

Reconstructing asymmetrical reproductive character displacement in a periodical cicada contact zone

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Keywords:

asymmetrical character displacement;
contact zone;
cross-mating experiments;
hybridization;
mate choice;
Magicicada;
reproductive character displacement.

Abstract

Selection against costly reproductive interactions can lead to reproductive character displacement (RCD). We use information from patterns of displacement and inferences about predisplacement character states to investigate causes of RCD in periodical cicadas. The 13-year periodical cicada *Magicicada neotreddecim* exhibits RCD and strong reproductive isolation in sympatry with a closely related 13-year species, *Magicicada treddecim*. Displacement is asymmetrical, because no corresponding pattern of character displacement exists within *M. treddecim*. Results from playback and hybridization experiments strongly suggest that sexual interactions between members of these species were possible at initial contact. Given these patterns, we evaluate potential sources of selection for displacement. One possible source is 'acoustical interference', or mate-location inefficiencies caused by the presence of heterospecifics. Acoustical interference combined with the species-specificity of song pitch and preference appears to predict the observed asymmetrical pattern of RCD in *Magicicada*. However, acoustical interference does not appear to be a complete explanation for displacement in *Magicicada*, because our experiments suggest a significant potential for direct sexual interactions between these species before displacement. Another possible source of selection for displacement is hybrid failure. We evaluate the attractiveness of inferred hybrid mating signals, and we examine the viability of hybrid eggs. Neither of these shows strong evidence of hybrid inferiority. We conclude by presenting a model of hybrid failure related to life cycle differences in *Magicicada*.

Introduction

Reproductive character displacement (RCD) is a biogeographical pattern in which sexual character differences of species (or incipient species) are accentuated in response to secondary contact (Brown & Wilson, 1956). Explanations for RCD fall into two broad categories (Howard, 1993; Noor, 1999; Lemmon *et al.*, 2004). 'Reproductive interference' hypotheses posit that reproductive traits diverge in response to interference or competition, in much the same way that resource competition may lead to ecological character displacement (Grant, 1972; Losos, 2000); for examples (see Pfennig, 2000; Hettyey & Pearman, 2003). In such cases, the involvement of

reproductive characteristics is incidental, because no direct sexual contact is required, and the interacting taxa need not be closely related or even capable of hybridizing. For example, 'acoustical interference' hypotheses suggest that competition for signal space may cause sexual signals that are confusingly similar or that are mutually obscuring to diverge until they no longer interfere with each other (Gerhardt & Huber, 2002; Wollerman & Wiley, 2002).

In contrast, the second broad category of explanations explicitly invoke sexual contact and the costliness of heterospecific sexual interactions (see Butlin, 1987, 1989). Examples of RCD that are attributable to hybrid costs are of particular interest for understanding processes of speciation. An emerging consensus from experimental studies (Hostert, 1997; Noor, 1999; Morgan-Richards & Wallis, 2003; Pfennig, 2003), model simulations (Sanderson, 1989; Liou & Price, 1994; Servedio & Kirkpatrick, 1997; Sadedin & Littlejohn,

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2003) and literature reviews (Littlejohn, 1981; Howard, 1993; Rice & Hostert, 1993; Noor, 1999; Servedio & Noor, 2003) is that gene flow between populations decreases the likelihood that displacement will occur. Species or incipient species in secondary contact are in '... a race between the enhancement of prezygotic isolation and the fusion of populations' (Coyne & Orr, 2004; also see Dobzhansky, 1940; Wilson, 1965; Walker, 1974; Hewitt, 1988; Harrison, 1990). Which of these fates prevails depends in large part on the nature of initial barriers to gene flow, because genetic exchange has an autocatalytic quality that tends to erode such barriers. The challenge in developing explanations for any given example of RCD is that displacement is a process that erases its own causes, and thus, contemporary, post-displacement populations may not provide accurate information about the causes of displacement. Complicating matters, species meeting in a contact zone may be displaced to different degrees; such asymmetries suggest precontact differences in the nature or intensity of selection on the species involved (Grant, 1972; Howard, 1993; Pfennig & Murphy, 2003; J.R. Cooley & D.C. Marshall unpublished). Reconstructing the conditions and interactions prevailing at the formation of any given contact zone is the only way to tease apart the roles of hybridization costs, gene flow and competitive interactions in promoting RCD.

The periodical cicadas of eastern North America (*Magicicada* spp.) exhibit an asymmetrical pattern of RCD (Marshall & Cooley, 2000) in a contact zone that likely formed within the last 10 000 years (Simon *et al.*, 2000). These insects are notable for their long life cycles (13 or 17 years), dense periodical emergences (Marlatt, 1923; Williams & Simon, 1995) and lek-like mating systems (Alexander, 1975; Cooley & Marshall, 2004) involving a complex acoustical and tactile courtship sequence (Alexander, 1968, 1975; Dunning *et al.*, 1979; Cooley & Marshall, 2001). Within a broad contact zone between the two 13-year species, *M. neotreddecim* and *M. treddecim* (Fig. 1, Marshall & Cooley, 2000; Cooley *et al.*, 2001), *M. neotreddecim* male call pitch and female pitch preferences are displaced upward ($+ \approx 0.4$ kHz, Marshall & Cooley, 2000; Cooley *et al.*, 2001), but displacement is asymmetrical, since *M. treddecim* shows no comparable change. Female *M. neotreddecim* in the contact zone discriminate against male *M. treddecim* (Marshall & Cooley, 2000), and genetic data suggest that strong reproductive isolation characterizes the contact zone today (Simon *et al.*, 2000; Cooley *et al.*, 2001). *M. neotreddecim* likely originated as an allochronic isolate of 17-year *M. septendecim*, from which it is indistinguishable in morphological characteristics and known mitochondrial and allozyme markers (Martin & Simon, 1990; Marshall & Cooley,

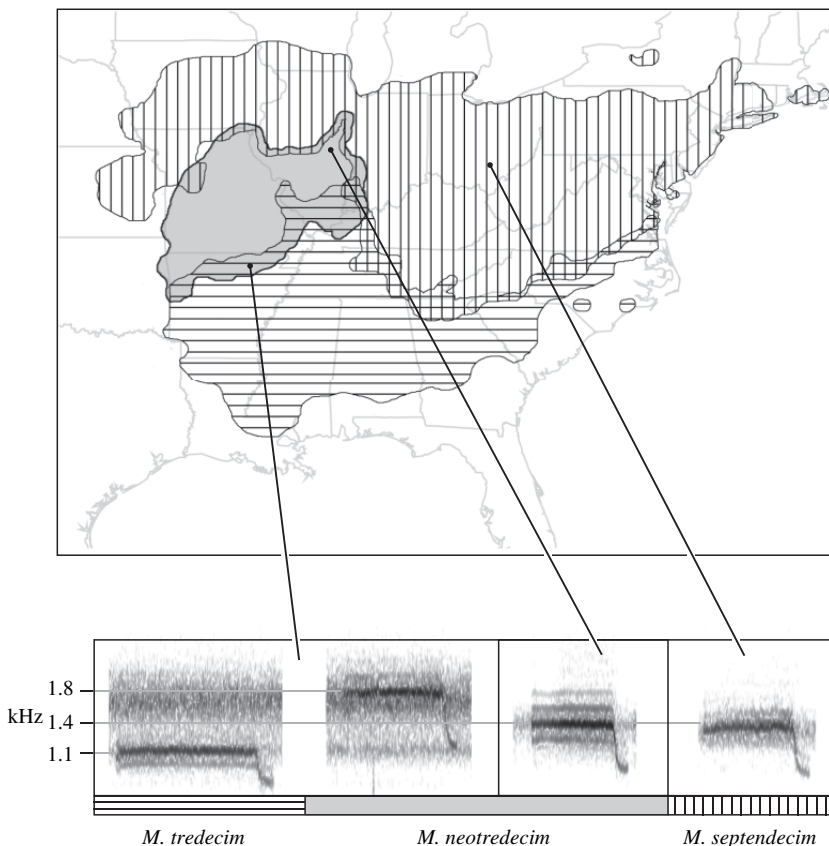


Fig. 1 The distribution of *M. neotreddecim* (shaded), *M. treddecim* (horizontal lines) and *M. septendecim* (vertical lines), in the eastern United States, with appended sonograms [graphs of song pitch (kHz) vs. time], for typical *M. septendecim* and *M. treddecim*, and for displaced and undisplaced *M. neotreddecim*. The three species are largely parapatric. Reprinted with permission from Cooley *et al.* (2001); copyright AIBS.

2000; Simon *et al.*, 2000; Cooley *et al.*, 2001; J.R. Cooley, D.C. Marshall & C. Simon, unpublished). Thus, the *Magicicada* -decim species group of periodical cicadas provides a unique opportunity for reconstructing the initial conditions within a contact zone, because the characteristics of undisplaced *M. neotreddecim* are directly observable in *Magicicada septendecim*.

In this study, we infer the nature and extent of heterospecific sexual interactions at the formation of the *Magicicada* contact zone and, using this information, we evaluate the relative merits of 'hybrid cost' and 'acoustical interference' hypotheses for the observed pattern of RCD. Specifically, we characterized contemporary patterns of song and preference variation in all *M. -decim* species, including allopatric and sympatric populations of *M. neotreddecim*. Using this information, combined with the results of playback experiments, we demonstrated that the signals of these species overlapped at the formation of the contact zone, and that there were likely substantial opportunities for interbreeding. Given this possibility, we investigated some potential fitness costs of interbreeding – the hatching success of hybrid eggs and the attractiveness of intermediate ('hybrid-like') signals to potential mates from either parental species. We conclude that the acoustical interference hypothesis cannot fully account for RCD in *Magicicada*, and that some post-hatching fitness costs may be ultimately responsible.

Materials and methods

General methods

The *Magicicada* 'decim' species – *M. neotreddecim*, *M. septendecim* and *M. treddecim* – have 13, 17, and 13-year life cycles, respectively (Marshall & Cooley, 2000). The *M. neotreddecim/M. septendecim* clade diverged from *M. treddecim*

over 1mya (Martin & Simon, 1988, 1990). Recently, most likely within the past 10 000 years, 13-year *M. neotreddecim* diverged from 17-year *M. septendecim* and became synchronized with *M. treddecim*; *M. neotreddecim* and *M. treddecim* today overlap in a zone running through western Kentucky, southern Indiana, southern Illinois, northern Missouri and northern Arkansas (Fig. 1; Marshall & Cooley, 2000). Thus, the *M. neotreddecim/M. treddecim* contact zone represents secondary contact between two lineages that were once allochronically and geographically isolated.

Different regional populations of periodical cicadas emerge on different schedules and are termed 'broods', each designated by a Roman numeral reflecting life cycle and order of appearance (Marlatt, 1923). With the exception of the pitch and preference differences associated with the pattern of RCD in *M. neotreddecim*, there are no known behavioural distinctions among broods within either the *M. treddecim* or the *M. neotreddecim/M. septendecim* lineages. Because of the brood structure, periodical cicadas of one or both life cycles are available for study for the majority of years in any 17-year period.

Evaluating contemporary reproductive isolation

The acoustic signals and female preferences of *M. neotreddecim* are displaced where this species is sympatric with *M. treddecim*, and playback experiments to captive females demonstrate that these signal differences are sufficient to isolate these species (Marshall & Cooley, 2000). We verified assortative mating in the field by comparing the frequency of mixed-species mating observed with the frequency expected under random mating. Within the *M. treddecim/M. neotreddecim* overlap zone, we preserved naturally occurring mating pairs in absolute ETOH (18 pairs, 1998 Brood XIX, Harold Alexander Wildlife Management Area; 25 pairs, 2002 Brood XXIII, Giant City State Park; study and collection locations listed in Table 1).

Table 1 Research site locations 1998–2002. Unmated female *Magicicada* were collected from these sites for use in experiments, and chorus recordings were made on the dates indicated.

| Year | Brood | State | County | Location | Latitude | Longitude | Recording date |
|---|-------|-------|-------------|---------------------|----------|-----------|----------------|
| <i>M. septendecim</i> | | | | | | | |
| 2001 | VII | NY | Onondaga | Onondaga nation | 42°58' | –76°10' | 6/12/01 |
| 2002 | VIII | WV | Hancock | Tomlinson run SP | 40°33' | –80°35' | 5/31/02 |
| 2003 | IX | NC | Burke | Private property | 36°22' | –81°07' | 5/20/03 |
| Allopatric <i>M. treddecim</i> | | | | | | | |
| 2001 | XXII | MS | Wilkinson | Homochitto NF | 31°18' | –91°08' | 5/9/01 |
| 2002 | XXIII | MS | Franklin | Homochitto NF | 31°26' | –90°49' | 5/6/02 |
| Allopatric (undisplaced) <i>M. neotreddecim</i> | | | | | | | |
| 1998 | XIX | IL | Piatt | Allerton park | 39°40' | –88°40' | 5/30/98 |
| 2002 | XXIII | IL | DeWitt | Weldon springs SP | 42°07' | –88°56' | 6/11/02 |
| Contact zone (displaced) <i>M. neotreddecim</i> and <i>M. treddecim</i> | | | | | | | |
| 1998 | XIX | AR | Sharp | H. E. Alexander WMA | 36°15' | –91°28' | 5/23/98 |
| 2002 | XXIII | IL | Jackson | Giant city SP | 37°36' | –89°11' | 5/25/02 |
| 2002 | XXIII | IN | Vanderburgh | Univ. S. Indiana | 37°58' | –87°40' | 5/28/02 |

At both locations we also used chorus recordings to estimate the relative abundance of the two species (see Marshall & Cooley, 2000). Species identity of mating individuals was determined on the basis of a 350 bp fragment of domain III of the mitochondrial SSU (12S) rRNA gene (Table 2), using methods described in Cooley *et al.* (2001). Polymorphism in this mitochondrial gene region is congruent with song pitch, female pitch preferences and abdomen colour differences that distinguish *M. tredecim* from the *M. neotreddecim*/*M. septendecim* clade (Cooley *et al.*, 2001). We used a Monte-Carlo resampling algorithm to estimate the number of mixed-species mating pairs expected in each population under random mating. On the basis of our mating pair collection data (see *Results* below) we modelled the Brood XIX mating pair collection as 16 : 1 *M. neotreddecim* : *M. tredecim* with a total of 17 females, and the XXIII overlap zone as 10 : 15 *M. neotreddecim* : *M. tredecim* with a total of 25 females. Both populations were modelled as having unlimited numbers of males in the same proportions, because individual female mating decisions are effectively independent due to the large numbers of individuals present and the likely male-biased operational sex ratio (Cooley & Marshall, 2001, 2004). Thus, the null expectation of random mating is that, for any given female, the probability of mating with a male of either species is proportional to the representation of that species in the population. For each of 10 000 iterations, the Monte-Carlo algorithm randomly assigned each female a mate under this null model and tabulated the number of simulated cross-species mating pairs, generating a cumulative frequency distribution that we used to identify one-tailed critical values (95th and 99th percentiles) for the frequency of heterospecific matings expected by chance.

Contemporary signal and preference variation in *M. -decim*

The pattern of RCD in *M. neotreddecim* was described on the basis of signal variation within Brood XIX (Marshall & Cooley, 2000). So that we could more fully characterize the nature and extent of displacement in this species, we constructed 'preference histograms' describing female mate acceptance criteria in several populations of each *M. -decim* species, using playback experiments modelled after those in Marshall & Cooley (2000). Individual *M. -decim* calls consist of a 1–3 s steady-pitch and nearly pure-tone 'main element' followed by a quieter 0.5 s frequency 'downslur' that terminates approximately 0.5 kHz lower; this downslur is a timing cue for receptive

females, who respond with a 'wing-flick signal' (Cooley & Marshall, 2001). In each playback session, 2–6 individually marked, mature, unmated females were placed in a screened test chamber and presented with pure-tone, synthetic *M. -decim* calls (see Cooley & Marshall, 2001) ranging in dominant pitch from 0.773 to 2.35 kHz. To each group of females, we played a randomly ordered series of the synthetic calls with each call repeated three times in sequence, and we noted any female wing-flick signals (indicative of female acceptance; see above). For each test female, a weighted average pitch preference (P) was calculated, where t = number of test pitches and n = number of responses to pitch i :

$$P = \frac{\sum_{i=1}^t (\text{kHz call}_i)(n_i)}{\sum_{i=1}^t n_i}$$

For each population the weighted average preferences of individual females were compiled into a 'preference histogram' representing the relative attractiveness of different call pitches. We chose histograms of individual preferences, because this method represents all tested females equally. An alternative method, constructing an aggregate histogram of all pooled females responses, produces histograms of similar shape, but the representation of females is unequal, since more responsive females have greater impact on the overall shape (see also Murphy & Gerhardt, 2000). Our preference histograms are similar in concept to the 'preference functions' of Ritchie (1996), except that Ritchie used cubic splines to construct his curves.

To verify that female *M. -decim* call pitch preferences match local conspecific male call phenotypes, we analyzed a recording of the natural *Magicedada* chorus from each study site. The choruses were recorded at a distance to minimize the possibility that any single male would dominate; thus, each recording is a sample of 100s (perhaps 1000s) of males. The recordings were digitized (48 kHz sampling rate) and ≈ 60 s sound samples from each were prepared by excising noisy portions and filtering out sound below 0.5 kHz and above 3 kHz. We used these samples to derive a power spectrum and a dominant pitch for each chorus. No temperature corrections were made, since *M. -decim* song pitch and female responses are relatively invariant over a wide range of temperatures (Marshall & Cooley, 2000; Cooley & Marshall, 2001). Female pitch preferences and male call phenotypes were compared using a Wilcoxon Signed Ranks test.

Estimates of premating isolation at contact

Based on our characterizations of contemporary signal and preference variation within *M. -decim* (see above) we used two proxies for predisplacement *M. neotreddecim* to make inferences about interactions at the formation of the *M. neotreddecim*/*M. tredecim* contact zone. First, we conducted playback experiments (described above) using

Table 2 12 s primer sequences (5'-3').

| | |
|------------|---------------------------|
| SR-J-14232 | taagagcgcacggcgatgtg |
| SR-J-14233 | aagagcgcacggcgatgtg |
| SR-N-14588 | aaactaggattagataccctattat |
| SR-N-14757 | ggacaaaattcgtgccagcag |

M. neotreddecim from contemporary allopatric populations. Caveats that apply to this strategy are: (1) northern Brood XXIII populations of *M. neotreddecim* may not be sufficiently isolated to rule out influences of the contact zone, while (2) Brood XIX *M. neotreddecim* and *M. treddecim* populations far from the contact zone are from such different latitudes that experiments using them may be confounded by adult age (i.e., maturity) differences. Our second strategy was to use the closely related 17-year species *M. septendecim* as a proxy for pre-displacement *M. neotreddecim*. The sexual behaviours and call characteristics of *M. septendecim* and allopatric *M. neotreddecim* are indistinguishable (Marshall & Cooley, 2000); thus, while using *M. septendecim* as a proxy in behavioural experiments has the risk that there is some unknown difference, it has the advantage of allowing us to avoid influences of the contact zone as well as potentially confounding maturity differences.

In order to assess the likelihood of hybridization at the formation of the contact zone, we conducted cross-mating experiments between *M. treddecim* and *M. septendecim* (proxy for pre-displacement *M. neotreddecim*). During the 2002 co-emergence of 13-year Brood XXIII and 17-year Brood VIII (which do not overlap), we collected teneral *M. treddecim* from within the overlap zone and *M. septendecim* from a WV population far from any 13-year cicadas (Table 1). After allowing the cicadas to mature, we placed 10 or 11 (see Table 3) individually marked females and 20 individually marked males of each species simultaneously in a large screened cage. We monitored the cage for 3 h, separating all mating pairs as they formed and returning separated cicadas to different parts of the cage; thus, some females were involved in the initiation of more than one mating pair. We conducted two replicates of this experiment, using different individuals each time.

Because the numbers of matings in the replicates were small, we combined them for analysis. Considering only first mating initiations, we used a Monte-Carlo resampling algorithm to model the null expectation of random mating in these experiments. Since some females never mated and thus may have been damaged or disturbed by handling, the experimental population was modelled as being equivalent to the actual number of females that mated (see Table 3), not the total of 20 females placed in the cage. The algorithm randomly assigned each female in the population a mate of either species and then

tabulated the total number of heterospecific pairings. After repeating this process 10 000 times, the algorithm generated 95th and 99th percentiles for the null expectation of heterospecific matings.

Although, females do not normally mate more than once (Cooley & Marshall, 2004), our experimental protocol permitted females to initiate multiple matings; thus, we could examine the consistency of their mating decisions. We modified the resampling algorithm to generate the null expectation for the number of mixed-species matings expected from females initiating more than four matings (fewer would not allow meaningful evaluation). As above, the algorithm resampled the data 10 000 times to construct one-sided 95th and 99th percentiles. We then compared the mating histories of the experimental females to these percentiles. We also examined female mating decisions for general biases using a Sign test, and we examined the consistency of individual females using a Wald-Wolfowitz runs test.

Assessing hybrid hatching success

Post-zygotic incompatibilities, manifested as egg hatching failure, are another possible explanation for RCD in *Magicalcada*. White (1973) provided evidence of hybrid failure between *M. septendecim* and *M. cassini*, in the form of reduced hatching success of hybrid vs. purebred eggs (65 vs. 76% hatching success). Because of the possibility of hatching failure, we compared the hatching rates of purebred *M. neotreddecim* eggs vs. hybrid *M. neotreddecim*/*M. treddecim* eggs (note, however, that *M. neotreddecim* and *M. treddecim* are more closely related than *M. septendecim* and *M. cassini*; Williams & Simon, 1995). To collect purebred eggs, we captured mated female *M. neotreddecim* from allopatric populations and placed them in a cage with branches for oviposition. To collect hybrid eggs, we collected unmated female *M. neotreddecim* from within the contact zone, cross-mated them with *M. treddecim* males, and allowed them to oviposit. After the eggs hatched (approximately 6 weeks), we dissected a subset of the eggnests under a microscope, dissecting each half of each eggnest separately (*Magicalcada* eggnests are bifurcated). Hatched eggs leave behind an empty translucent 'shell' or chorion that has a distinctive anterior split (Marlatt, 1923; White, 1973). Failed eggs are yellow, red, or otherwise darkened, filled with crystalline, waxy, or other deposits, and their breaches differ from the

Table 3 First matings in female free-choice experiments. The number of individuals included was: Replicate A: 10 females *M. septendecim*, 11 females *M. treddecim* and 20 males of both species; Replicate B: 10 females of both species and 20 males of both species. See text for explanation.

| | | Replicate A | | Replicate B | | Total | |
|--------|--------------------|------------------|--------------------|------------------|--------------------|------------------|--------------------|
| | | <i>treddecim</i> | <i>septendecim</i> | <i>treddecim</i> | <i>septendecim</i> | <i>treddecim</i> | <i>septendecim</i> |
| Female | <i>Treddecim</i> | 5 | 7 | 2 | 1 | 7 | 8 |
| Male | <i>Septendecim</i> | 6 | 3 | 0 | 3 | 6 | 6 |

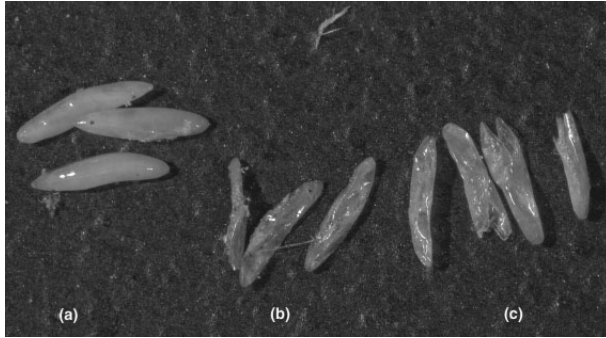


Fig. 2 Photographs of *Magicicada* eggs. (a) Normal, unhatched *Magicicada* eggs; (b) dead, unhatched eggs; and (c) normal, hatched eggs.

stereotypical anterior splits of hatched eggs (Fig. 2; White, 1980; White & Lloyd, 1981; Stoetzel & Russell, 1991). Because our experiment was designed to assess the proportion of eggs that fail for intrinsic reasons, we counted only the eggnest halves in which at least one egg hatched and we excluded eggnest halves in which mortality was due to obvious extrinsic factors such as parasitism or predation (indicated by the presence of frass or foreign insect parts in the eggnest), or due to an extensive plant wounding response that may have trapped or damaged eggs.

Assessing acoustical interference

A female periodical cicada's ability to perceive and respond to a call's terminal downslur is a prerequisite to mating (Cooley & Marshall, 2001). Within a loud chorus of conspecifics, since most call and chorus sound energy is at the main call pitch, not the downslur frequencies, downslurs are not masked by the chorus, and females are able to perceive them. But while the allopatric calls of *M. neotreddecim* are higher-pitched than those of *M. treddecim*, the call pitches overlap in such a way that lower-pitched chorus of *M. treddecim* could acoustically mask *M. neotreddecim* downslurs, reducing females' ability to perceive males and respond. The 'acoustical interference' hypothesis makes two specific predictions about interactions between *M. treddecim* and undisplaced *M. neotreddecim*. First, since undisplaced *M. neotreddecim* calls contain little sound energy at frequencies typical of *M. treddecim* calls and contain no sound energy at the lowest frequencies of *M. treddecim* downslurs, the acoustical interference hypothesis predicts a specific, asymmetrical pattern of RCD, with *M. neotreddecim* showing more displacement than *M. treddecim*. No other explanation for the asymmetry of displacement in specifically predicts greater displacement in *M. neotreddecim* than in *M. treddecim* rather than vice-versa. Second, the acoustical interference hypothesis predicts that for *M. neotreddecim*, an *M. treddecim* background chorus should shift female preferences upwards, favouring calls whose downslurs

end just above the pitch of the interfering chorus (see Wollerman & Wiley, 2002).

Our surveys of male signals/female preferences in allopatry and in the contact zone (see above) test the first prediction of the acoustical interference hypothesis. We tested the second prediction of this hypothesis during the 2003 emergence of Brood IX in NC using a series of playback experiments conducted under three different background chorus conditions: (1) no background chorus present; (2) conspecific background chorus present; and (3) heterospecific (*M. treddecim*) background chorus present. In these experiments, we used *M. septendecim* females as a proxy for predisplacement *M. neotreddecim*. We used a bandpass-filtered (removing all frequencies except 0.65–2.0 kHz) *M. septendecim* chorus recording from the 2003 emergence of Brood IX as our simulated conspecific background chorus (pitch ≈ 1.4 kHz); we used the same recording to create a simulated chorus of *M. treddecim* by shifting the pitch to one typical of *M. treddecim* (≈ 1.11 kHz). By playing these choruses in the background during playback experiments, we could uncover the effects of different background chorus conditions on female acceptance behaviours.

We conducted these playbacks in a quiet, open field away from any natural choruses. For each experimental trial, we placed five individually marked mature female *M. septendecim* in a test cage, randomized the order of three different background treatments (no background, unmodified *M. septendecim* background and pitch-shifted *M. septendecim* background), and played synthetic male calls (see above) in random order. The background was set to ≈ 70 db, and the simulated calls to ≈ 68 –71 db. In all trials one or more females responded during each of the playback series, indicating that conditions remained at least minimally appropriate for testing throughout. We used Kruskal-Wallis one-way ANOVAs, with mean weighted average pitch preference as the dependent variables, to examine the effect of background treatment on each of the song pitches. A Kolmogorov-Smirnov test of the shapes of the female preference curves was used to corroborate shifts in preferences under different background treatments.

Results

Evaluating contemporary reproductive isolation

On the basis of 12 s mtDNA haplotype, no mixed *M. neotreddecim*/*M. treddecim* mating pairs were collected in the 1998 Brood XIX and 2002 Brood XXIII overlap zones. Of the individuals collected in the Harold Alexander WMA (1998), 32 were *M. neotreddecim* and two were *M. treddecim*, while the collection made near Giant City SP (2002) consisted of 20 *M. neotreddecim* and 30 *M. treddecim*. These relative abundances are consistent with local chorus recordings (Fig. 3). Mating in both populations was highly assortative, since no mixed-species mating

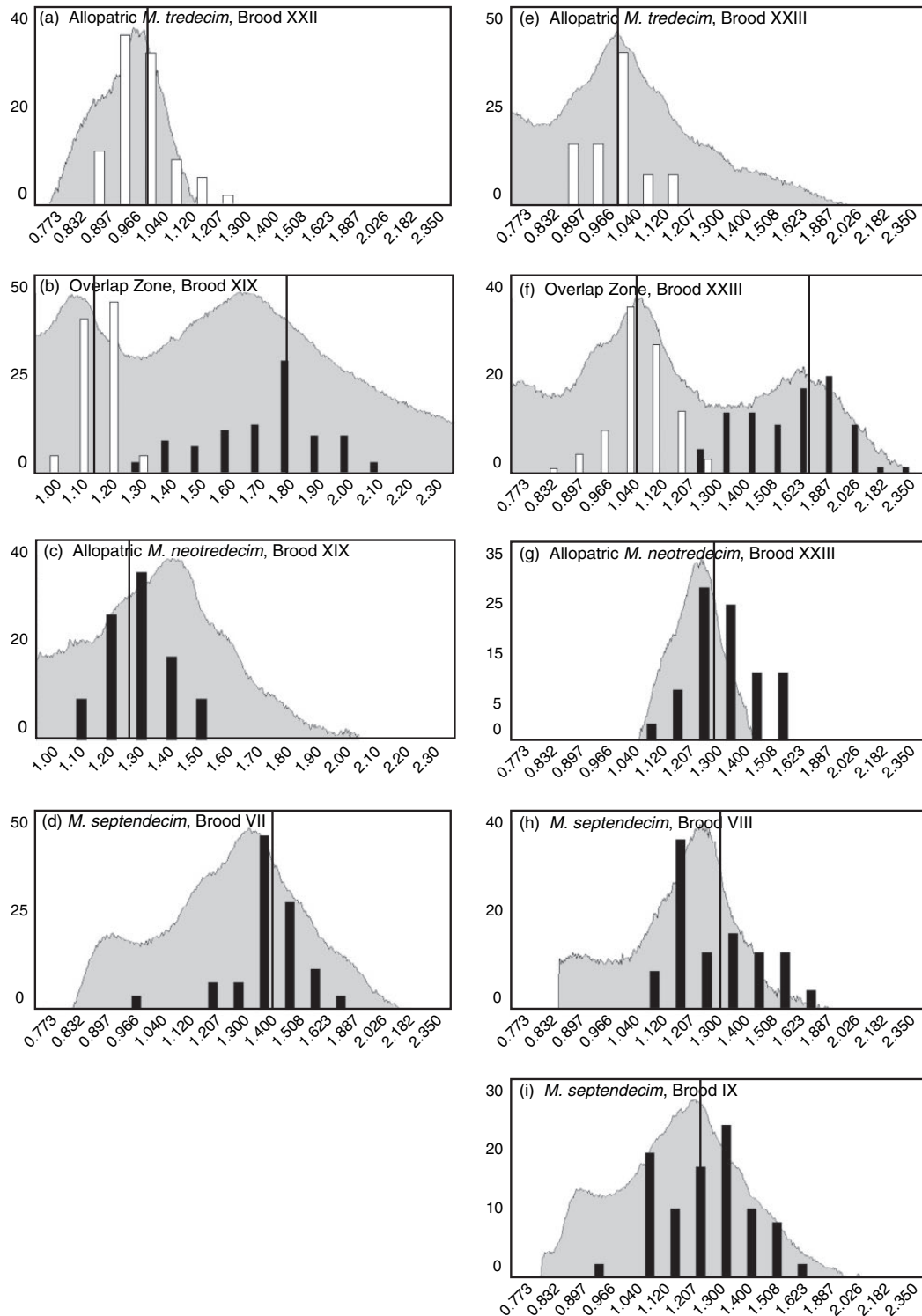


Fig. 3 (a–i) Histograms of female weighted average preferences superimposed on acoustical power spectra of male chorus pitch for allopatric populations and for populations within the *M. tredecim*/*M. neotreddecim* overlap zone. *M. tredecim* female preferences are indicated by open bars, *M. neotreddecim* and *M. septendecim* by solid bars; mean population female preferences are indicated by a vertical line. Logarithmic power spectra of male chorus pitch (shaded) are indications of relative acoustical power scaled in dB.

Table 4 Female sample sizes in playback experiments, mean weighted female pitch preferences and local chorus pitch.

| Species | Year | Brood | State | Biogeography | N | F preference (kHz) | M chorus pitch (kHz) |
|------------------------|------|-------|-------|--------------|----|--------------------|----------------------|
| <i>M. septendecim</i> | 2001 | VII | NY | | 30 | 1.43 ± 0.15 | 1.39 |
| | 2002 | VIII | WV | | 25 | 1.36 ± 0.17 | 1.32 |
| | 2003 | IX | NC | | 44 | 1.34 ± 0.17 | 1.32 |
| <i>M. tredecim</i> | 1998 | XIX | AR | Contact zone | 21 | 1.16 ± 0.08 | 1.14 |
| | 2001 | XXII | MS | Allopatric | 51 | 1.02 ± 0.09 | 1.05 |
| | 2002 | XXIII | MS | Allopatric | 22 | 1.03 ± 0.09 | 1.06 |
| <i>M. neotreddecim</i> | 2002 | XXIII | IL/IN | Contact zone | 92 | 1.08 ± 0.09 | 1.12/1.13 |
| | 1998 | XIX | AR | Contact zone | 67 | 1.76 ± 0.16 | 1.69 |
| | 1998 | XIX | IL | Allopatric | 11 | 1.31 ± 0.11 | 1.38 |
| | 2002 | XXIII | IL | Allopatric | 72 | 1.39 ± 0.13 | 1.33 |
| | 2002 | XXIII | IL/IN | Contact zone | 29 | 1.70 ± 0.23 | 1.69/1.63 |

pairs were collected at either site. The Monte-Carlo resampling algorithm for random mating produced a 95th percentile of two or more mixed matings for the H. Alexander WMA sample, and eight or more mixed matings for Giant City SP sample.

Call and preference phenotypes

Across all populations, average female pitch preferences were statistically indistinguishable from male chorus pitches (Fig. 3, Table 4) (Wilcoxon Signed Ranks Test, $Z = -0.535$, $P > 0.5$; local chorus and preference treated as pairs). Although, statistically indistinguishable, the matches were not exact, and we are unable to determine whether the mismatches result from actual trends or methodological artifacts. *M. septendecim* (proxy for undisplaced *M. neotreddecim*) and *M. tredecim* have substantially overlapping preference and signal pitches while *M. tredecim* and displaced (contact-zone) *M. neotreddecim* overlap only slightly, with only *M. neotreddecim* exhibiting pitch displacement (Fig. 3, Table 4).

Estimates of premating isolation at contact

We found no evidence that the *M. -decim* species were reproductively isolated at the formation of the contact zone. In the combined replicates, there were 27 total first matings, 14 of which were mixed-species (Table 3). These results fall well within the 95th percentile Monte-Carlo random mating expectation of at least four mixed-species mating pairs, suggesting that the *M. septendecim* and *M. tredecim* intermixed freely. Females initiating four or more matings also showed little evidence of consistency in mate choice. Our resampling algorithm suggests that, among these females, the null expectation for random mating is at least one heterospecific pairing. The Sign test revealed no general mating biases among these females, and the Wald-Wolfowitz runs test of individual mating decisions suggested that only a single female *M. tredecim* appeared to discriminate against heterospecifics, although the statistical power of this experiment is low due to small sample size (Table 5).

Table 5 Individual mating histories of females that initiated (but were not allowed to complete) four or more matings. The sign test suggests that females did not bias mating decisions to favour conspecifics ($P \geq 0.1$), and the Wald-Wolfowitz runs test suggests that only one female (11) consistently favoured conspecific mates.

| | Female ID* | Conspecific mates (N) | Heterospecific mates (N) | Z | P (two-tail) |
|-------------------------------|------------|-----------------------|--------------------------|-----------|--------------|
| <i>M. septendecim</i> females | 4 | 3 | 1 | -1.000 | 0.317 |
| | 10 | 2 | 2 | -1.225 | 0.221 |
| | 89 | 2 | 2 | 0.000 | 1.000 |
| <i>M. tredecim</i> females | 2 | 2 | 2 | 0.000 | 1.000 |
| | 5 | 1 | 3 | 1.000 | 0.317 |
| | 8 | 4 | 2 | -1.768 | 0.077 |
| | 9 | 3 | 3 | -0.913 | 0.361 |
| | 11 | 6 | 1 | -9999.000 | ≤0.001 |
| | 14 | 2 | 3 | -0.436 | 0.663 |

*Each experimental female was assigned an identification (ID) number.

Assessing hatching success

Hatching success (number of hatched eggs/total number of eggs) following a hybrid female *M. neotreddecim*/male *M. tredecim* mating was not statistically different from the hatching success of eggs laid by wild-caught *M. neotreddecim* females (Table 6). Our results are roughly comparable to those of White (1973), although our hybrid eggs are from much more closely related species.

Acoustical interference

Both background treatments reduced female responsiveness compared to the no-background treatment. The no-background treatment generated 542 female responses, while playbacks against a simulated 1.4 kHz background (characteristic of undisplaced *M. neotreddecim*/*M. septendecim*) and 1.11 kHz background (typical of *M. tredecim*) generated 129 and 134 female responses, respectively (Kruskal-Wallis one-way ANOVA, dependent variable: number of responses to a given pitch; test statistic = 10.253, $P < 0.01$). The background choruses

Table 6 Hatching success of hybrid *M. -decim* eggs from cross-mating experiment relative to eggs laid by wild-collected *M. neotreddecim* females. Wild-caught females were mated females captured from an allopatric population with no other *M. -decim* species available as mating partners. There was no statistical difference in hatching success for the two groups of eggs (Fisher's Exact Test, $P \geq 0.90$).

| Female | Male | n Females | n Eggs | Hatching success |
|---|---------------------|-----------|--------|------------------|
| <i>M. neotreddecim</i> | <i>M. treddecim</i> | 14 | 4112 | 83.5% |
| <i>M. neotreddecim</i> (wild-caught) | | 5 | 635 | 83.7% |

also appeared to shift the mean weighted pitch preference of females (Kruskal-Wallis one-way ANOVA, dependent variable: pitch preference; test statistic = 22.397, $P < 0.001$). Female *M. septendecim* pitch preferences were shifted from 1.34 ± 0.17 with no background, up to 1.49 ± 0.20 kHz with a heterospecific background simulating *M. treddecim* and down to 1.20 ± 0.23 kHz with a conspecific background chorus of *M. septendecim* (Fig. 4). Further corroboration of these results comes from pairwise Kolmogorov-Smirnov comparisons (two-sided) of the shapes of the female preference histograms under the three background treatments, which show that preferences under all three background conditions differ from each other (no background vs. 1.4 kHz: test statistic = 0.419, $P \leq 0.01$; no background vs. 1.11 kHz: test statistic = 0.420, $P \leq 0.002$; 1.11 vs. 1.4 kHz: test statistic = 0.615 $P \leq 0.001$; see Fig. 4).

Discussion

Why does RCD occur? At its simplest, RCD is attributable to differences, present at the initiation of contact, that make hybrid sexual interactions costly. Sometimes the crucial differences are prezygotic, as when mating signals distinguish species even before displacement. For example, allopatric populations of *Drosophila serrata* and *Drosophila birchii* have distinctive cuticular hydrocarbons, but even so, these distinctions are accentuated when these two species are placed into experimental contact (Blows, 1998; Higgie *et al.*, 2000; Servedio & Noor, 2003). The frog species *Hyla cinerea* and *Hyla gratiosa* have calling songs that differ in pitch and duration even in allopatry, but the differences are accentuated in sympatry (Höbel & Gerhardt, 2003). In each of these examples, the allopatric populations suggest that prezygotic differences distinguished the species before contact – but just as with our data on *M. neotreddecim* and *M. treddecim*, these initial differences may not have been sufficient to fully isolate them.

Many of the fitness costs typically implicated in RCD are post-zygotic, manifested as deficits in hybrid offspring quantity or quality (Butlin, 1989; Hostert, 1997; Kirkpatrick & Ravigne, 2002), including hybrid 'behavioural sterility' or mate-location inefficiency (Coyne *et al.*, 2002; Höbel & Gerhardt, 2003). Increased parental mortality following a heterospecific mating (Servedio, 2001) is another potential cost of hybridization, because even if hybrid offspring show no fitness deficits, increased parental mortality effectively dilutes the

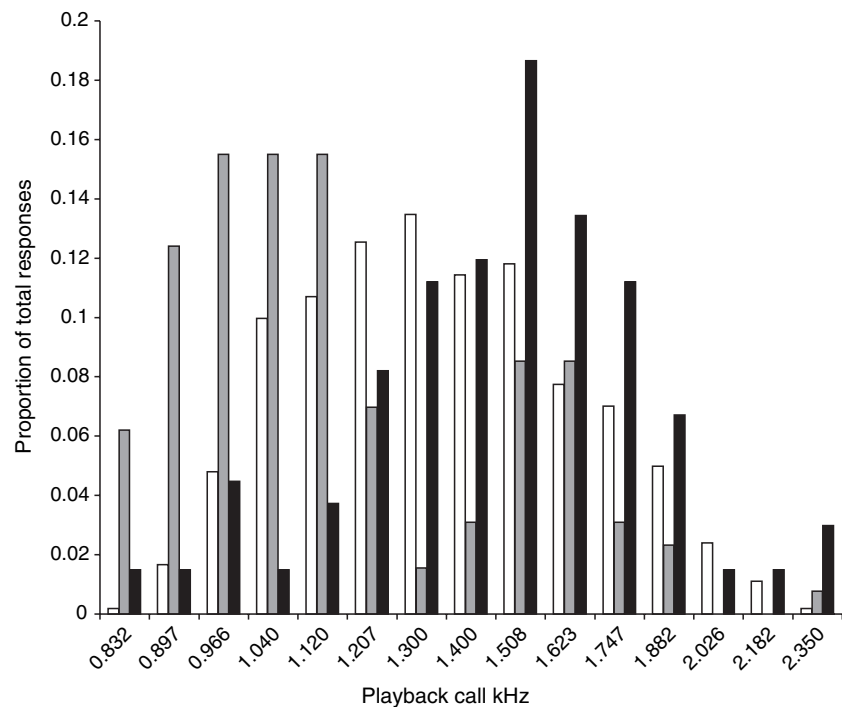


Fig. 4 Histogram of female *M. septendecim* wing-flick signal responses to call playbacks of various pitches under different background chorus conditions. open bars: female responses with no background chorus; grey bars: female responses under simulated *M. treddecim* chorus; and black bars: female responses under simulated *M. septendecim* chorus.

fitness of hybrid offspring by reducing future reproduction. Other examples include genetic barriers such as karyotypic incompatibilities, which lead to hybrid failure in New Zealand wetas (*Hemideina thoracica*, Morgan-Richards & Wallis, 2003) or incompatibilities related to Haldane's Rule (the sterility or absence of heterogametic F_1 hybrid offspring (Haldane, 1922; Coyne, 1985).

The net evolutionary change in the *Maglicada* contact zone has been the nearly complete reproductive isolation of *M. tredecim* and *M. neotreddecim* via directional exaggeration of pre-existing sexual signal differences. These signal changes appear to be the results of selection for RCD (rather than byproducts of some other process such as runaway sexual selection) because they are no more extreme than is necessary to prevent call overlap in the contact zone. There is no evidence of ongoing selection for signal displacement, because of the apparent stabilizing selection suggested by the general match between male calls and female call preferences (see Ritchie, 1996; Houde & Hankes, 1997; Blows, 1999), and the bell-shape of *Maglicada* preference histograms. Following the establishment of the contact zone, stabilizing selection must have given way, at least temporarily, to directional selection, but how these two modes of selection have interacted is beyond the scope of this paper. Even so, for stabilizing selection to have been superseded, even temporarily, the selective pressures responsible for RCD in *Maglicada* must have been relatively strong. Which potential causes of RCD are most compatible with our experimental results for *Maglicada*?

Could acoustical interference alone explain displacement in *M. neotreddecim*? The asymmetrical pattern of character displacement (all change occurring in *M. neotreddecim* and none evident in *M. tredecim*) is consistent with a specific prediction of the acoustical interference hypothesis (that an *M. tredecim* background chorus impedes the ability of *M. neotreddecim* females to respond to males, but that the reciprocal is not true). Our playback experiments demonstrate that, for an *M. -decim* female with a pitch preference of 1.3–1.4 kHz (typical of *M. septendecim* and undisplaced *M. neotreddecim*), the presence of an *M. tredecim*-like background chorus attenuates responsiveness to lower-pitched calls, both by shifting the mean weighted female preference from values of ≈ 1.3 –1.4 kHz up to $\approx 1.49 \pm 0.20$ kHz and also by changing the pitch eliciting the greatest number of responses. This temporary distortion of female responses (which is distinct from the permanent, greater magnitude evolutionary changes associated with the contact zone) is consistent with results presented in Cooley & Marshall (2001). Marshall (2000) reported similar results, except that in his study, the single pitch eliciting the most responses was not altered by a background chorus. We suspect that these slightly different outcomes may be attributable to methodological differences (signal/noise ratio or the presence/absence of a natural ambient chorus). We do not suggest that the shifts in female

responsiveness revealed by these experiments are adaptive; rather, we suggest that these shifts reveal the selective pressures on female perception that may underly adaptive evolution of female responsiveness. Thus, our experimental alteration of *M. -decim* female acceptance behaviour demonstrates that in *M. neotreddecim*, a lower-pitched background chorus could potentially impose directional selection on preference phenotypes. Over evolutionary time this selection could lead to the permanent, exaggerated signals and preferences characteristic of *M. neotreddecim* in the contact zone. Furthermore, the presence of a simulated *M. septendecim* background chorus appears to cause female responses to assume a bimodal distribution; this bimodality suggests one explanation for why conspecific choruses do not, or perhaps cannot, impose directional selection on call phenotypes.

Yet acoustical interference can only be a partial explanation for displacement in the *Maglicada* contact zone. First, this hypothesis leaves unresolved an important point: the call downslurs of high-pitched, mutant *M. neotreddecim* individuals would also be partially obscured by the calls of undisplaced conspecifics, and there is no simple way to discount the effects of a potentially interfering chorus of undisplaced conspecifics. Some additional factor – perhaps scarcity of *M. neotreddecim* relative to *M. tredecim*, or perhaps the 'fit' of displaced *M. neotreddecim* downslurs between the calls of undisplaced *M. neotreddecim* and *M. tredecim* – must have made it possible for the calls of mutant, high-pitched *M. neotreddecim* males to have an advantage in eliciting female responses.

In addition, the acoustical interference hypothesis cannot account for the possibility of direct sexual contact, because it addresses only competition for signal space. Our data suggest that sexual contact was possible, given the typical, multispecies nature of *Maglicada* choruses and the overlap of *M. neotreddecim* and *M. tredecim* signals at the formation of their contact zone. In our playback experiments, female *M. septendecim* (proxy for undisplaced *M. neotreddecim*) were only slightly less responsive to model calls of 1.1–1.2 kHz (the upper *M. tredecim* call range) than to 1.3–1.4 kHz calls typical of their own species, suggesting that prior to contact with *M. tredecim*, undisplaced *M. neotreddecim* females possessed the latent ability to respond to all male call phenotypes present in a population of *M. tredecim*. Even though no *M. tredecim* male would have been more likely to elicit responses from undisplaced *M. neotreddecim* females than would the lowest-pitched (≈ 1.2 kHz) *M. neotreddecim* male, the overlap of female pitch preferences and heterospecific male call pitches could have given heterospecific courtships some chance of success. Although, the pattern of RCD meets a key prediction of the acoustical interference hypothesis as applied to *Maglicada* (only *M. neotreddecim* displaced), this prediction is not exclusive to this hypothesis; for example, the relative abundance of the two species at the formation of the contact zone could also

explain the asymmetry of displacement (see below). Furthermore, if RCD in *Magicalcada* were based *only* on signal interference, signal *convergence* may have been a plausible outcome in the contact zone. For these reasons, the possibility (perhaps even likelihood) of cross mating indicates that the acoustical interference hypothesis by itself may not be sufficient to explain RCD in *Magicalcada*.

An alternative possibility is that our experiments were biased and overestimated the jeopardy of cross mating, perhaps because our study (like all previous studies of *Magicalcada* behaviour) lacks a temporal component. If sexual receptivity in *Magicalcada* females has a gradual onset, so that females respond only to maximally stimulating calls initially, and if operational sex ratios in *Magicalcada* choruses are typically such that even marginally receptive females are quickly located and courted by males, then even slight differences in call pitch preference might isolate populations to a greater degree than our current data suggest.

Nevertheless, it remains possible that barriers to sexual pairing between *M. tredecim* and undisplaced *M. neotredecim* were incomplete at first contact, so the potential costs of hybridization deserve further scrutiny. Our dissections of eggnecks give no reason to suspect early-acting viability defects in hybrid offspring; hybrid egg hatching success from female *M. neotredecim*/male *M. tredecim* crosses is indistinguishable from hatching success of eggs from wild-caught *M. neotredecim* females. From other studies, hybrid sterility appears common, and one possibility (outside the practical scope of this study) is that hybrid *Magicalcada* offspring are sterile. Another possibility is 'behavioural sterility', or inappropriate sexual behaviours in hybrid offspring. Although, we have no information about the calls of hybrid *M. -decim* individuals, it is likely that they would have the same general pure-tone call structure as normal *M. -decim* calls, and it is possible that their pitch would be intermediate to both parental species. Comparisons of female preference histograms indicate that calls intermediate to those of *M. tredecim* and undisplaced *M. neotredecim* would be acceptable to, although perhaps not preferred by, most individuals in the contact zone, and calls across the entire range of pitches tested would have some chance of being accepted by females of either species (Fig. 3). Thus, hybrid behavioural sterility, mediated by unattractive songs, is not likely to be a strong isolating factor in *Magicalcada*.

The life cycle architecture of 13- and 17-year cicadas suggests an alternative testable hypothesis for RCD in *Magicalcada*. Seventeen-year *Magicalcada* nymphs have been reported to undergo a 4-year 'dormancy period' that is not shared by their 13-year congeners (White & Lloyd, 1975). Because *M. neotredecim* is recently derived from a 17-year ancestor, while *M. tredecim* is not, it is possible that the genetic or developmental architecture of the 13-year life cycle differs between these species. Such a mismatch in life cycle genetics could cause hybrid

M. tredecim-*M. neotredecim* offspring to develop according to variable schedules that would allochronically isolate them. Because periodical cicadas rely on dense, superabundant populations to reduce the individual risks of predation, low-density 'straggler' populations are quickly annihilated by predators (Alexander & Moore, 1958; Lloyd & Dybas, 1966; Karban, 1982; Williams *et al.*, 1993). Thus, hybridization could be costly, because it would interfere with strict adherence to majority life-cycle phenotypes enforced by predation. Tests of this 'life cycle incompatibility' hypothesis include ongoing studies of the underground developmental rates of hybrid nymphs from the mating experiments outlined above, as well as close monitoring of *M. tredecim*/*M. neotredecim* along their contact zone (and *M. tredecim*/*M. septendecim* along theirs) in order to detect and observe the fates of any unusual off-cycle emergences.

How can 'hybrid quality' hypotheses account for the asymmetry in displacement? Although, asymmetries in displacement may actually be quite common (Howard, 1993), it is difficult to judge their prevalence, since not all studies of RCD explicitly test for displacement in both species. Asymmetrical body size displacement has been reported in crayfish (*Orconectes* spp. Butler, 1988), and asymmetrical displacements in call temporal and spectral properties have been reported in both the frog genera *Hyla* (Loftus-Hills & Littlejohn, 1992) and *Pseudacris* (Fouquette, 1975). The causes of displacement asymmetries are unclear, but asymmetries ultimately must reflect some biases in the costs, consequences, or capacities for interbreeding of the species involved (Grant, 1972; Howard, 1993; Pfennig & Murphy, 2003).

Even if there are no hybridization cost asymmetries for two species, the relative abundances of species interacting in contact zones may lead to displacement asymmetries. *Nasonia longicornis* (Hymenoptera) is a jewel wasp species with a range entirely embedded within the range of a similar species *Nasonia vitripennis* (Bordenstein *et al.*, 2000). In this example, *N. longicornis*, the species with the smaller range, has undergone displacement, presumably because it also has the greatest jeopardy of mating with heterospecifics. Similarly, Kaneshiro's (1976, 1980, 1983) models of asymmetrical mating preferences among Hawaiian *Drosophila* species rely on abundance differences among populations to explain these asymmetries. Noor (1995) explicitly attributes asymmetrical displacement in the *Drosophila pseudoobscura* group to rarity of one of the involved species. In these examples, unequal abundance is the ultimate explanation for asymmetries in gene flow or displacement; when one species is rare relative to another, interspecific encounter rates and opportunities for hybridization are unequal. Since displacement is a product of both the costs of hybridization and the risk of heterospecific pair formation, and since members of a rare species are confronted by many more potential heterospecific mates than are

members of more common species, relative abundance may play a central role in shaping the asymmetry of displacement.

Our study uncovered no evidence of asymmetrical costs, barriers or conflicting selection associated with direct sexual interactions that could explain the asymmetry of displacement in *Magiccada*. However, it is possible that *M. neotrededim* was initially rare in the contact zone relative to *M. trededim*. In some contemporary populations, this species remains rare; Simon *et al.* (2000) found that in Brood XXIII, *M. neotrededim* was rarer than *M. trededim* in all five contact populations examined. Initial *M. neotrededim* rarity is compatible with models suggesting that *M. neotrededim* formed outside the contact zone (in parapatry) and later expanded into the range of *M. trededim* (Marshall & Cooley, 2000; Cooley *et al.*, 2001; Marshall *et al.*, 2003).

When two populations come into secondary contact, will they diverge or amalgamate? The key to making such predictions seems to be an understanding of the possibilities and likelihood of sexual contact and gene flow. *Magiccada* present a difficult case: although, the acoustical interference hypothesis is consistent with many aspects of RCD in *M. neotrededim*, the significant possibility of interbreeding at the formation of the *Magiccada* contact zone suggests that some costs (currently unknown) of hybridization are also involved. These costs must have been substantial to counteract stabilizing selection and to effect rapid displacement, without leaving any evidence of mtDNA introgression. There is no evidence that post-mating, prezygotic barriers to gene flow apply – hybrid eggs hatch, and at no lower frequency than purebred eggs. No conspecific sperm precedence seems applicable (reviewed in Howard, 1999) since female *Magiccada* generally mate once, and earlier studies show that hybrid matings between even more distantly related *Magiccada* species do not trigger remating (Cooley, 1999). Yet because of the extremely long life cycles involved, we were only able to test limited ways in which hybrids fail, and possible hybrid deficiencies manifested later in development remain largely unexplored. These deficiencies may be extremely difficult to detect – for example, the life cycle incompatibility hypothesis posits that *Magiccada* hybrids fail relatively late in development, not due to any intrinsic quality deficits, but due to the action of an external factor – extreme predator pressure (see Servedio & Noor, 2003). Such a situation would make hybridization extremely costly – and these costs would not be evident in any experiments that failed to replicate the ecological context of these animals.

It is difficult to pinpoint any single difference isolating *Magiccada* species at contact. The most reasonable possibility, and the one most congruent with current theory, is that at first contact, gene flow between *M. neotrededim* and *M. trededim* was insufficient to oppose the effects of some as-yet unknown genetic differences or combination of differences separating these species. Alternatively, and

contrary to current theory, RCD in *Magiccada* may have occurred in the face of substantial gene flow – although, it would be premature, without additional study, to embrace the latter possibility.

Acknowledgments

We thank the owners and managers of the study sites listed in Table 1 for permission to conduct research on their properties. Funding for 1998–2000 field data collected by JC and DM was provided by the Frank W. Ammermann Endowment of the University of Michigan Museum of Zoology Insect Division. Funding for data collected in 2002–2003 was provided by National Science Foundation DEB 99-74369 and University of Connecticut grants to CS. Travel for KH was supported by ISAT grant 02-CSP-38-HILL from the Royal Society of New Zealand. Special thanks are also due T. Lardaro and N. Yielding for their assistance during the 2002 field season. R. D. Alexander and anonymous reviewers provided helpful comments on earlier drafts. Correspondence and requests for materials should be addressed to John Cooley, Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269-3043. All specimens, audio recordings, and sequence data used in this research are deposited at the University of Connecticut.

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Received 05 July 2005; revised 23 September 2005; accepted 26 September 2005