed (e.g., Gerhardt 1982, 1987). If mismating has negative consequences, sympatric females should choose conspecific calls even when confounding variables such as intensity favor heterospecific calls. We therefore performed playbacks that tested the preference strength of females. Here the SPL of the *H. gratiosa* stimulus was held constant at 85 dB SPL, and the SPL of the standard conspecific stimulus was lowered in 3 dB steps. Preference strength was defined as the greatest difference in SPL at which a significant proportion of females chose the conspecific stimulus. In this experiment we used synthetic calls that had the same duration (160 ms) and envelope characteristics (rise time: 25 ms, inverse exponential; fall time: 50 ms, inverse exponential), but differed in their spectral composition: *H. cinerea* (0.9 + 2.7 + 3.0 kHz); *H. gratiosa* (0.5 + 1.5 + 2.0 kHz). The call rate of both signals was equalized at 75 calls $\times$ min^{-1}, with the calls exactly alternating with each other.

Estimates of preference functions

A more subtle effect of sympatry with *H. gratiosa* might be a shift in the form of the preference function for signals that differ in their spectral properties. Finding that females from sympatric areas show a shift in preference functions towards higher frequencies, or asymmetric functions, in which mean values are more strongly preferred to lower frequencies than to higher frequencies, would constitute evidence for reproductive character displacement.

The temporal characteristics (call duration, rise-fall characteristics, and call rate) of the alternatives used in this experiment were the same as described above. Each stimulus had three components of equal amplitude, with a fixed pattern of frequency differences ([lowest frequency peak = LFP] + [3 $\times$ LFP + 0.3 kHz] + [3 $\times$ LFP + 0.6 kHz]). The frequency difference between the two higher-frequency components was always 300 Hz. The resulting 300-Hz periodicity (beats) are typical of the advertisement calls of *H. cinerea*, and differences in this temporal property can affect the relative attractiveness of synthetic calls (Gerhardt 1978). The SPL of the alternatives was equalized at 85 dB SPL at the release point of the female.

Discrimination against calls representative of interspecific hybrids

We tested the relative attractiveness of hybrid advertisement signals to female green tree frogs by conducting playback trials where we gave females a choice between the standard conspecific call (0.9 + 2.7 + 3.0 kHz) and a synthetic call with a spectrum representative of a *H. cinerea* $\times$ *H. gratiosa* hybrid (0.65 + 1.95 + 2.60 kHz; for call data of field-recorded hybrids, see Gerhardt et al. 1980). The temporal parameters of the two alternatives were the same as described above, and the stimuli were equalized at 85 dB SPL at the release point of the female.

Calling Perches

Males of *H. cinerea* usually call from elevated perches in or around ponds, whereas *H. gratiosa* males typically call while floating in the water (Mecham 1960). Calling males and satellites of *H. cinerea* attempt to mate with other frogs that they detect in their vicinity (Perrill et al. 1978). Lamb and Avise (1986) suggested that this satellite behavior may contribute to the directional hybridization (their allozyme data indicated that most females in mismated pairs were *H. gratiosa*) observed at their study site. By using higher calling perches in localities were *H. gratiosa* is also calling, males of *H. cinerea* might reduce the chances of inadvertently contacting and mating with a female of *H. gratiosa*.

We measured perch height to the nearest 1 cm in male *H. cinerea* from five allopatric and six sympatric populations during the 1999–2001 breeding seasons. We used the water level as baseline for measuring perch height (e.g., a male sitting on the ground of a steep shoreline would be recorded not as sitting 0 cm above ground but X cm above water level). Additionally we noted the highest available perch within a 50-cm radius around the frogs to make sure that all study sites had similar proportions of elevated call perches available. Some study sites, especially some fish hatcheries, had large numbers of frogs that were calling from ponds with mowed edges or plastic pond liners. These sites (A3, A4, S1) were not included in our comparisons because of the lack of elevated call perches.

We compared the call-perch heights of *H. cinerea* males from different populations by calculating nested ANOVAs. In one comparison, study site was nested within allopatric and sympatric distribution. In the other comparison, study site was nested in syntopic sites and sites where *H. gratiosa* was absent (allopatric and sympatric).

RESULTS

Geographic Variation in Advertisement Calls

We did not find the predicted pattern of higher dominant call frequencies in sympatry (nested ANOVA: $F_{1,9} = 2.9$, $P < 0.10$) (Fig. 2). However, males of *H. cinerea* from syntopic ponds did have significantly higher-frequency calls than did males that called from ponds without *H. gratiosa* (nested ANOVA: $F_{1,9} = 8.91$, $0.01 < P < 0.025$).

The principal component analysis yielded two principal components with eigenvalues above 1.0. The first principal component, which mainly represented spectral properties (waveform periodicity, LFP and HFP) had an eigenvalue of 2.52 and accounted for 50.5% of the total variance. The second principal component, which mainly represented call duration, had an eigenvalue of 1.37 and accounted for 27.4% of the variance. Plotting the first against the second principal component showed a separation of allopatric and sympatric populations; furthermore, the two syntopic populations grouped farthest from the allopatric group (Fig. 3). PC 1 was also significantly different between allopatric and sympatric populations (nested ANOVA: $F_{1,9} = 19.5$, $P < 0.001$), as well as between syntopic populations and those that did not have *H. gratiosa* present (nested ANOVA: $F_{1,9} = 12.9$, $P <$
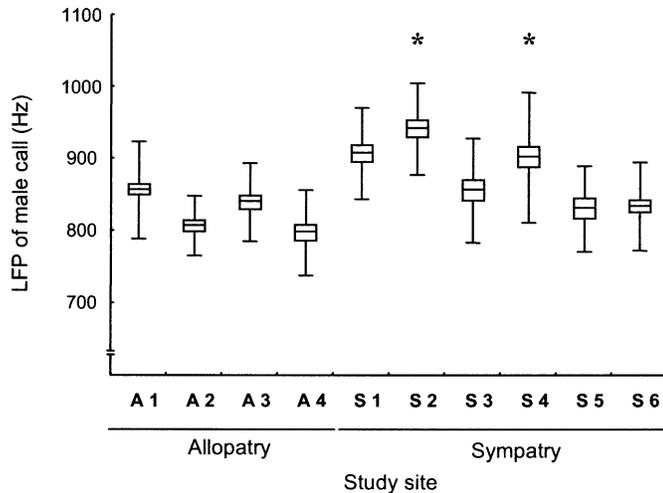
FIG. 2. Dominant frequency of the advertisement calls of male *Hyla cinerea* from four allopatric (A1-A4) and six sympatric (S1-S6) populations. Note the nonclinal nature of the variation in call dominant frequency, and the jump towards higher values at the border from allopatry to sympatry. Although there was no consistent effect of geographic sympatry, males from syntopic ponds (S2 and S4, indicated by asterisks) had higher frequency calls than did males that called from ponds without *H. gratiosa*. Shown are average values, the SE (box) and the SD (whiskers) for each population. See Figure 1 for details about the location of the study populations.

FIG. 3. Plots of principal component values (± SE) for four allopatric (open symbols) and six sympatric (filled symbols) localities. Call characteristics associated with spectral parameters (LFP, HFP, waveform periodicity) loaded mainly in PC1; call duration loaded mainly in PC 2. Lower PC 1 values indicate that the frequency components of the calls were higher, lower PC 2 values indicate that the duration of the calls were shorter. Allopatric populations group on the right, sympatric populations on the left. Syntopic populations (S2 and S4, filled squares) group furthest left. See Figure 1 for details on location of the study populations.

0.001), while PC 2 was not ($F_{1,9} = 3.45$, $P > 0.10$; $F_{1,9} = 2.68$, $P > 0.10$, respectively).

Female Preferences

Preference strength for conspecific stimuli

Females from sympatric localities maintained their preference for the conspecific signal over a larger range of intensity differences than did females from allopatric localities, including differences exceeding 20 dB (Fig. 4). As expected there was a tradeoff between conspecific signal characteristics and signal amplitude: with increasing amplitude differences favoring the heterospecific stimulus, the proportion of females that chose this stimulus instead of the conspecific standard call increased. However, this effect was much less pronounced in samples of females from sympatry. At amplitude differences of −6 dB or less all of the sympatric females chose the standard call; the first mistakes occurred when the difference was increased to −9 dB. By comparison, at an amplitude difference of −6 dB, substantial proportions of females from three of the four allopatric populations choose the heterospecific stimulus. Females from Beechwood SFH (A3) were an exception; preference strength was comparable or even slightly better than that of females from some sympatric localities.

Frequency preference functions

Females of *H. cinerea* from sympatric localities did not show the predicted shift of preference away from low-frequency calls (Fig. 5). In all test populations females strongly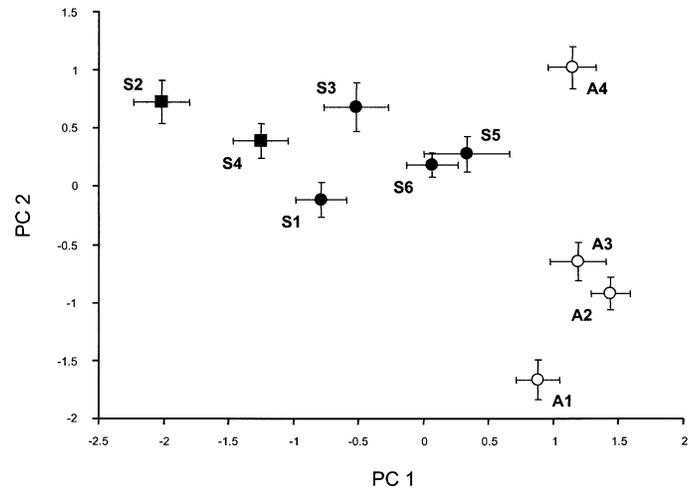 rejected high frequency stimuli, choosing the 0.9 kHz alternative to alternatives of 1.2 kHz and 1.1 kHz; many females also preferred the standard call to a 1.0-kHz alternative. Low frequency alternatives (0.6 kHz, 0.7 kHz, and 0.8 kHz) were as attractive as the standard stimulus (0.9 kHz) or even more attractive, suggesting directional selection towards low frequency calls. Only in Georgia did females discriminate against the lowest alternative (0.6 kHz), giving rise to an overall stabilizing selection on male call frequency in that population.

Discrimination against hybrid signals

We gave 107 females (20 from A1; 15 from S1; 14 from A2, A4, and S6; and 10 from A3, S2, and S4) a choice between synthetic calls representative of conspecific and hybrid calls. Females from all four populations in sympatric areas showed a significant preference for the conspecific over the hybrid stimulus. The proportions of females choosing the conspecific stimulus (and the lower confidence limits) were 0.87 (0.64), 1.0 (0.74), 0.9 (0.61), and 0.86 (0.61). Females from two of the four populations in allopatry also preferred the conspecific over the hybrid signal [A1: 0.95 (0.78); A3: 0.9 (0.61)], whereas the two other allopatric populations did not show a significant preference [A2: 0.71 (0.46); A4: 0.64 (0.39)].

Variation in Call-Perch Height

Males of *H. cinerea* used a wide variety of elevated calling perches, but also called from ground level close to the edge of ponds or from floating vegetation. Males only rarely called while sitting in shallow water.

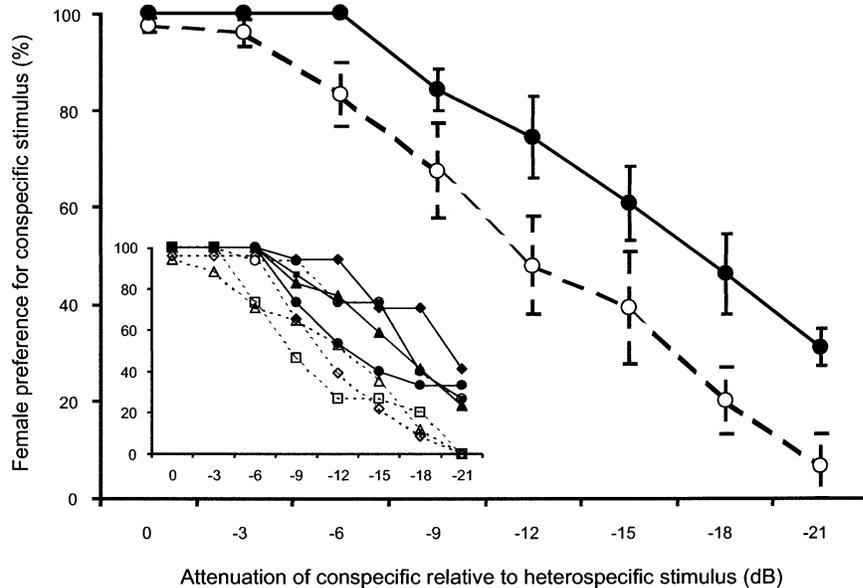When comparing the eight study sites used in the analysis

FIG. 4. Preference strength for the conspecific call of females of *H. cinerea*. A series of stimuli pairs with increasing amplitude differences favoring the heterospecific call were presented to *H. cinerea* females. Preference strength of allopatric (open symbols) and sympatric females (filled symbols) were calculated as the mean percentage (± SE) of females choosing the conspecific over the heterospecific stimulus in the respective stimulus pairs. The smaller inset shows the same dataset, but individual populations are shown separately (Allopatry: open diamond A1, open square A2, open circle A3, open triangle A4; Sympatry: filled circle S1, filled diamond S2, filled triangle S4, filled square S6). Even though females from Beechwood SFH (A3) showed a preference strength that was comparable or even slightly better than that of females from some sympatric localities, the overall pattern shows greater preference strength in sympatry over allopatry. We tested a total of 134 females in this experiment (23 from A1, 15 from A2, 15 from A3 17 from A4, 15 from S1, 17 from S2, 17 from S6, and 15 from S8).

(Fig. 6), we did not find a significant difference between perch heights in allopatric versus sympatric sites (nested ANOVA: $F_{1,7} = 1.91$, $P < 0.10$). However, males from syntopic populations called from significantly higher perches than did males from ponds that had no calling *H. gratiosa* present (nested ANOVA: $F_{1,7} = 7.19$, $0.025 < P < 0.05$).

DISCUSSION

Sympatry with *Hyla gratiosa* appears to have influenced the mate recognition system of *H. cinerea*, resulting in reproductive character displacement in female preference strength for signals with conspecific spectral properties vis-à-vis calls representative of *H. gratiosa*. There were also subtle but statistically significant differences in male advertisement calls and marginally significant differences in male calling perches (if alpha level is adjusted for multiple comparisons).

As we expected, the differences between frogs from allopatry and sympatry were more pronounced on the receiver (female) side of the communication system. In the experiments that estimated preference strength some individual females from allopatric localities chose the heterospecific stimulus when its amplitude was only 6 dB higher than that of the conspecific signal. In small and modest-sized choruses, a conspecific call is likely to be audible above chorus background if it is only 6 dB lower than that of a nearby heterospecific call. The difference in selectivity between females from sympatry and allopatry in our experiments involving a 6 dB difference in call levels would thus be likely to translate into a difference in the probability of choosing to approach

a heterospecific male in nature. This may not be true for the much larger differences in amplitude (≥ 12 dB) we tested because the less intense conspecific calls would probably be masked (Gerhardt and Klump 1988). Yet we found striking differences in the selectivity of females from sympatry and allopatry under these test conditions, suggesting an evolutionary effect of selection on the auditory system. Similarly enhanced receiver selectivity in sympatry has also been found in two species of fish (Rundle and Schluter 1998; Gabor and Ryan 2001), gray tree frogs (Gerhardt 1994), Darwin's finches (Ratcliffe and Grant 1983), *Drosophila* (Noor 1995) and mosquitoes (McLain and Rai 1986).

Contrary to our predictions we did not find a general shift of the female preference functions towards higher-frequency values (away from those of *H. gratiosa*) in sympatric populations. Rather, low frequency alternatives (0.6 kHz) were preferred by females in some localities. This attractiveness is even more surprising given that the hybrid call (0.65 kHz) and the heterospecific call (0.5 kHz) used in other experiments had quite similar low-frequency peaks, but were nevertheless rejected by females in most populations. Because the high-frequency peaks of all three stimuli overlapped, one possibility is that the difference in the waveform periodicity, which was held constant at the preferred 0.3 Hz-periodicity in the series of conspecific calls but not in the hybrid and heterospecific calls, maintained the relative attractiveness of the 0.6 kHz alternative.

Geographic differences in male calls and calling behavior are more difficult to interpret. Significant advertisement call differences between allopatric and sympatric localities
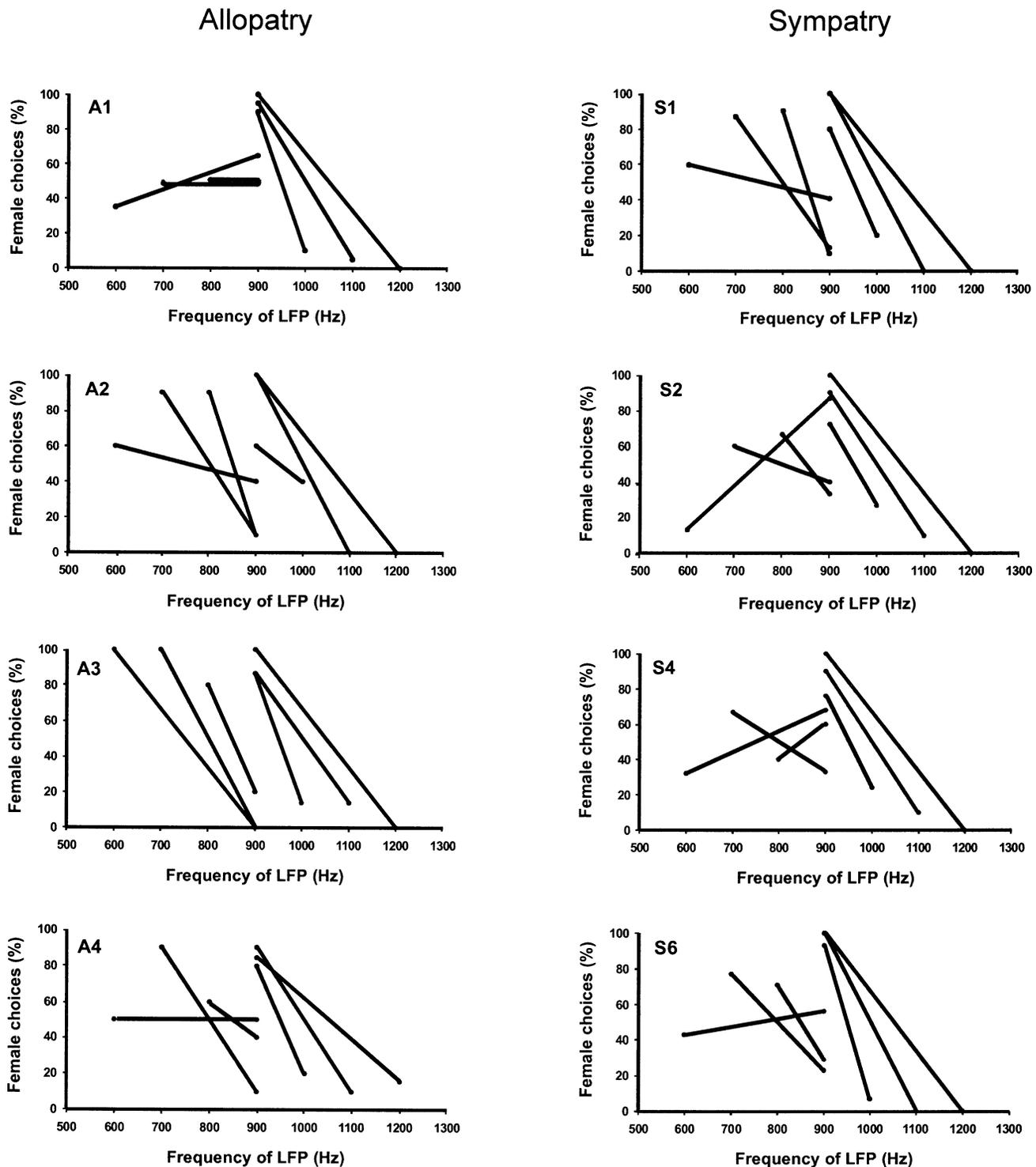
FIG. 5.   Frequency preferences of allopatric (left column) and sympatric (right column) females of *Hyla cinerea*. Shown is the percentage of females choosing each of the two alternatives. Points connected with a line indicate the pair of stimuli tested against each other. We tested 264 females from four allopatric populations (45 females from A1, 19 from A2, 46 from A3, and 34 from A4) and four sympatric populations (35 females from S1, 32 from S2, 36 from S6, and 17 from S8). Most of the females were tested with a subset of the six possible combinations. The choice of frequencies in the standard call (LFP = 0.9 kHz) was based on previous studies of populations of *H. cinerea* in eastern Georgia, for which the preference functions and the average advertisement call properties were already available (Gerhardt 1987). These values, in turn, were chosen because the mean low-frequency peak in the calls of males in these populations was about 0.9 kHz. We initially lacked sufficient data to reliably estimate the spectral properties of the calls of males in the localities considered in this paper. Subsequently, after such data became available, we selected two populations, one in which the mean carrier frequency was close to 0.9 kHz (S4) and another in which the mean carrier frequency was just over 0.8 kHz (S6). There we repeated part of the tests, but this time we used the 0.8 kHz stimulus as the standard call against which we tested other alternatives. Using standard calls whose frequencies were significantly (> 1 SD) lower or higher than those in the calls of the average male in the population did not influence the shape of female preference functions (unpubl. data).
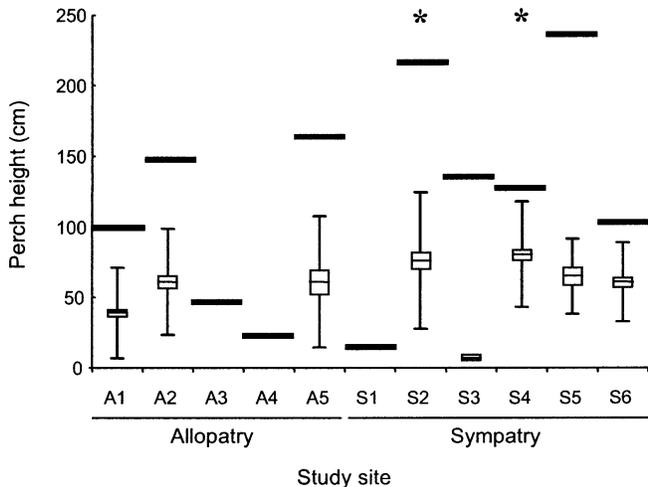
FIG. 6. Average value of maximum perch heights that were available to calling males in the 11 study populations (dark lines), and average perch heights (boxplots) used by male *Hyla cinerea*. Sites A3, A4, and S1 had almost no elevated call perches available and were therefore eliminated from further comparisons. The boxplots show the average values, the SE (box), and the SD (whiskers) of the perches used by male *Hyla cinerea* in the remaining three allopatric (A1, A2, A5) and five sympatric (S2–S6) study populations. Perch heights were significantly different among the eight sites used in the analysis. There was no significant difference between perch heights in allopatric versus sympatric sites, but males from syntopic populations (S2 and S4, indicated by asterisks) called from significantly higher perches than males from ponds that had no calling *H. gratiosa* present. See Figure 1 for details on location of the study populations.

only emerged when we considered combinations of several call characters in a principal component analysis. Interestingly it was PC 1, in which mainly spectral parameters loaded, that showed significant differences between allopatric and sympatric sites, whereas PC 2, in which mainly temporal parameters loaded, did not. Given that the calls of *H. cinerea* and *H. gratiosa* differ mainly in their frequency composition, this result may reflect the importance of spectral call parameters in the mate choice of *H. cinerea*. Also, we did find the predicted shifts towards higher values of call dominant frequency and calling perch height when we compared these traits in frogs from syntopic populations with those in localities where *H. gratiosa* was absent. Thus, two important conclusions concerning male traits emerged: (1) Spectral components of the advertisement call are under stronger selection during interspecific interactions than temporal parameters, and (2) the divergence is strongest at syntopic breeding sites, suggesting that direct behavioral interactions may enhance the process. It would be interesting to learn if the shift to higher calling perches within sympatric areas is facultative.

Criteria for Demonstrating Reproductive Character Displacement

Studies claiming to present evidence for reproductive character displacement and reinforcement have met considerable skepticism in the past (Paterson 1982; Butlin 1987). Some of this criticism is valid because of failures to address

important criteria necessary to demonstrate the expected pattern and process. In the present study we paid special attention to these concerns. First, we verified that the observed displacement has not occurred for other, especially ecological reasons, by testing and rejecting the hypothesis that clinal variation in body size (which is linked to call dominant frequency in this species) may explain the geographic variation in male advertisement call. Furthermore, we simultaneously evaluated geographic variation in both signal characters and receiver preferences when studying reproductive character displacement, which, except for Higgie et al. (2000) and Gerhardt et al. (unpubl. ms; see review by Gerhardt 1999) has not been done in other studies. Our results, however, underscore the importance of doing so because the lack of signal variation does not necessarily imply a similar lack in variation in receiver selectivity (see Kelly and Noor 1996; Noor 1999). Many instances of reproductive character displacement may have gone undetected simply due to a bias in the characters sampled. Another important point raised by Gerhardt and Huber (2002), and underscored by data on geographic variation in reproductive character displacement by Gabor and Ryan (2001), is the necessity to evaluate as many populations as possible in both sympatry and allopatry. Variation in behavior occurs within sympatry and allopatry, and testing only a few populations may obscure the overall pattern (Gabor and Ryan 2001). We tested female preferences in four allopatric and four sympatric populations, and our sample size for evaluating male calls and calling perches was even larger. We found considerable variation in behavior within allopatry and within sympatry in female preference strength, and also found one allopatric population in which female selectivity was comparable to that of sympatric populations. However, this population was located closer to the zone of sympatry than the other allopatric populations, which suggests that there may have been gene flow between this population and nearby sympatric ones, or that this populations was formerly sympatric and that *H. gratiosa* has recently become extinct in that area.

Selection against Hybrids

When mismatings occur in nature and there is selection against the resulting hybrids, reinforcement is almost certainly the process responsible for the pattern of reproductive character displacement (Howard 1993). Abundant evidence exists for mismatings, hybrids, and backcrosses between *H. cinerea* and *H. gratiosa* (Gerhardt 1974a; Gerhardt et al. 1980; Mecham 1960; Lamb and Avise 1986; Schlefer et al. 1986). Evidence for selection against the resulting hybrids is not as straightforward. *Hyla cinerea* and *H. gratiosa* are more genetically compatible than most other combinations of North American hylids: several combinations of mixed parental, hybrid × parental, and hybrid × hybrid crosses yielded similarly high fertilization rates, and the development of the cleaved eggs was normal (Mecham 1965). Only some hybrid crosses (F_1 female + *H. cinerea* male) resulted in about 10% reduction in egg cleavage (Mecham 1960). However, lower than expected percentages of hybrids and backcrosses in an introgressing population in Alabama suggest that

low-level genetic incompatibility (Lamb and Avise 1986; Schlefer et al. 1986). Hybrids of both sexes may also be at an ecological disadvantage (Schlefer et al. 1986). Barking tree frogs are most common in xeric habitats, and are more desiccation-tolerant than green tree frogs, which typically stay close to permanent bodies of water (Farrell and MacMahon 1969). Hybrids and backcrosses have intermediate body proportions (Gerhardt et al. 1980) and are less desiccation tolerant than *H. gratiosa* (Layne and Romano, unpubl data; in Schlefer at al. 1986) and thus might be at a disadvantage in the typical nonbreeding habitats of both parental forms. Heterospecific matings may therefore result in fewer offspring that may have lower fitness, and natural selection is expected to act on senders and receivers to reduce the frequency of interspecific matings.

Mate choice during interspecific interactions in sympatry can also lead to a pattern expected of reproductive character displacement (Coyne and Orr 1989; Noor 1999; Servedio 2001). The fitness of otherwise fully viable and fertile hybrids can be severely reduced if they have lower mating success (Coyne and Orr 1989), and this form of indirect sexual selection is also likely to contribute to the divergence of communication behavior of the parental species. Several cases of sexual selection against hybrids have been reported (Davies et al. 1997; Noor 1997; Wells and Henry 1998; Vamosi and Schluter 1999; Naisbit et al. 2001), as well as cases of reduced mating intensity for certain hybrids (Buckley 1969; Noor 1997; Davies et al. 1997; Vamosi and Schluter 1999). Our playback trials clearly show the relative unattractiveness of synthetic hybrid signals in all sympatric and some allopatric localities, where female green tree frogs strongly rejected advertisement signals representative of interspecific hybrids. Gerhardt (1974a) also found that in playback experiments females of both parental species preferred prerecorded conspecific calls to those of two prerecorded, putative hybrids. Thus, hybrid males are likely to have reduced mating success, unless hybrid females preferred hybrid males, as has been reported for hybrids of certain parrots (Buckley 1969). Whether this is the case has not yet been tested experimentally, but it seems unlikely because at another site hybrids mated with parentals and hybrids in the stochastic manner predicted by the proportion of parentals and hybrids present at the breeding pond (Schlefer et al. 1986).

Conclusion

We have demonstrated that the features of the communication system of *H. cinerea* that are used in species recognition are more accentuated where they live in sympatry with their sister species *H. gratiosa*, and that the resulting pattern is consistent with the pattern of reproductive character displacement. Selection against the production of less ecologically fit and sexually unattractive hybrids is likely to have driven selection for increased mate discrimination, which suggests reinforcement. We are aware that difficulties in interpreting the data arise from the fact that we are ignorant about the precontact status of the interacting taxa, the extent to which their interactions might have contributed to speciation, or the extent to which their ranges of distribution might

have changed. These problems, however, apply to nearly all studies of reproductive character displacement. Regardless of whether or not interspecific interactions contribute to divergence in communication systems prior to or during the establishment of stable sympatry, we emphasize that the presence of another species with structurally similar signals and with which hybridization occurs and has adverse effects is likely to be a consistent constraint on the direction of change in signals, receiver preferences, or both.

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