Auditory sensitivity of an acoustic parasitoid (*Emblemasoma* sp., Sarcophagidae, Diptera) and the calling behavior of potential hosts

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Abstract

Using field broadcasts of model male calling songs, we tested whether *Tibicen pruinosa* and *T. chloromera* (Homoptera: Cicadidae) are candidate hosts for acoustic parasitoid flies. The model calling song of *T. pruinosa* attracted 90% of the flies (Sarcophagidae: *Emblemasoma* sp.; all larvapositing females) when broadcast simultaneously with the model *T. chloromera* song, a phonotactic bias reconfirmed in single song playbacks. In paired broadcasts of model *T. pruinosa* songs with different relative amplitudes (3 dB or 6 dB), significantly more flies were attracted to the more powerful song, a result consistent with the responses predicted by a model proposed by Forrest and Raspet [1994]. Using intracellular recordings and dye injections, we characterized the sensitivity of auditory units in sound-trapped flies. Intracellular recordings from six auditory units (5 interneurons, 1 afferent) revealed best sensitivity for frequencies near 3-4 kHz, matching the predominant spectral components of the calling songs of both species of cicada. Interestingly, although flies could be attracted to *T. pruinosa* broadcasts throughout the day, hourly censuses of singing males revealed that calling occurred exclusively at dusk. Furthermore, the duration of the dusk chorus in *T. pruinosa* was significantly shorter than the midday chorus of the less attractive song of *T. chloromera*. We propose that the tight temporal aggregation of the dusk chorus time could function to reduce risk from attracted parasitoids.

Keywords

Cicada; Diel Behavior; Parasitoid; Cricket; Chorus; *Tibicen*; *Ormia*; *Emblemasoma*; phonotaxis; interneuron; frequency tuning

Introduction

In some species of acoustically signaling insects male calling songs not only attract conspecifics, but also acoustically orienting parasitoids [Cade, 1975; Soper et al. 1976; Lehmann, 2003]. For example, several species of ormiine flies (Tachinidae) are acoustic parasitoids of a variety of singing orthopterans [Burk, 1982; Allen, 1995; Lakes-Harlan and Heller, 1992]. Although the larvae of ormiines will successfully develop in several potential host species [Wineriter and Walker, 1990; Walker and Wineriter, 1991; Shapiro, 1995], host

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searching female flies exhibit acoustic selectivity that is based on the spectral and temporal parameters of the hosts' songs [Fowler, 1987; Walker, 1993]. With respect to song frequency, the interspecific variance in host songs for ormiines is large, ranging over 4 octaves [2.7 kHz - ~40 kHz; Fowler, 1987; Stumpner and Lakes-Harlan, 1996; Lehmann and Heller, 1998; Stumpner et al., 2007]. So far, sensitivity in ormiine flies is shown to track this variation with tuning matching the spectral range of hosts' songs in one of two general patterns: relatively sharper tuning to a host frequency range (*O. ochracea*, 4-8 kHz) [Robert et al., 1992; Oshinsky and Hoy, 2002] or broader tuning that encompasses a range of host songs with very different spectra, facilitating host switching (e.g., *Therobia leonidei* and *Homotrixa alleni*) [Stumpner and Lakes-Harlan, 1996; Stumpner et al., 2007].

Acoustic parasitism in Diptera is not limited to the orthopteran-tachinid system, however. Members of the family Sarcophagidae (Emblemasomatini) use an independently evolved ear [Edgecomb et al., 1995; Robert et al., 1999; Lakes-Harland et al., 1999] to carry out an analogous strategy for finding their hosts, singing cicadas [Soper et al., 1976]. Like that for ormiines, there appears to be interspecific variance in host choice, as several species of flies, found either as larvae or reared to adults, have been collected from various parasitized cicada taxa [Lopes, 1971, 1981; Soper et al., 1976]. All *Emblemasoma* examined so far express the inflated prosternum known to be the tympanal organ [Lopes, 1971; Lakes-Harlan et al., 1999; Robert et al., 1999], suggesting that host choice is based on auditory cues. At present, although there are limited data regarding the auditory mechanisms, behavior and sensitivity of sarcophagids, two interesting differences with ormiines have emerged. First, the mechanical modes of vibration of the tympanal organ differ from those in ormiines [Robert et al., 1999]. Second, sensitivity in *Emblemasoma auditrix* (genus formally *Colcondamyia*; Pape, 1990] differs from the two patterns in ormiines, as whole nerve recordings from interneurons in the neck connectives show evidence for a tuning mismatch: best responses occur at 5 kHz, nearly an octave below the dominant frequency of its host's song (9 kHz), suggesting discrimination between songs of different cicada species is not based on spectral cues [Köhler and Lakes-Harlan, 2001]. In addition to the tuning mismatch, *E. auditrix* also exhibits a sharp decrease in physiological sensitivity to low frequencies (<5kHz) [Lakes-Harlan et al., 1999; Köhler and Lakes-Harlan, 2001]. Having data on the sensitivity of only one sarcophagid species, of course, leaves the question open as to the extent of convergence with ormiines; that is, whether the mechanisms and patterns of sensitivity consistently differ between the analogous traits. To explore this question, we investigated the sensitivity and behavior of parasitoids of cicadas that produce low frequency calls.

Using field broadcasts of calling songs, our study shows that the relatively low frequency (3.5 kHz) call of *Tibicen pruinosa* (Cicadae) is attractive to acoustic parasitoid flies (*Emblemasoma* sp.). Because most of the spectral energy in the *T. pruinosa* call is below the tuning previously shown for *E. auditrix*, specialization for sensitivity to this low frequency song was measured in the auditory units of attracted flies using intracellular recordings and dye injections. In addition, we show that *T. pruinosa* exhibits a tightly timed and short duration chorus, making simultaneous comparisons important to phonotactic host choice. Thus, we used paired broadcasts of the *T. pruinosa* song that varied in relative intensity and spatial separation to assess the behavioral threshold and phonotactic decision rules employed in the field by host searching flies [Forrest and Raspet, 1994].

Methods

Census of Cicada Community

To assess the potential hosts for sarcophagid flies near our field site, we made hourly censuses of calling male cicadas from 08:00 to 20:00 hours (Central Daylight Time) during ten days between 24 July and 24 August 1992 on the University of Mississippi campus, Lafayette

County, Mississippi. Average sunrise and sunset times during the census were 05:42 (±0.15) and 19:56 (±0.21) hrs, respectively. For each census at least one of us walked a seven station circular route through an area of large mixed hardwood trees (distance from station to station was ca. 90 m). At the beginning and end of each census, temperatures were measured in direct sunlight and in the shade using a Fluke 8062 multimeter and a Fluke 80T-150U temperature probe. Mean ambient temperature for each hourly observation was calculated by averaging these four temperature measures. At each of the seven stations, relative light intensity was measured on a 12 unit logarithmic scale with a General Electric light meter. One minute was spent at each station counting the number of individual cicadas calling within earshot. A complete circuit of the census took approximately 20 minutes.

During the census, visual observation of individual cicadas was impossible because they called high in the treetops. There were three species, however, *Tibicen pruinosa, T. chloromera* and *Neocicada hieroglyphica*, that were easily distinguished by differences in their calling songs, allowing individual calling males to be singled out and censused by ear. Although the songs of *T. pruinosa* and *T. chloromera* cover similar bandwidths, their temporal structure is very different (see fig. 1a,b). Whereas *T. pruinosa* produces an FM burst at a relatively slow rate (1.4 - 1.6 burst / sec), *T. chloromera* produces a noisy broadband burst without FM at a faster rate (10 - 13 bursts / sec). The song of *Neocicada hieroglyphica* is easily distinguished from the other two as it exhibits both a higher frequency band and faster temporal structure (peak freq.: 4.8 ± 0.12 kHz; harmonic: 9.11 ± 0.25 kHz; 200 minimum - 375 maximum pulses/s; Parameters for *N. hieroglyphica* are from 2 randomly selected 12 second sections from 1 song each from 3 individuals recorded at the field site).

Because some males may have flown to different perches as we walked the census circuit, individuals may have been counted more than once in an hourly census. Therefore, we normalized the hourly census for each day as a proportion of the maximum hourly count for that day. Thus, each day's maximum hourly census was 1.0 and all other hourly counts ranged between 0 and 1.0. This technique does not give an accurate measure of the number of calling cicadas but does reflect relative calling diversity and activity per hour.

We determined the autocorrelation function of the census data to test for significant periodicity in the series of hourly observations [Chatfield, 1989]. Certain assumptions were made in using this method of analysis. Because there was no evidence of cicada singing during the nighttime hours between 2100 and 0700, the census was not taken during this time. For these hours, cicada broadcast activity was designated a census level of 0. The relationships between temperature and sunlight intensity and cicada calling activity were analyzed using logistic regression [Hosmer and Lemeshow, 1989]. This method of analysis allows for the input of a binomial dependent variable (e.g., weighted number of calling males) and compares the data to a χ^2 distribution with 1 degree of freedom. A two-tailed t test was used to compare the mean temperatures for the times with the maximum amount of male calling in both species. Comparison of the distribution of calling times between *T. chloromera* and *T. pruinosa* was done using a variance ratio test [Zar, 1984].

Sound Synthesis for field Broadcasts

All acoustic stimuli were generated using a personal computer and custom written software. The digital stimuli were played back using a Tucker-Davis Technologies (TDT) 16-bit digital-to-analog converter (sample period 40 μsec) with the output low-pass filtered at 10 kHz using a TDT filter (75 dB/octave) to prevent aliasing.

The model *T. pruinosa* and *N. hieroglyphica* broadcasts consisted of the continuous playback of a digitally sampled portion of an exemplar call (fig. 1). The sampled portion was the most prominent repeated element in the middle of the calls [Alexander 1960]. The exemplar was

taken from recordings of singing males near our broadcast site (<6.4 km) using an E.P.M. model p650 parabolic microphone, a Sony TCD5 Pro2 analog cassette recorder and a TDK SA-X tape. The spectrum of the calling song was analyzed using the complex FFT routine in Mathcad (Cambridge, MA). Any effects of temperature on song structure were not included, as it was impossible to measure the ambient temperature at the positions of the calling males (note that ambient temperature may or may not affect the carrier frequency of the calling song; Josephson and Young, 1979].

Unlike the *T. pruinosa* song, which contains varying intensity across frequency bands and frequency modulation, the *T. chloromera* song consists of the repetition of a broadband burst. Thus, the *T. chloromera* model song was constructed by digitally synthesizing a song pulse (fig. 1) with the 40 dB bandwidth and temporal properties of those recorded from a singing male.

There are experimental limitations in using the model calls. First, the models represent only a portion of an actual male calling songs and do not include "wind-up" or "wind-down" segments. Although these segments could be relevant to flies, their variance (particularly in duration and intensity) would have created times during playback in which song intensity differed (a parameter important to phonotaxis, see below [Wyttenbach and Farris, 2005]). Second, the models also eliminate between-male variance, which could also affect fly attraction; the models thus are valid for testing the effects of single variables such as intensity, as broadcasts of actual male calls would vary across several acoustic parameters. Third, when broadcasting a single model or actual call, the results may not generalize to all songs.

The model calling songs were broadcast through Motorola piezoelectric speakers. All digital song buffers had the same rms amplitude. By broadcasting a 3.5 kHz tone, sound pressure levels (SPL re. 20 μPa) were calibrated 10 cm above each speaker (± 1.0 dB) before each broadcast using a custom built microphone holder rigidly mounted to the face of the speaker. After calibration to the tone, the output was switched to the song buffers and the calibration verified. SPL was measured using a Fluke 8060A multimeter (calculating root-mean-square amplitude; integration time: 100 ms), a Bruel & Kjær (B&K) 4135 1/4 inch microphone (grid on), a B&K 2639 preamp and a B&K 2804 microphone power supply. The calibration system was checked using a B&K 4230 pistonphone calibrator.

Sound Trapping Flies

Sound traps, consisting of a speaker mounted inside of a 19-liter mesh bucket, were placed in an area of mixed hardwoods at the University of Mississippi Biological Field Station, Lafayette, Co., MS, U.S.A.. Similar to the slit traps designed for trapping *O. ochracea* [Walker, 1989], a 12 cm × 3 cm tapered slit in the lid of the bucket trap allowed flies (*Emblemasoma* sp.: Sarcophagidae) to enter but not to escape. Broadcasts were carried out during September 10-20, 1996-1998. During this time, males of all three cicada species were heard calling near the broadcast site.

Preliminary data showed no fly attraction to the model *N. hieroglyphica* song (see below). Thus, field experiments focused on the response of flies to broadcasts of the *T. pruinosa* or *T. chloromera* model songs. First, to assess relative attractiveness, model calling songs were broadcast from either of two separate traps (109 dB, 5 m trap separation). The effects of the intensity of the calling songs and the distance between sources on the attraction of parasitoid flies were subsequently tested by broadcasting the model *T. pruinosa* song from both traps at two different relative intensities (Δ6 and Δ3 dB, 5 m separation) and separations (5 and 10 m separation, Δ6 dB). Broadcast conditions were: 109 vs. 103 dB and 109 vs. 106 dB, 5 m separation and 109 vs. 103 dB, 10 m separation. The sound traps were checked every 30 minutes and the flies in each trap were collected. Note, as shown previously, flies often sat on the outside

of the trap for several minutes [Schniederkötter and Lakes-Harlan, 2004; cf. *O. ochracea*, Walker, 1993]. Whereas some subsequently entered the trap, those that did not were captured by hand in vials and included in the data. There was no recognizable anatomical difference between those captured outright and those by hand.

Broadcasts began in the morning prior to the mid-day chorus of *T. chloromera* (see below) and continued until after sunset. To avoid the effect of trap position on relative attraction, the two songs in each broadcast condition were alternated between the two trap positions until approximately the same number of flies was attracted in both configurations. A Tukey-type test for multiple comparisons between proportions compared the relative responses to field broadcasts (proportion of the total attracted flies at each trap [Zar, 1984]). A chi-square goodness-of-fit test was used to test whether the relative response proportions differed from random or model predictions.

Physiological Recordings

Cold-anesthetized female flies (*Emblemasoma* sp; sound-trapped with *T. pruinosa* song) were mounted on a platform dorsal side up in a foam lined Faraday cage that reduced acoustic and electrical noise. Using a dorsal dissection so as not to interfere with ventrally located hearing organ [Lakes-Harlan et al., 1999; Robert et al., 1999], the fly's flight muscles and gut were removed to reveal the fused thoracic ganglion. Electrical activity in auditory units in the fused ganglion was recorded using thin-walled (1.0 mm o.d.) borosilicate glass, micropipette electrodes filled at the tip with 2.5% Lucifer Yellow (Sigma) and backfilled with 0.1 mol 1^{-1} LiCl. Electrode resistance varied from 70-140 MΩ. The indifferent electrode was inserted into the thorax. The search stimulus used to locate auditory units consisted of a 10 ms tone pulse (1 ms ramps) of 5-7 kHz. After amplification (AM systems model 1600 DC amplifier), the neural responses were digitized (10 µs sampling period) using a TDT AD3 and System II Array Processor and custom written software. By injecting hyperpolarizing current (0.2-1.2 nA) after recording, cells were stained with Lucifer Yellow for anatomical identification. After staining, the ganglia were dissected and fixed (16-24 hrs.) in 4% Paraformaldehyde (pH 7.4), dehydrated in an ascending ethanol series and cleared using methyl salicylate. Stained cells were photographed and digitized as whole mounts using a Leitz Dialux 20 and BioRad MRC-600 confocal microscopes, respectively. Cell identification (i.e., interneuron or afferent) was accomplished by comparing micrographs of the Lucifer fills to those produced by backfilling the chordotonal auditory organ with Neurobiotin / ABC kit (vector labs) or fluorescent dextrans (Molecular Probes Inc. Texas Red D-3328 or FITC D-3306), a procedure which only illuminates afferents. Although all cells were partially filled, this distinction could still be made.

After penetrating a cell, its frequency tuning was determined by measuring the minimum sound pressure level (±3 dB) required to elicit at least 1 action potential in 3/5 stimulus presentations. Stimuli were pure tones that varied in frequency from 2-35 kHz (20 ms pulses, 1 ms ramps, 100 ms ISI) and were presented in ascending order. During measurement of the tuning curve, input / output (I/O) functions at each cell's characteristic frequency were measured by calculating the mean number of spikes elicited for the five repetitions presented at each intensity step. Tuning curves showed linear change in sensitivity around the best frequency (see results) and thus were analyzed using a simple linear regression.

Results

Census of Available Cicada Hosts

The census was necessary to catalog which potential hosts were present and when they were at risk of attracting larvapositing flies. In the area of our field site we identified 3 species of singing cicadas, *N. hieroglyphica, T. chloromera* and *T. pruinosa*. Whereas *N. hieroglyphica*

called throughout the day, *T. pruinosa* and *T. chloromera* called at distinct times. For *T. chloromera*, 87% (n = 360 individuals) of singing was observed between 0800 and 1100 hours (mean temperature: 23±3°C), whereas 92% (n = 105) of singing by *T. pruinosa* occurred between 1900 and 2000 hours (mean temperature: 23°C ±3)(fig. 2). Mean temperature for these two times did not differ significantly (P=0.5, t = 2.0, n = 50). Chorus duration for *T. chloromera* is significantly broader than that in *T. pruinosa* (variance ratio test; p<0.001). For both species, autocorrelation analysis of male signaling times revealed consistent 24 hr. periodicities, as the highest correlations between observations of singing males were 24 hours apart (fig. 3). Thus, the broader duration chorus time of *T. chloromera* cannot be the result of a shift in chorus time across the days of our census.

The calling activity of both species was significantly correlated to changes in light intensity between censuses (table 1). In *T. chloromera*, the species with the pre-noon chorus, singing was also correlated to changes in ambient temperature. However, for *T. pruinosa* the species which choruses primarily at dusk, calling activity was not influenced by temperature, but was significantly correlated to the levels of ambient light (table 1).

Attraction of Flies

Two of the model cicada songs attracted larvapositing female sarcophagid flies (*Emblemasoma* sp.; genus determined from Aldrich, 1916). Unfortunately, attraction of only females prevented their specific identification, as the taxonomy of this group is based in part on male genitalia [Pape, 1990; personal communication]. Single speaker broadcasts over 32 hours of the *N. hieroglyphica* call failed to attract any flies (data not shown). However, in 31 hrs. of simultaneous broadcast (over 6 days) of the model *T. pruinosa* and *T. chloromera* calling songs, 90.3% of the flies were attracted to the *T. pruinosa* trap (n=82, χ^2=29.8, P < 0.0001). This preference for the *T. pruinosa* call did not appear to be due to a relative difference in attractiveness, as broadcast of the *T. chloromera* song alone from both traps for 3.5 hrs. on one day failed to attract any flies, even though flies were present; switching one of the traps to the *T. pruinosa* song attracted two flies within one hour. Because of this attraction to the *T. pruinosa* song, field broadcasts were subsequently limited to experiments using the *T. pruinosa* song only. Continuous broadcast of this song revealed that, in contrast to diel calling patterns shown above, fly phonotaxis occurred throughout the day. On days with continuous uninterrupted broadcasts of the model *T. pruinosa* song, flies were captured from 0900 hr. until just prior to sunset (fig. 2).

Effects of Song Intensity and Spacing

The relative attractiveness of two traps broadcasting the model *T. pruinosa* song depended on their relative intensity. For intensity differences of 3 dB and 6 dB, the more intense song attracted 75-80% of the flies (table 2). Increasing the intensity difference from 3 dB to 6 dB did not change the relative attractiveness of the more intense song (q = 0.804, p > 0.5), however. There was no effect of trap separation on the relative attraction of flies to the two songs. For songs that differ by 6 dB, the less intense song attracted 24.1% and 21.2% of the flies for the 10 m and 5 m separations, respectively (q = 0.777, p > 0.5).

Auditory Sensitivity in Emblemasoma sp

In contrast to interneurons recorded in the neck connectives of *E. auditrix* [Lakes-Harlan et al., 1999], there was no evidence of a tuning mismatch in flies attracted to the *T. pruinosa* song. Recordings from 5 interneurons, which exhibited intersegmental projections, and one afferent (1 cell each from 6 individuals) revealed consistent tuning to frequencies near 3 kHz, the dominant frequencies of the cicadas' calling songs (figs. 4, 5). For the interneurons, tuning was sharp due to the steep reduction in sensitivity from 3 kHz to 2 kHz (20 ± 5 dB). The reduced sensitivity for frequencies above 3 kHz was also steep (linear slope: 3.1 ± 0.6 dB/kHz up to

10 kHz; $r^2 = 0.92$, P <0.004), with little sensitivity above 6 kHz and only one recording showing minimal sensitivity above 10 kHz. This sensitivity is similar to that of the receptor, but the single sample prevents statistical comparison or general description of receptor tuning. Thus, based on the center frequency and tuning sharpness of these auditory units, spectral sensitivity in these flies appears to match that of the most attractive song.

Although sound trapping data show that these flies could be making phonotactic choices based on relative intensity [table 2; but see Forrest and Raspet, 1994], the response to single pulses of 3 kHz suggests that the interneurons recorded here do not contribute to intensity coding as both the latency and I/O functions varied little with pulse intensity (the single afferent offers anecdotal evidence of such coding, however. fig. 6)

Discussion

Stemming from the difficulty in both capturing and rearing the host cicadas, sarcophagid acoustic parasitoids continue to be a largely inaccessible system, with most data collected using indirect methods. So far, studies of males, mating behavior, effects of parasitism on host reproductive behavior and risk of parasitism have been impossible. Given that the cicadas in our study area are very difficult to observe in the field, we also used an indirect method to assess host choice and sensitivity in a member of this group. Our data are compared to those of the only other homologous, fly-cicada system in which auditory behavior has been studied, *Emblemasoma auditrix - Okanagana rimosa*.

Fly Phonotaxis

At our study site, at least three species of cicadas are potential hosts for *Emblemasoma* sp.: *N. hieroglyphica*, *T. pruinosa* and *T. chloromera*. Assuming the model songs are accurate, *T. pruinosa* appears to be at least one host for *Emblemasoma* larvae: phonotactic host choice was biased to the *T. pruinosa* call and attracted only larvapositing females. Similar to the response of *E. auditrix* to artificial signals [Köhler and Lakes-Harlan, 2001], the success of the model *T. pruinosa* song also shows that the "wind-up" and "wind-down" sections of the call are not necessary for fly phonotaxis. The large response to the *T. pruinosa* call not withstanding, these data are not conclusive as to host specificity. Indeed, the data suggest that *T. chloromera* is also a potential host, as the model call attracted ~10% of the flies in paired broadcasts with *T. pruinosa*. In addition, flies were attracted throughout the day, overlapping the chorus time of *T. chloromera* and preceding the start of *T. pruinosa* chorus (see below). The data regarding song preference could also result from inaccuracies in the model calls, themselves. Thus, to conclusively answer the question of acoustic host specificity, measurement of the responses to playbacks of numerous calls of different males would be required [Kroodsma et al., 2001].

The spectra of the *T. chloromera* and *T. pruinosa* songs exhibit large overlap. Thus, in addition to providing insight into host choice, the relatively small response to the broadcast of the *T. chloromera* model song also shows that frequency band *per se* is not sufficient to elicit comparable levels of fly phonotaxis. The temporal and frequency modulation within the *T. pruinosa* song thus appears to have an effect.

Effects of Relative Song Intensity and Separation on Fly Attraction

Given the high potential of temporal overlap for *T. pruinosa* calls during the short duration chorus, it is predicted that decision rules for host choice that employ simultaneous comparisons of call parameters, such as intensity, are important. Numerous studies have shown that relative song intensity affects the phonotactic response of attracted insects [Forrest and Raspet, 1994; Wyttenbach and Farris, 2005], including parasitoid flies [Cade, 1979; Walker and Wineriter, 1991; Walker, 1993; Wagner, 1996]. There are four phonotactic decision rules that are based

on song intensity which could explain such results: active choice based on relative intensity at the receiver (loudness), active choice based on absolute intensity at the source, choose the closest male and respond randomly (i.e., go to any song above threshold; sometimes called passive attraction). A model of the responses generated by these decision rules shows that they can be distinguished by varying source separation and relative song power output [Forrest and Raspet, 1994]. Whereas varying the power output of the broadcasts is simple, varying source separation must be normalized to the phonotactic threshold of the receivers so that the amount of overlap in active space changes with separation. We used the most sensitive physiological threshold (69.2 dB SPL at 3 kHz) to set the separation of the two traps so that for the 10 m separation, the 109 dB SPL song had attenuated to threshold at the second speaker producing a normalized separation of 1.0 [normalized 5 m separation = 0.5; Forrest and Raspet, 1994]. For the 1.0 separation (and greater), the predicted response proportions are identical for all decision rules [Forrest and Raspet, 1994]. Thus, the response proportion at 10 m separation, $\Delta 6$ dB is important because it serves as a control for use of the threshold to set the normalized separation to 1.0. The response proportion for this condition (0.241 to quieter source; table 2) is nearly identical to that predicted by the model (0.25 to quieter source; $\chi^2=0.011$, P = 0.915), suggesting that, like *E. auditrix* [Köhler and Lakes-Harlan, 2001], ~69 dB SPL is indeed the response threshold in the field.

Unlike for larger relative separations (≥ 1.0), the predicted response proportions for the decision rules differ as the call sources are moved closer together [Forrest and Raspet, 1994]. For these conditions we have only two data sets, limiting our conclusions. Although both response proportions at the 0.5 relative separation are in good agreement with those predicted for phonotaxis based on loudness ($\Delta 3$ dB, 0.212 vs. predicted 0.17, P=0.5; $\Delta 6$ dB, 0.242 vs predicted 0.28, P=0.6) and appear to differ with those predicted for passive attraction ($\Delta 3$ dB, 0.212 vs predicted 0.5, P=0.003; $\Delta 6$ dB 0.242 vs predicted 0.353, P=0.089), more data are required to resolve which decision rule is employed.

Fly Auditory Physiology

A potential mechanism for improving signal detection is an increase in the signal-to-noise ratio through spectral filtering, in which best sensitivity is matched to the dominant frequencies of the signal [Green and Swets, 1974; Capranica, 1978]. This null hypothesis, is predictive, as a matched filter is commonly exhibited across vertebrates and insects [Gerhardt and Schwartz, 2001; Gentner and Margoliash, 2003; Schildberger, 1984]. Our results are consistent with this hypothesis, showing that the frequency sensitivity of interneurons in flies attracted to the *T. pruinosa* song is well matched to that of the call (fig. 5). As with *E. auditrix* [Lakes-Harlan et al., 1999], tuning in *Emblemasoma* sp. is not due to mechanical tuning of the tympanal membrane. Robert et al. [1999] used a subset of the flies in this paper to measure the mechanical response in the tympanal organs, revealing a broadband resonance from 1-30 kHz. Thus, the tuned physiological response shown here suggests that there is either intrinsic (e.g., physiological) tuning or subsequent mechanical mechanisms that filter stimuli around the *T. pruinosa* spectrum [see Lakes-Harlan et al., 1999]. Considering our results, the mismatch in tuning found in *E. auditrix* is not a characteristic of this taxon and, at least with respect to the *O. rimosa* song, phonotactic decisions by *E. auditrix* are made with less than optimal frequency information [Hennig et al., 2004]; such an interpretation is consistent with the broad tuning of the *E. auditrix* behavioral response [Köhler and Lakes-Harlan, 2001].

Although behavioral thresholds between *Emblemasoma* sp. and *E. auditrix* are similar (see above), comparisons between physiological thresholds are difficult, as those previous studies used different stimulus and threshold protocols [Lakes-Harlan et al., 1999, 2000; but see Stumpner and Lakes-Harlan, 1996]. *E. auditrix* is reported to be ~18 dB more sensitive at the best frequency. However, in contrast to the methods used here, *E. auditrix* thresholds were

calculated based on averaged responses to 20 repetitions and a longer duration stimulus (50 ms, producing 4 dB more energy / stimulus pulse).

Diel Singing Patterns and Parasitoids

Several abiotic and / or biotic factors vary with the diel behavior patterns of singing insects [Forrest, 1998; Loher, 1989; Walker, 1983; Römer, 1998]. In this study we found that chorus times in *T. chloromera* (pre-noon) and *T. pruinosa* (dusk) were correlated with certain environmental conditions (table 1). As shown for other members of the genus *Tibicen* [Hastings and Toolson, 1991], such correlations may reflect differences in the ability to thermoregulate, with cicadas that are endothermic singing at different times than those that are ectothermic, such as *T. chloromera* [Sandborn, 2000; Sanborn et al., 1995; 2003]. Whether singing in *T. pruinosa* and *T. chloromera* is physiologically restricted by environmental conditions or not, correlations between chorus timing and environmental conditions may also reflect use of these conditions as cues for timing behavior appropriately [Loher, 1972; Loher and Orsak, 1985; Alexander, 1960; Crawford and Dadone, 1979].

Diel variation in noise levels and signal transmission through the environment may also constrain singing times [Forrest, 1994; Römer, 1998]; increasing the reception of an acoustic signal by potential mates may be facilitated by signaling at times when there is less background noise [Greenfield, 1988; Doolan and Young, 1989; Walker, 1983; Sueur, 2002] or when signal propagation is maximum [Henwood and Fabrick, 1979; Young, 1981]. Indeed, noise in the form of concurrent signaling by heterospecifics creates acoustic interference [Latimer and Broughton, 1984; Bailey and Morris, 1986], causing choruses to shift to quieter times [Greenfield, 1988]. Although the temporal properties of the *T. pruinosa* and *T. chloromera* songs are quite different, their spectral ranges are similar (overlapping from ca. 2.5 to 6.0 kHz; fig. 1), potentially causing congeneric acoustic interference. Hypothetically, such a cost would favor the diel displacement in singing times observed in this study.

In addition to physiological and acoustical constraints, our data add a factor correlated to differences in diel chorus times: risk of attracting parasitoid flies. For the hosts of acoustic parasitoids, costs include reductions in reproductive behavior and increased mortality [Cade, 1984; Adamo et al., 1995; Allen, 1995]. Consequently, selection from acoustic parasitoids has affected male singing behavior in a variety of taxa [Kolluru, 1999; see Zuk and Kolluru, 1998 for review]. One potential counter-adaptation is a shift in the diel patterns of male singing behavior to times with reduced risk [Walker, 1983; Zuk et al., 1993; Allen, 1998; Bertram et al., 2004]. A second potential defense against acoustic parasitoids (and predators) is a reduction in the distribution of singing times, in which increased temporal aggregation (i.e., greater diel synchrony) may reduce the risk of parasitism through an encounter-dilution effect [Mooring and Hart, 1992; Alexander, 1975; Walker, 1983]. Several species of cicada are known to exhibit tightly timed, short duration choruses (e.g., 10-25 min; Alexander, 1960; Crawford and Dadone, 1979; Gogala and Riede, 1995]. Previously, however, there were few data describing any biotic correlates to these diel signaling patterns in cicadas, such as the attraction of acoustic parasitoids.

Our observations of fly attraction and the diel chorus patterns of *T. pruinosa* and *T. chloromera* suggest that selective pressure from *Emblemasoma* sp. may contribute to the daily timing of sexual signaling in male cicadas. Although our field study cannot resolve a cause-effect relationship between diel chorus timing and parasitoid attraction, the short duration chorus in *T. pruinosa* should at least function to avoid longer exposure to parasitoids that are clearly phonotactic throughout the day. Also, the significantly shorter duration of the *T. pruinosa* chorus relative to that of *T. chloromera* could represent the temporal analog of a selfish herd and reduce individual risk by increasing chorus size [Ryan et al., 1981].

Acknowledgements

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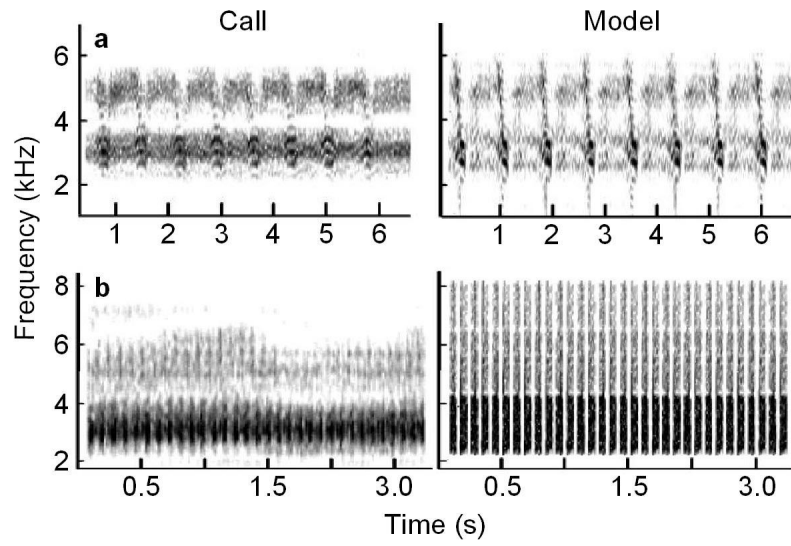
Figure 1.
Spectrograms of recorded and model calling songs of **a**) *T. pruinosa* and **b**) *T. chloromera*.
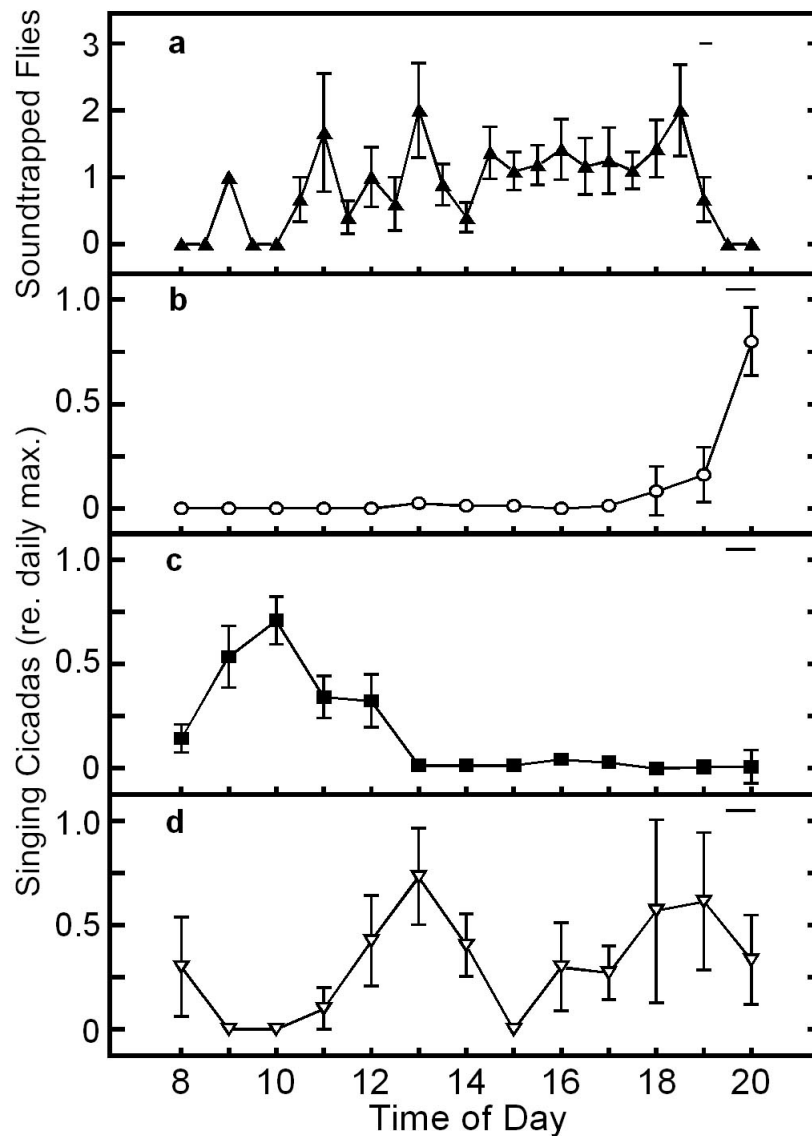
Figure 2.
a Mean (±S.D.) number of flies trapped at the continuous broadcast of a model *T. pruinosa* calling song at half-hour intervals on two different days (n = 44 flies). **b-d** Arithmetic mean proportion (±S.D.) of singing cicadas (**b** *T. pruinosa*; **c** *T. chloromera;* **d** *N. hieroglyphica*) relative to the time of day. No census was taken from 2100 to 0700 hrs.. Solid horizontal lines represent the range of sunset times for the data.
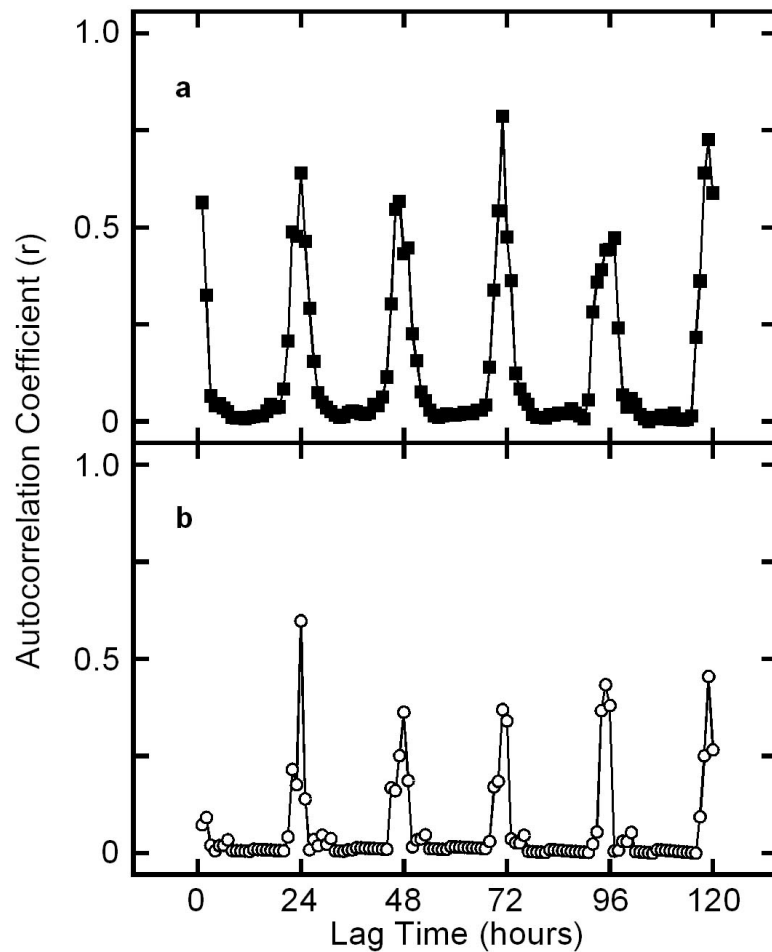
Figure 3.
Correlograms of the time series data for diel calling in **a**) *T. chloromera* and **a**) *T. pruinosa*. Each point represents the autocorrelation coefficient for each 1 hour lag of the first 120 hours of the observations (lags begin at 1 hr). The ten days of census were combined to form a continuous time series. To complete the 24 hours of observations, census levels of 0 were given to the hours of 2100 to 0700, when no cicadas called. Autocorrelation coefficient (r) values are greatest at a period of 24 hours for both species.
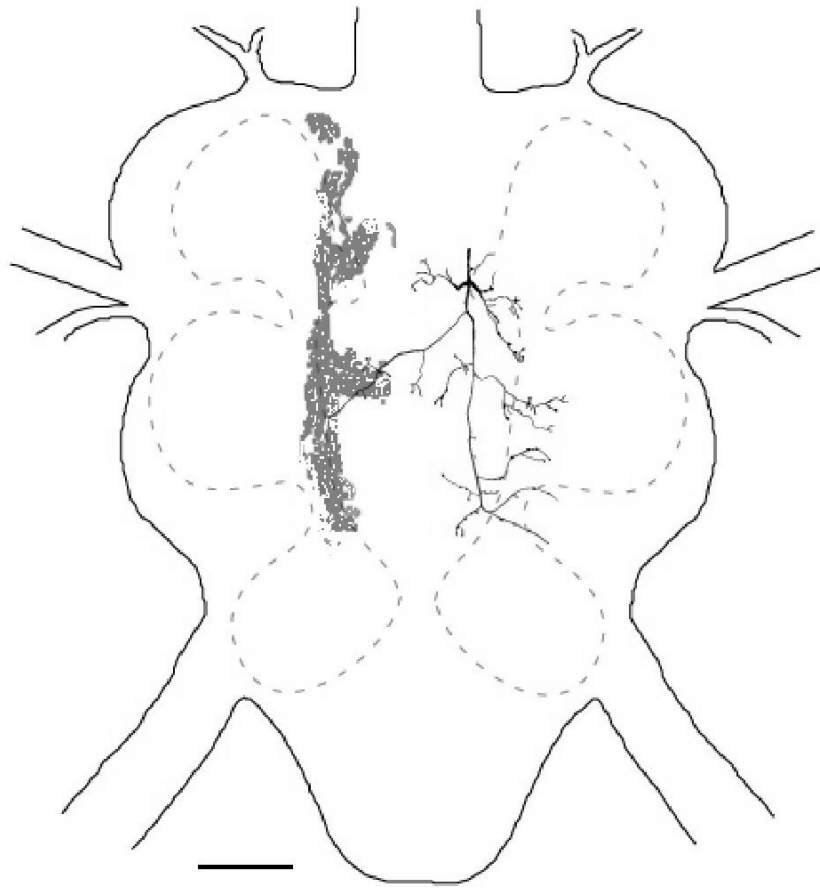
Figure 4.
Anatomy of an auditory interneuron *Emblemasoma* sp. (ventral view; top is anterior). Grey shaded fill is a trace from the backfill of the auditory nerve. Interneurons were identified by any contralateral projections. Scale bar is 100 µm.
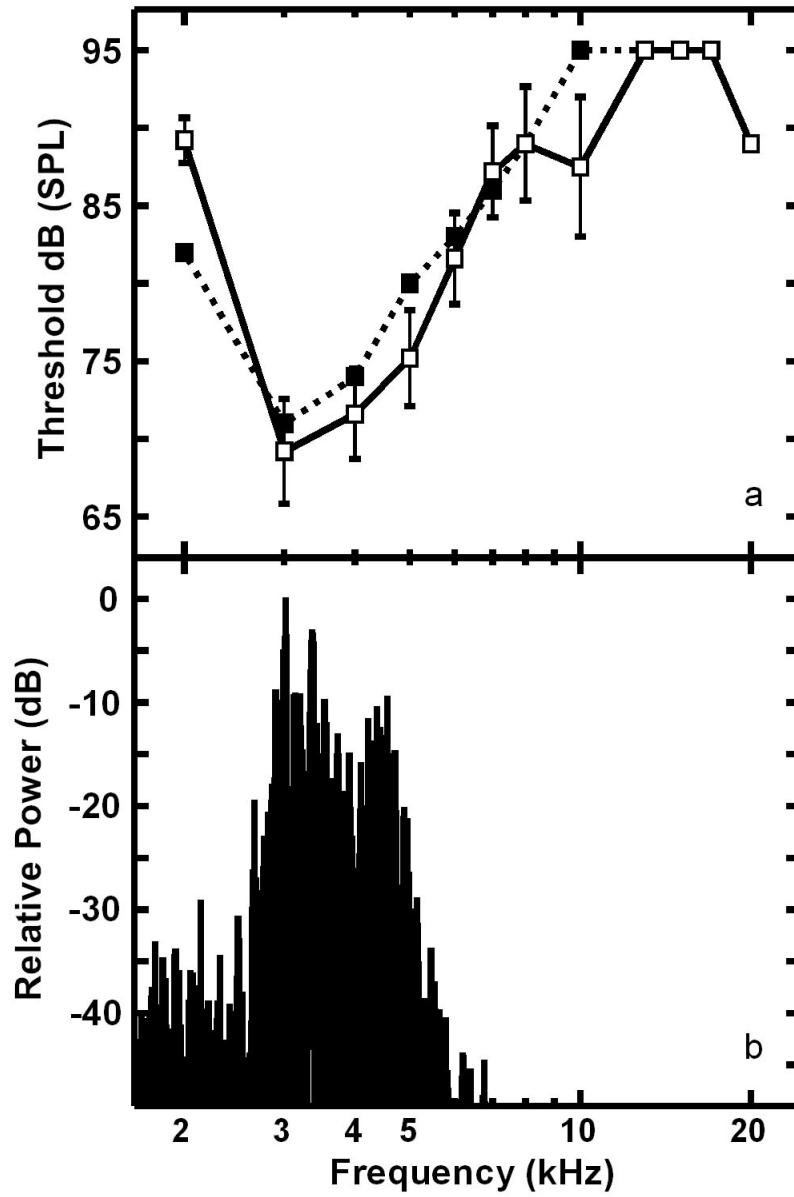
Figure 5.
Auditory tuning curves for *Emblemasoma* sp. **a** Mean stimulus amplitude (±S.E.) required to elicit a response (one action potential) in 3 / 5 presentations. Open and closed symbols are data from interneurons (n = 5) and afferents (n = 1), respectively. Thresholds for interneurons above 10 kHz are responses from only one cell. **b** Power spectrum of the *T. pruinosa* call.
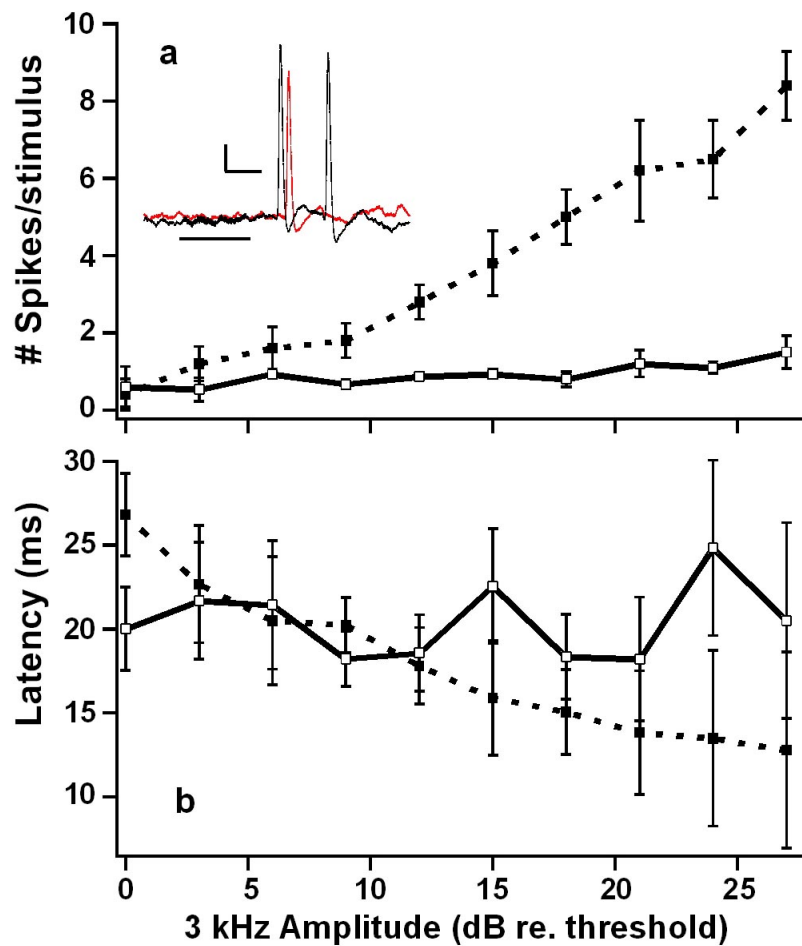
Figure 6.
Input / Output curves for 3 kHz stimuli. Mean (±S.D.) number of spikes (**a**) and latency (**b**) to first spike. Open and closed symbols are data from interneurons (n = 5) and afferents (n = 1), respectively. Inset: example of interneuron response to varying stimulus amplitude. Stimulus is a 20 ms pulse of 3 kHz at 71 dB SPL (red) and 95 dB SPL (black). Horizontal bar represents stimulus time. Scale: 10 mV, 10 ms.

Table 1

Correlations between environmental variables and proportion of males singing. Columns are the: species and environmental variable, the logistic regression parameter estimate (slope), the chi-square value and the *P*-value relative to a chi-square distribution with 1 df

Species variable	Parameter Estimate	χ^2 Value	p
T. chloromera (mid-day chorus)			
Light	0.0196	0.34	0.5586
ΔLight	0.6606	138.00	<0.0001
Temp	-0.0110	0.18	0.6741
ΔTemp	0.2111	20.19	<0.0001
T. pruinosa (dusk chorus)			
Light	-1.3307	121.04	<0.0001
ΔLight	0.5360	15.22	<0.0001
Temp	0.0534	0.69	0.4060
ΔTemp	0.0858	0.95	0.3302

Table 2

Relative attraction of flies to model *T. pruinosa* calling songs. Columns are the: calling song separation, calling song (CS) levels for the two songs (S.1 vs. S.2), relative intensity difference between the two songs (ΔdB), sample size of attracted individuals (N), proportion attracted to the quieter song (P(S.2)) and comparison of the response proportions to a random response using a chi-square goodness of fit test (1 df)

Separation (meters)	CS dB SPL @ 0.10 m S.1 vs. S.2	ΔdB	N	P(S.2)	Response comparison to random, χ^2 value	p
5	109 vs. 103	6	33	0.212	10.939	<0.001
5	109 vs. 106	3	33	0.242	8.758	<0.005
10	109 vs. 103	6	29	0.241	7.759	<0.01