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RESEARCH ARTICLE

Recognition of variable courtship song in the field cricket *Gryllus assimilis*

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SUMMARY

We analyzed the courtship song of the field cricket *Gryllus assimilis*. The song comprises two elements: groups of ca. 10 pulses (chirps) with low fundamental frequency (3.5–3.7 kHz) alternating with high-frequency (15-17 kHz) pulses (ticks) that usually occur as doublets. Some elements of courtship song are quite variable (high coefficient of variation) both within and between males, whereas others are more stereotypical. In experiments with playback of synthesized courtship songs, we studied the importance of several song parameters for mating success, which we evaluated as the probability with which females mounted muted, courting males. Altering some features that show little variability, such as chirp-pulse rate or carrier frequency of ticks, resulted in significant decreases in mounting frequency, consistent with the notion that trait values showing little variability are constrained by stabilizing selection exerted by females. However, alteration of one invariant trait, the occurrence of both song components, by omitting either component from test songs only slightly affected female responsiveness. Alteration of a variable song trait, the number of ticks per song phrase, had no effect on female response rate, thus failing to provide support for the idea that variable traits provide a substrate for sexual selection. An unusual characteristic feature of the song of *G. assimilis* is that chirp pulses often contain substantial high-frequency power, and indeed may entirely lack power at the fundamental frequency. Playback experiments showed that such songs are, nevertheless, behaviorally effective. To understand the neural basis for this, we recorded the responses of the two principal ascending auditory interneurons of crickets, AN1 and AN2. Our results suggest that the frequency selectivity of the neurons is sufficiently broad to tolerate the spectral variability of courtship chirps.

Key words: mating behavior, playback, stabilizing selection, auditory interneuron.

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INTRODUCTION

In singing Orthoptera, song is an important component of reproductive isolation. Bush-cricket, cricket and grasshopper males attract females from afar with species-specific calling songs (e.g. Heller, 1988; von Helversen and von Helversen, 1994; Ragge and Reynolds, 1998; Gerhardt and Huber, 2002). In most cricket species, the calling-song pattern is relatively simple, consisting of sequences of similar sound pulses. The songs of sympatric species usually differ in temporal parameters (e.g. number of pulses per sequence, pulse rate and pulse duration) and/or dominant carrier frequency, and numerous behavioral experiments in a variety of species have shown that both the characteristic temporal pattern and the carrier frequency play important roles in calling-song recognition (e.g. Walker, 1957; Elsner and Popov, 1978; Pollack and Hoy, 1979; Doherty and Hoy, 1985; Stout and McGhee, 1988; Hennig and Weber, 1997). It has been suggested that the calling songs of Orthoptera are subject to stabilizing selection (Fitzpatrick and Gray, 2001; Ferreira and Ferguson, 2002; Vedenina, 2005; Heller, 2006) for species identification and localizability. The calling song should provide species-specific identification and ease of localization, which often results in most song traits being relatively stable. Functional requirements may limit the variability of many song traits [static traits (*sensu* Gerhardt, 1991)]. At the same time, some calling-song traits, e.g. the chirp rate in chirping cricket species, may be highly variable. These parameters, 'motivational' according to Popov and Shuvalov (1977), or 'dynamic' according to Gerhardt (1991), have been suggested to be important for intraspecific competition.

In crickets and many other Orthoptera, distinct close-range signals including courtship song and chemical and tactile signals induce females to mate. Courtship songs are often more complex than calling songs. For example, in many cricket species, courtship songs contain two elements that differ in both temporal and frequency characteristics, whereas calling songs comprise only one element (Elsner and Popov, 1978). Calling songs of grasshoppers of the subfamily Gomphocerinae have at most two elements, but courtship songs of some species include five to seven different elements (Otte, 1972; Ragge and Reynolds, 1998; Vedenina and von Helversen, 2009). Courtship song may also be more variable than calling song (e.g. Zuk et al., 2008), and this can form the basis for female choice (Rebar et al., 2009).

Until now, only a few studies on only two cricket species (*Gryllus bimaculatus* and *Teleogryllus oceanicus*) have examined courtship song recognition (Burk, 1983; Libersat et al., 1994; Balakrishnan and Pollack, 1996; Rebar et al., 2009). In the present study, we characterize the spectral and temporal properties of courtship song in the field cricket *Gryllus assimilis*, and we assess the behavioral importance of various song parameters using synthesized song models in playback experiments. We focus in particular on the relationship between the variability of individual song parameters, both within and between males, and their importance for song recognition. We also recorded the responses to courtship songs of the two main ascending neurons

of crickets, AN1 and AN2, to learn how they might contribute to a female's decision of whether to accept a courting male.

MATERIALS AND METHODS

Crickets

Crickets, *Gryllus assimilis* (Fabricius 1775), were reared in plastic containers (60×40×44 cm) at 27°C, on a 14 h:10 h light:dark cycle, with *ad libitum* access to Purina cat chow and water. Males and females were separated before the final moult. One to two days before experiments, the crickets were individually isolated in inverted mesh-covered plastic cups, with food and water *ad libitum*. We used 3- to 10-day-old virgin females and 1- to 2-week-old males in all experiments.

Song recording and analysis

A male and a female were placed into a cylindrical (14×13 cm) open-top arena, the floor of which was a 15 cm Petri dish covered by a paper towel, and the walls of which were formed from aluminum screening. A microphone (Brüel and Kjær, type 4134, 0.5 inch; Nærum, Denmark) was placed at a height of 5–6 cm from the top of the arena. The output of a measuring amplifier (Brüel and Kjær 2610) was digitized (100 kHz sampling rate) using a National Instruments multi-function interface (USB-6212, Austin, TX, USA), controlled by MATLAB (MathWorks, Natick, MA, USA) programs. Temporal parameters and power spectra of the songs were analyzed with CoolEdit (Syntrillium, Seattle, WA, USA) and TurboLab 4.0 (Bressner Technology, Gröbenzell, Germany). We analyzed songs of 21 males. We measured 11 temporal and three frequency parameters (Table 1), each measured for 10 instances of the relevant parameter in the same song.

Behavioral experiments

Trials were performed within the first 4 h of scotophase in an anechoic chamber at 24–26°C, illuminated by a red light. Courtship consists of a series of stereotypical behaviors, including production of a courtship song, that, if successful, culminates in mounting of the male by the female (Loher and Dambach, 1989; Adamo and Hoy, 1994). We introduced a male into the arena and after he appeared calm (in 3–5 min), we introduced a female. If no contact occurred within 5 min, or if the male failed to produce courtship song within 5 min after contact, the trial was discarded. If the female failed to mount the male within 5 min after the beginning of courtship the trial was scored as 'no mounting'. We used each female only once; males were used in up to four trials. After each trial, the arena was rinsed with 70% ethanol and the paper towel was replaced to remove any olfactory cues that might have been left by the crickets.

Intact males were used as a positive control and muted males without playback were used as a negative control. Males were muted the day before trials by severing the right forewing proximal to the stridulatory file. In playback experiments, each time the muted male attempted to sing (as indicated by movements of the remaining forewing), a touch of the keyboard commanded the computer to play the test song from a loudspeaker mounted above the arena at a height of 54 cm. All trials were video recorded.

Courtship song structure and sound stimuli

The structures of test songs were based on recorded courtship songs of *G. assimilis*. Detailed description of the courtship song is presented in the Results. As in many species of *Gryllus* (Alexander, 1961; Zhantiev and Dubrovin, 1974; Rheinlaender et al., 1976; Fitzpatrick and Gray, 2001), the courtship song of *G. assimilis* consists of two distinct elements: pulses of low fundamental frequency (3.5–3.7 kHz) that are grouped into series ('chirps') and isolated pulses of higher fundamental frequency (16–17 kHz; 'ticks'). In a typical song, several chirps alternate with a pair of ticks (Fig. 1). The ticks are usually of higher amplitude than the chirps. We tested female responsiveness to 12 synthesized songs (see Figs 3–5), in which we altered the number of song elements, the pulse rate, the structure of the chirps, the number of ticks per phrase and the carrier frequency of both chirps and ticks.

Test songs were synthesized in MATLAB, emitted by a National Instruments multi-function interface (USB-6212) and routed through a calibrated attenuator (Tucker-Davis PA4, Tucker-Davis Technologies, Alachua, FL, USA) before being amplified and broadcast through a loudspeaker (CHR-70, Markaudio, Hong Kong, PRC). Peak intensities of chirps and ticks were 88 and 96 dB SPL, respectively; these values are 6–8 dB greater than the average amplitudes of these components in recorded songs.

Electrophysiology

Females, aged 7–12 days after the final molt, were anesthetized on ice and their wings, middle legs and hind legs were removed. They were mounted on a wax support, ventral side up, and the fore femora were fixed parallel to the body with wax. The prothoracic ganglion and cervical connectives were exposed by ventral dissection, supported on a metal platform and bathed in physiological saline

Table 1. Mean, standard deviation (s.d.) and coefficient of variation (CV) of courtship song parameters in *Gryllus assimilis*

		CV	
Song parameter	Mean ± s.d. (*N*=21)	Among individuals (%)	Within individual (%) (min.–max.)
Pulse duration (s)	0.007±0.0009	12	23 (11–41)
Pulse period (s)	0.014±0.0014	10	16 (7–27)
Pulse number per chirp	10.5±3.3	31	39 (13–71)
Chirp duration (s)	0.172±0.050	29	46 (16–77)
Chirp period (s)	0.431±0.070	16	22 (6–60)
Number of chirps per phrase	5.4±0.9	17	38 (0–101)
Chirp/tick amplitude	0.330±0.306	93	58 (0–104)
Tick duration (s)	0.008±0.001	13	18 (0–30)
Time between subsequent ticks (s)	0.109±0.041	38	20 (0–50)
Phrase period (s)	2.85±0.61	21	31 (0–85)
Tick number in a phrase	1.8±0.4	23	25 (0–49)
Tick dominant frequency (Hz)	16018±1295	8	6 (2–15)
Chirp fundamental frequency (Hz)*	3731±233	6	4 (2–9)
Chirp dominant frequency (Hz)*	4601±1148	25	44 (2–88)

*For these parameters, *N*=13.

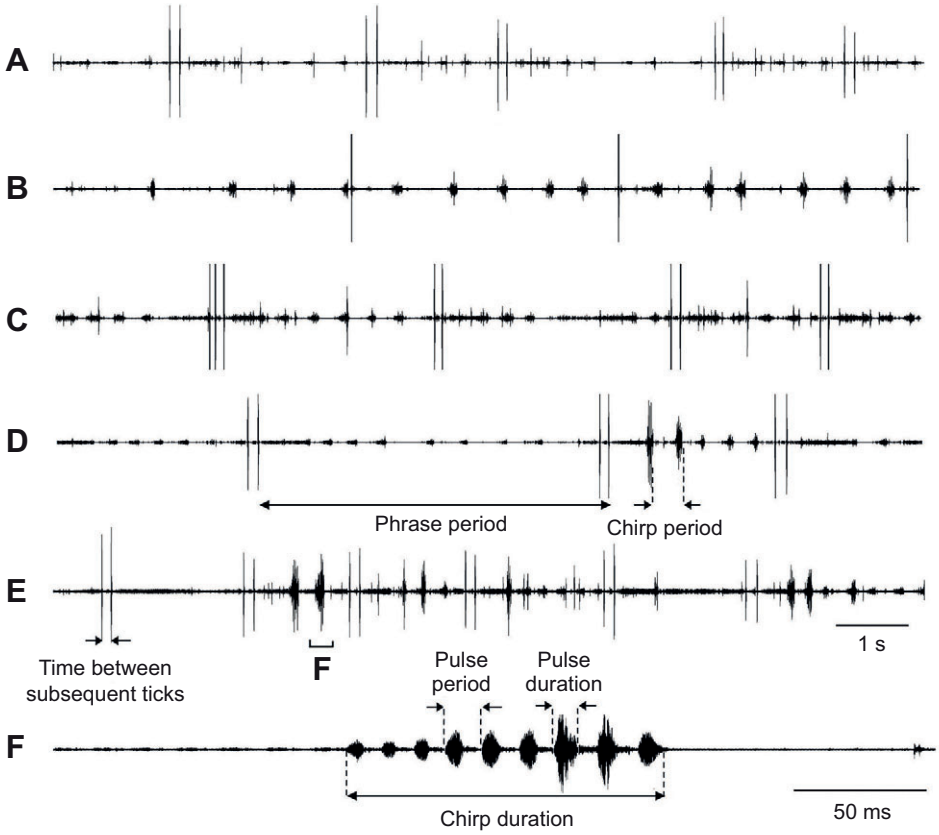

Fig. 1. Courtship songs recorded in five males (A–E) of *Gryllus assimilis*, illustrating its variability. Temporal parameters analyzed are shown in D–F. A fragment of the song shown in E is shown in F at an expanded scale.

(Strausfield et al., 1983). AN1 and AN2 were recorded extracellularly from the cervical connectives with tungsten hook electrodes. Extracellular spikes of AN1 are small in amplitude. To better resolve these, we separated the connective into medial and lateral bundles (after removal of the surrounding sheath) and recorded from the medial portion, which contains the axons of both AN1 and AN2. We also sectioned the ascending and descending connectives from the prothoracic ganglion to minimize background activity. Recordings were amplified with a Grass P15 amplifier (Astro-Med, West Warwick, RI, USA) and digitized for off-line analysis (16 bits, 10 kHz sampling rate; PCI-6251 analog-to-digital board, National Instruments) using custom MATLAB programs. Stimuli were broadcast from loudspeakers situated perpendicular to the cricket's longitudinal axis, ipsilateral to the recorded axons. To record responses to natural courtship song, we placed an arena containing a pair of crickets such that the courting male was approximately 3 cm from the electrophysiology preparation, a distance comparable to that between a courting male and an unrestrained female.

We quantified the strength of AN1 and AN2 responses to song models as the difference in the number of spikes produced in 100 ms windows before and after stimulus onset. To evaluate whether spike timing reflected the temporal pattern of the song model, we calculated the synchronization coefficient, which reflects the degree to which spikes tend to occur at fixed phases within the pulse–interpulse cycles of the chirp (Goldberg and Brown, 1969).

RESULTS

The courtship song of *G. assimilis*

The courtship song of *G. assimilis* consists of two components, which we refer to as chirps and ticks. Chirps are groups of low-amplitude sound pulses with mainly low dominant sound frequency, and ticks are single, higher-amplitude pulses with high dominant frequency. On average, a song phrase consists of five chirps followed by a pair of ticks, although this structure is quite variable (Fig. 1, Table 1).

Quantification of the song parameters, including both within-male and among-male variability, is presented in Table 1. The least variable among the temporal traits were the durations of chirp pulses and ticks, and the pulse period. For frequency parameters, variability was lowest for the dominant frequency of ticks and the fundamental frequency of chirps. The most variable character was the ratio of chirp to tick amplitude. Note that for the majority of traits, within-male variability was higher than among-male variability (Table 1).

The spectra of chirp pulses deserve special attention. Dominant frequency is quite variable among males (CV=25%) and even higher within individual males (44%; Table 1, Fig. 2). Some chirps consist of pulses with low (ca. 3.7 kHz) dominant frequency and less intense higher harmonics (Fig. 2A), whereas in others high frequencies were far more prominent (Fig. 2C,D). Pulses with substantial high-frequency components were also characterized by an irregular amplitude envelope. Frequency composition of pulses could even vary between successive pulses of the same chirp. For example, the third and fifth pulses of the chirp shown in Fig. 2D had a fundamental frequency of 3.8 kHz, with harmonics at 7.6, 11.4 and 15.2 kHz, whereas in the second and fourth pulses of the same chirp, the lowest frequency band was at 10 kHz. Nevertheless, in the 'average' chirp pulse, a low, fundamental frequency is dominant, with higher harmonics 10 to 15 dB less intense (Fig. 2E, filled squares).

In contrast with chirp pulses, the dominant frequency of ticks was quite consistent at approximately 16 kHz (Fig. 2B, Table 1).

Courtship song is required for female responsiveness

Males that were rendered mute by removing their right forewings showed normal courtship behavior. The latency between antennal

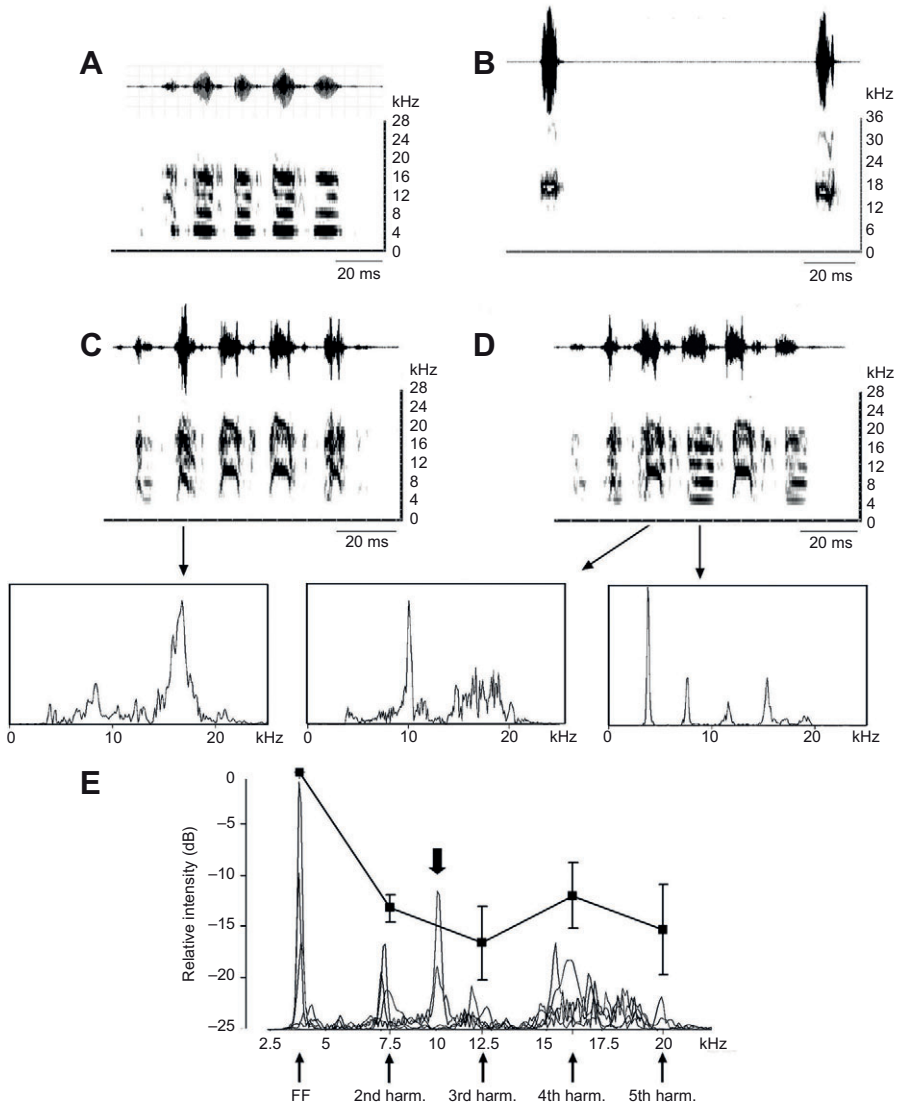

Fig. 2. Frequency characteristics of courtship song in *Gryllus assimilis*. (A–D) Sonograms of three chirps and two ticks of the courtship song; power spectra of three pulses are shown below. (E) Power spectra superimposed for all pulses of the chirp shown in D. The thick arrow indicates a peak at the dominant frequency of one of the pulse types in D, illustrated in the central power spectrum, which does not correspond to any of the harmonics of the dominant frequency of the other pulse type, illustrated in the right-most power spectrum. Squares in E show mean (±95% confidence intervals) values of the relative intensity levels of the harmonics for the songs of 13 males.

contact and body rocking, a component of courtship, did not differ between intact and mute males (Mann–Whitney *U*-test, U=3948, N intact=67, N mute=124, P=0.57), nor did latency between antennal contact and singing (U=3948; N intact=72, N mute=130, P=0.16). However, courting mute males had little success in eliciting mounting responses (Fig. 3A). The mounting frequency in response to mute males (28%) was significantly different from that to intact males (87%; Fisher's exact test, two-tailed, $P<0.001$). Mounting latency did not differ between females that mounted mute males (63±36 s) and those that mounted intact males (42±8 s; Mann–Whitney *U*-test, U=156, P=0.16).

We tested the importance of various song elements for female responsiveness in playback experiments using computer-generated song models. Female mounting frequency varied significantly among these models (χ^2=27.53, $P<0.004$ as determined by Monte Carlo simulation with 10,000 permutations). In the following, we assess the effectiveness of individual test songs through pair-wise comparisons of mounting frequencies with those of the positive and negative controls described above. For ease of comparison, the data for intact and mute males shown in Fig. 3 are replicated in Figs 4 and 5. We divide the 12 song models tested into two groups, depending on whether they manipulated mainly temporal or spectral song characters.

Importance of temporal parameters of courtship song

Stimulus 1 approximated normal courtship song. It comprised a phrase of seven chirps, each with seven low-frequency (3.5 kHz) pulses that alternated with two high-frequency (17 kHz) ticks (Fig. 3). The numbers of pulses per chirp and of chirps per phrase, although not equal to those of the mean song (Table 1), were well within the ranges of these parameters produced by courting males. The amplitudes of successive pulses within the chirp varied in a manner resembling that of the natural song. Playback of this stimulus to accompany courtship of muted males was as effective as courtship by intact males in eliciting mounting responses (83.3 *vs* 86.8%; Fisher's exact test, P=0.74; Fig. 3).

Stimuli 2 and 3 tested whether both song elements are required for successful courtship. Stimulus 2 was identical to stimulus 1 except that the ticks were omitted, and in stimulus 3 the chirps were omitted. Response rates to these stimuli were lower than that to the positive control (70.8 and 66.7%, respectively), although this was significant only for stimulus 3 (P=0.034). Responses to both stimuli were significantly greater than those to silent, courting males. Thus either song element can, by itself, elicit nearly normal levels of mounting.

Courtship song of *G. assimilis* differs from that of other *Gryllus* species described so far in that it typically contains a pair of ticks

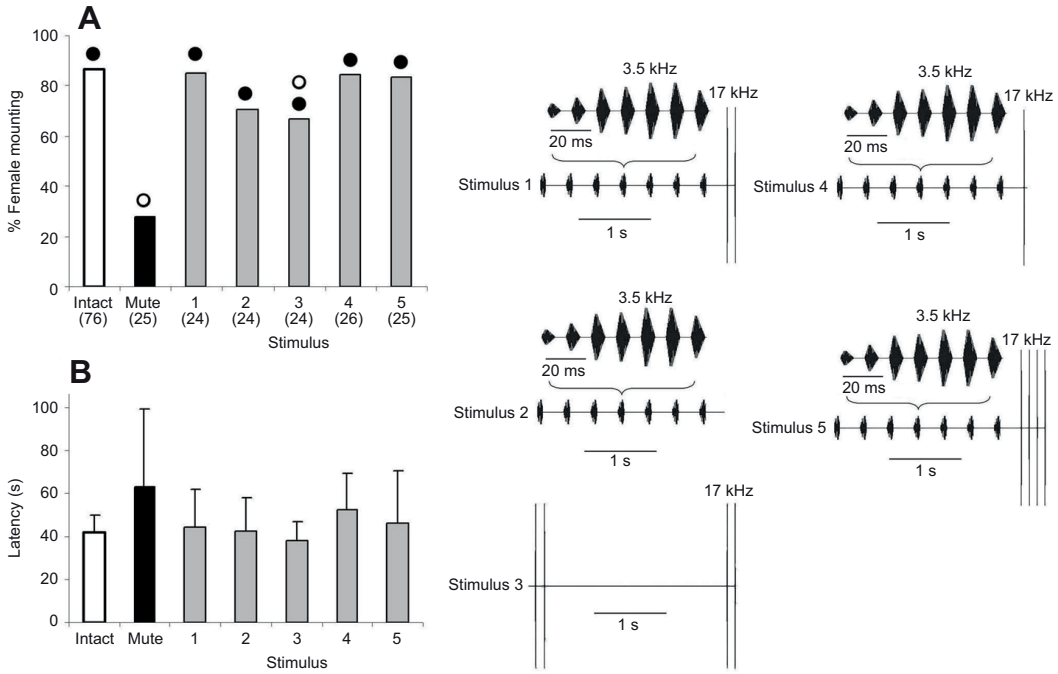

Fig. 3. The importance of chirps and ticks for female responsiveness in *Gryllus assimilis*. (A) Percent of females that mounted males. Black circles above the bars indicate significant differences from the negative control (mute males); open circles indicate significant differences from the positive control (intact males) (P<0.05). Sample sizes are shown in brackets. Response frequencies are shown for courtship by intact males, by muted males with no playback ('mute'), and by muted males accompanied by playback of the test songs shown at the right. (B) Latency (mean ± s.d.) from onset of courtship song to the mounting response.

per phrase, rather than only one. Stimuli 4 and 5 were tested to evaluate the importance of tick number; these contained, respectively, one and four ticks. Both of these stimuli were as effective as stimulus 1 and as courtship by intact males (Fisher's exact test, P>0.74 for all pair-wise comparisons; Fig. 3).

Mounting latencies did not differ for responses to the stimuli shown in Fig. 3, including those to the positive and negative controls (Kruskal–Wallis test, H=4.9, P=0.55; Fig. 3B).

The chirps of courtship song resemble the calling song of *G. assimilis*, although the pulse period in courtship song, 14 ms, is somewhat longer than that in calling song, ca. 12 ms (Weissman et al., 2009). In view of the general importance of pulse period for recognition of cricket calling songs (Gerhardt and Huber, 2002; Hedwig and Pollack, 2007), we tested how changes in the courtship-chirp temporal structure affect stimulus effectiveness. In stimulus 6, the pulse period was twice the normal pulse period. In stimulus 7, the pulsed structure of the chirp was eliminated by replacing the pulse sequence with a tone, the amplitude envelope of which resembles the normal pulse-to-pulse variation in amplitude. Stimulus 8 retained the normal pulse rhythm, but the pulses were continuous rather than being grouped into chirps (Fig. 4). All of these stimuli were intermediate in effectiveness; they were significantly less effective than the positive control (stimulus 6: 60.9%, P=0.013; stimulus 7: 54.2%, P=0.001; stimulus 8: 66.7%, P=0.03), but more effective than the negative control (P=0.04, 0.08 and 0.01, respectively). Mounting latencies did not differ for the stimuli illustrated in Fig. 4 (Kruskal–Wallis test, H=7.6, P=0.1; Fig. 4B).

Importance of frequency parameters of courtship song

Changing the frequency of ticks from their normal value of 17 to 3.5 kHz resulted in poor responsiveness, which did not differ significantly from the negative control (stimulus 9, P=0.08; Fig. 5A). Surprisingly, the mounting frequency to stimulus 9, with 'incorrect' ticks (52%), was lower than that to stimulus 2, which lacked ticks entirely (71%), although the difference was not significant (P=0.37). Similarly, changing the carrier frequency of chirps from 3.5 to 17 kHz (stimulus 10) resulted in a response level (33%) similar to that of the negative control and significantly lower than that of stimulus 3 (67%, P=0.04), which lacked chirps. These results suggest that songs with a spectrally 'incorrect' song component are less effective than songs that lack that component entirely.

As shown earlier, the spectra of natural chirp pulses are variable and complex, with differing relative contributions of low- and high-frequency components. Stimuli 11 and 12 mimic some of these

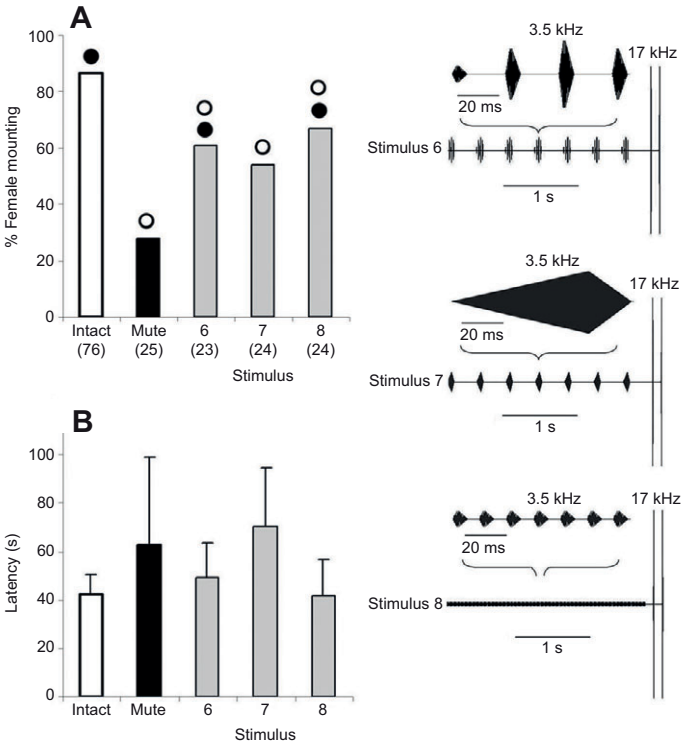

Fig. 4. The role of chirp structure in *Gryllus assimilis*. (A) Percent of females that mounted males. Symbols are as in Fig. 3. Data for intact and mute males are replotted from Fig. 3 for ease of comparison. Sample sizes are shown in brackets. The test songs are shown at the right. (B) Response latency.

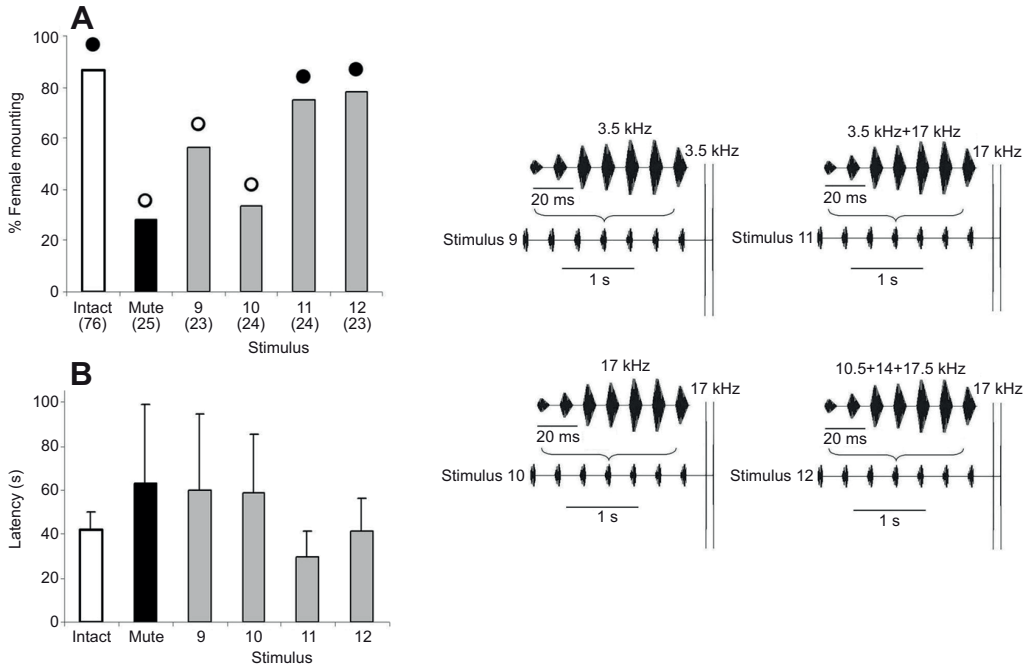

Fig. 5. The importance of frequency parameters for female responsiveness in *Gryllus assimilis*. (A) Percent of females that mounted males. Symbols are as in Fig. 3. Data for intact and mute males are replotted from Fig. 3 for ease of comparison. Sample sizes are shown in brackets. The test songs are shown at the right. (B) Response latency.

natural chirp-pulse types. In stimulus 11, chirp pulses include a low-frequency fundamental (3.5 kHz) and its fifth harmonic, and are spectrally similar to the pulses shown in Fig. 2A. Chirp pulses of stimulus 12 consist only of a series of higher harmonics of 3.5 kHz (third–fifth), with no power at the fundamental frequency, and are similar to some of the pulses shown in Fig. 2C,D. Both of these stimuli resulted in response levels (75 and 78%, respectively) that were similar to that elicited by intact courting males (P>0.2).

As for the previous stimuli, mounting latencies did not differ for responses to stimuli 9–12 (Kruskal–Wallis test, H=9.1, P=0.17; Fig. 5B). Nevertheless, when considered across all of our experiments, there was a nearly significant trend towards a negative correlation between mounting frequency and response latency (Spearman rank correlation, r=–0.52, N=14, P=0.06; Fig. 6).

Responses of auditory neurons

Our findings on the role of chirp-pulse spectrum in song effectiveness are puzzling. Songs with chirps that contain 3.5 kHz are highly effective (Fig. 3, stimuli 1–4), even if a 17 kHz component is also present (Fig. 5, stimulus 11), whereas songs with chirps containing only 17 kHz are ineffective (Fig. 5, stimulus 10). However, a song containing a series of harmonics from 10.5 to 17 kHz is highly effective, despite the absence of a low-frequency component. To understand the physiological basis for these findings, we recorded the responses of the two principal ascending auditory neurons of crickets, AN1 and AN2, which carry to the brain information about low and high sound frequencies, respectively (Wohlers and Huber, 1982; Hennig, 1988). Chirps with 3.5 kHz pulses (stimulus 1) evoked robust responses in both AN1 and AN2 when played at an intensity of 80 dB SPL, similar to that produced by courting males (Fig. 7A,D). Our manipulations of chirp temporal pattern (Fig. 4) showed that the pulse pattern is an important feature for song effectiveness, and indeed this is represented in the responses of both neurons as periodic variations in firing rate (Fig. 7D). Chirps composed of the series of higher harmonics (stimulus 12) when played at high intensity also elicited clear, pulse-coding responses from both AN1 and AN2, despite the absence of power at 3.5 kHz (Fig. 7B,E). Chirps with 17 kHz pulses (stimulus 10) evoked strong responses from AN2 that clearly captured the pulse pattern, whereas AN1 responded only weakly, with poor temporal coding (Fig. 7C,F). These results suggest that patterned activity of AN1, perhaps accompanied by that of AN2, is required for chirps to be attractive. Results similar to those shown in Fig. 7 were obtained for 11 of 13 specimens tested.

Although AN1 responses were clearly evident upon visual inspection, quantification was possible only for a subset of experiments, in which the relatively small AN1 spikes could reliably be separated from other activity (Fig. 8). With increasing intensity of stimulus 1, the response of AN1 remained constant, whereas AN2 showed an increasing response (Fig. 8A). By contrast, with increasing intensity of stimulus 12, responses increased for both neurons (two-way ANOVA, AN1, intensity effect, P=0.066; stimulus effect, P<0.005; interaction, P=0.025; AN2, intensity effect and stimulus effect, both $P<3\times10^{-6}$, interaction, P=0.384; Fig. 8B). At an intensity similar to that produced by courting males (80 dB SPL), AN1 responses did not differ for the two stimuli (paired t-test, P=0.48), whereas AN2 responded more strongly to stimulus 12 (P=0.016).

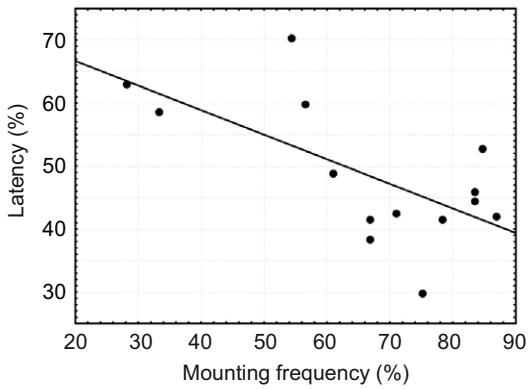

Fig. 6. Correlation between mounting frequency and response latency in *Gryllus assimilis*. Points show mounting frequencies and mean response latencies for the 12 test songs and for responses to intact males and to muted males with no playback.

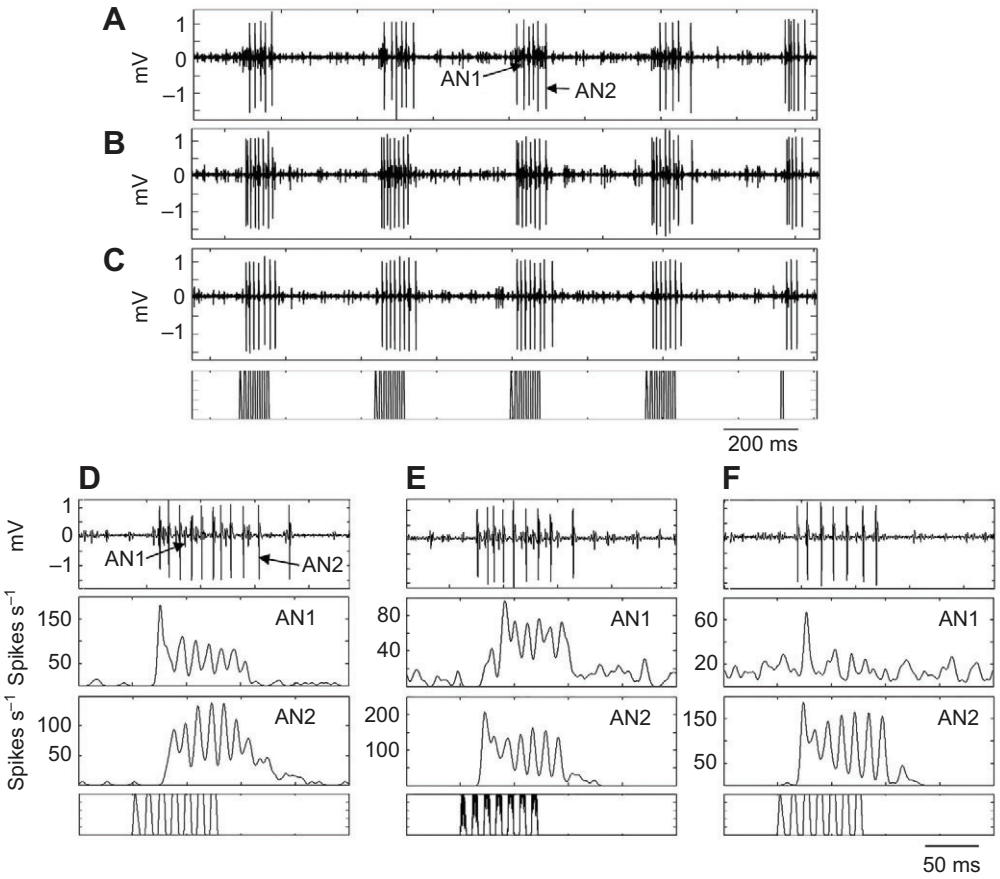

Fig. 7. Responses of the AN1 and AN2 of *Gryllus assimilis* to three test stimuli presented at 80 dB. (A–C) Example spike trains; the segment shown includes responses to the last four chirps and the first tick of the song phrase; the stimulus is shown below C. (A) Chirp-pulse frequency: 3.5 kHz; tick: 17 kHz; (B) chirp-pulse frequency: 10.5+14+17.5 kHz; tick: 17 kHz; (C) all pulses 17 kHz. (D–F) Example spike trains and firing rates of AN1 and AN2 shown at a faster time scale. Each panel shows the response to a single chirp (top), the firing rate averaged over 34 chirps for AN1 and AN2 (middle) and the stimulus envelope (bottom). Firing rate was calculated by converting each spike train to a series of ones (when spikes occurred) and zeros, and convolving this with a Gaussian kernel with standard deviation of 2 ms. Chirp-pulse frequency: (D) 3.5 kHz, (E) 10.5+14+17.5 kHz and (F) 17 kHz. Small and large spikes are produced by AN1 and AN2, respectively. The jagged stimulus envelope in E is the result of the periodicity produced by adding the three frequency components.

The synchronization coefficients (SCs) of responses of AN1 were lower than those for AN2 (three-way ANOVA on rank-transformed SCs, neuron effect: P=0.024; Fig. 8C). Although SCs were generally rather modest overall (the maximum possible value, indicating perfect synchronization, is 1.0), they were nevertheless statistically significant in most cases (Table 2).

The response of AN1 to the harmonics-only stimulus could reflect the breadth of its tuning, or it could be due to a low-frequency distortion product generated by the superposition of higher harmonics, such as has been described in other insect ears (e.g. Coro and Koessl, 1998; Warren et al., 2009). To examine this, we presented stimuli consisting of only one or the other of the third and fourth harmonics, i.e. 10.5 or 14 kHz. In five of six crickets tested, AN1 as well as AN2 responded to both of these stimuli presented at 80 dB SPL (Fig. 9C,D); in the sixth cricket, only AN2 responded. Thus, although we cannot rule out a contribution of distortion products to AN1's response, this is not necessary to account for the response to chirps composed of the third through fifth harmonics.

AN1 and AN2 also responded to natural courtship song produced by an intact, courting male positioned close to the neurophysiology preparation. Low-amplitude chirps elicited strong responses in AN1 but only occasional action potentials in AN2 (Fig. 10A), but both neurons responded well to high-amplitude chirps (Fig. 10B,D). Ticks elicited responses only from AN2 (Fig. 10C).

DISCUSSION

Variability of courtship song in *G. assimilis*

The courtship song of *G. assimilis* is more variable than the courtship songs of other cricket species so far described (Balakrishnan and Pollack, 1996; Fitzpatrick and Gray, 2001; Zuk et al., 2008). Seven of 14 courtship song parameters studied in *G. assimilis* had among-male CV values in excess of 20%. In contrast, only two of nine courtship characters studied in *G. texensis* and *G. rubens* and one of eight characters measured in *T. oceanicus* had CV values exceeding 20% (Balakrishnan and Pollack, 1996; Fitzpatrick and Gray, 2001). Those characters that are variable in *G. assimilis* are rather consistent in *G. texensis* and *G. rubens*. For example, the relative amplitude of chirps and ticks, the most variable character in *G. assimilis*, shows little variability in *G. texensis* and *G. rubens*. The number of pulses per chirp is more variable in *G. assimilis* than number of pulses per phrase in *G. texensis* and *G. rubens* (in which the low-frequency portion of the song is not organized into chirps). At the same time, there are similarities between the songs of these species. Interphrase interval in *G. texensis* and *G. rubens* and phrase period in the *G. assimilis* song (parameters that are likely to be correlated) are relatively variable, whereas pulse period is quite constant in all three species (CV≈10%), as well as in the courtship song of *T. oceanicus* (Balakrishnan and Pollack, 1996).

Factors affecting song recognition

Summarizing evidence from frogs, Gerhardt (Gerhardt, 1991) found that among-male variability of some acoustic properties of advertisement calls exceeded within-male variability, providing a possible substrate for sexual selection. Similarly, courtship songs of *T. oceanicus* are highly repeatable within individuals and playback experiments using recorded songs of successful and unsuccessful males show that females prefer the former (Rebar et al., 2009). In *G. assimilis*, CVs were generally greater within males than among males (Table 1), suggesting that courtship song in this species may play little if any role in female choice. Consistent with this, we found no evidence for directional selection, a signature of sexual selection, acting on highly variable traits. For example, the

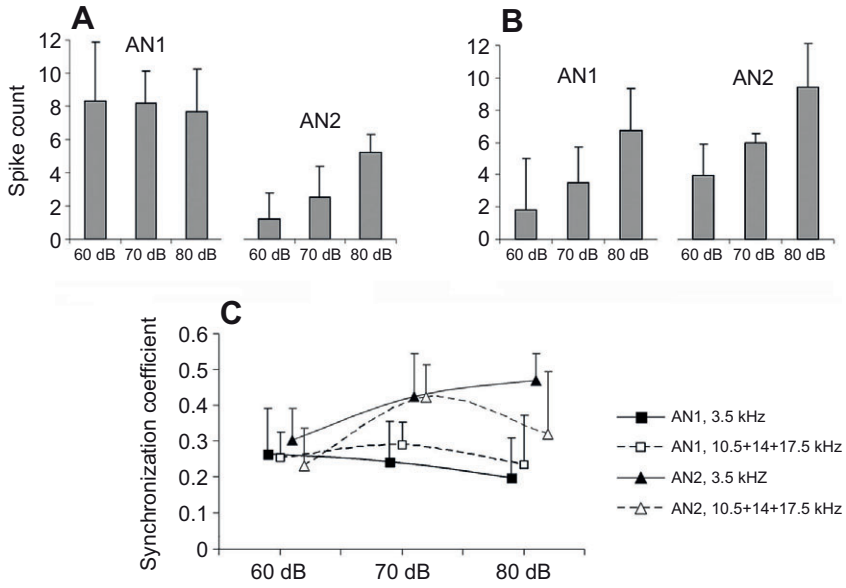

Fig. 8. Spike counts and synchronization coefficients (SCs) of responses of AN1 and AN2 in *Gryllus assimilis*. Results for each preparation (N=3–6) are the means ± s.d. of responses to 34 chirps. Spike counts are calculated as the difference between counts for 100 ms periods before and after the onset of each chirp. (A) Responses to the chirp-pulse frequency 3.5 kHz. (B) Responses to the chirp-pulse frequency 10.5+14+17.5 kHz. (C) SCs, calculated by assigning spikes to 2.3-ms-wide bins within the intra-chirp pulse–interpulse cycle. Responses and SCs are shown for AN1 and AN2 at three different intensities. In C, the data for SC are displaced slightly on the x-axis to enhance visibility.

number of ticks per phrase is quite variable (within- and among-male CVs were 23 and 25%, respectively), yet females responded nearly equally to songs containing zero, one, two or four ticks. Similarly, chirp duration is highly variable (among- and within-male CVs, 29 and 46%, respectively), yet both increasing this (stimulus 8; continuous pulses) and decreasing it to zero (stimulus 3, ticks only) resulted in modest decreases in responsiveness.

Variation of the dominant frequency among different pulses in a courtship chirp of *G. assimilis* is of special interest. Such variation has not been described for any other cricket species. In courtship songs of other species, chirps or pulses alternating with ticks are always of low dominant frequency, which is usually similar to the dominant frequency of the calling song. Higher harmonics in low-frequency courtship pulses were documented for *T. oceanicus* (Harrison et al., 1988; Libersat et al., 1994; Balakrishnan and Pollack, 1996) and in several species of *Gryllus* (Nocke, 1972; Rheinlaender et al., 1976; Fitzpatrick and Gray, 2001); however, in all cases, they were shown to be considerably less pronounced than the fundamental. In *G. assimilis*, the higher harmonics are not only pronounced in some pulses, but the low-frequency component may be absent entirely, yet this has no effect on female responsiveness.

The most constant characters in *G. assimilis* songs are the two frequency parameters, tick dominant frequency and chirp fundamental frequency. Changing the carrier frequency of the ticks from 17 to 3.5 kHz resulted in a significant decrease of mounting response, as did the converse change in frequency of chirp pulses from 3.5 to 17 kHz. However, as noted above, the low-frequency component is not an absolute requirement for successful courtship.

Among temporal traits, chirp-pulse period is the least variable character (among-male variation was 10% and within-male variation was 16%). In behavioral experiments, changing the pulse period (stimulus 6) or pulse structure (stimulus 7) resulted in significant decreases in mounting frequency, consistent with the idea that traits showing relatively little variability are important for song recognition.

Another very consistent character of *G. assimilis* song is the presence of both chirps and ticks; both elements occurred in all of the courtship songs that we analyzed. Nevertheless, either element could be omitted entirely from a test song with only slight detrimental effect. Similar results were reported previously for *T. oceanicus*, in which one song component, the chirp, was sufficient to evoke normal mounting, and the other component, the trill, was only partially effective (Balakrishnan and Pollack, 1996). In *G. bimaculatus*, high-amplitude, high-frequency ticks were shown to be a crucial component of a successful song, whereas the role of a less intense, low-frequency component, analogous to the chirps of *G. assimilis*, was not tested (Libersat et al., 1994). Balakrishnan and Pollack (Balakrishnan and Pollack, 1996) pointed out that although the acoustic components of a 'missing' song element are absent in playback experiments, other components, such as vibrations of the substrate or body, would still be presented by the muted, courting male, and that this might contribute to the effectiveness of these partial songs. This applies to our experiments as well.

Physiological mechanisms underlying recognition of the courtship song frequency parameters

There are two principal ascending auditory neurons in crickets: AN1, which is tuned to the fundamental frequency of calling song, and AN2, with broad tuning to high frequencies (e.g. Wohlers and Huber, 1982; Hennig, 1988). AN1 has been studied with respect to its role in relaying information about calling song to the brain (e.g. Schildberger and Hörner, 1988), whereas the behavioral roles proposed for AN2 are recognition of courtship song (Rheinlaender et al., 1976; Harrison et al., 1988) and bat evasion (Nolen and Hoy, 1984). With respect to courtship song recognition, the emphasis has

Table 2. Proportion of preparations with statistically significant (P<0.05) synchronization coefficients, as determined by Rayleigh's Z-test

Neuron	Stimulus (kHz)	Intensity (dB SPL)	No. significant/ no. tested
AN1	3.5	60	5/5
		70	4/4
		80	4/5
	10.5+14+17.5	60	2/3
		70	3/3
		80	5/5
AN2	3.5	60	2/3
		70	3/4
		80	5/5
	10.5+14+17.5	60	5/6
		70	4/4
		80	4/5

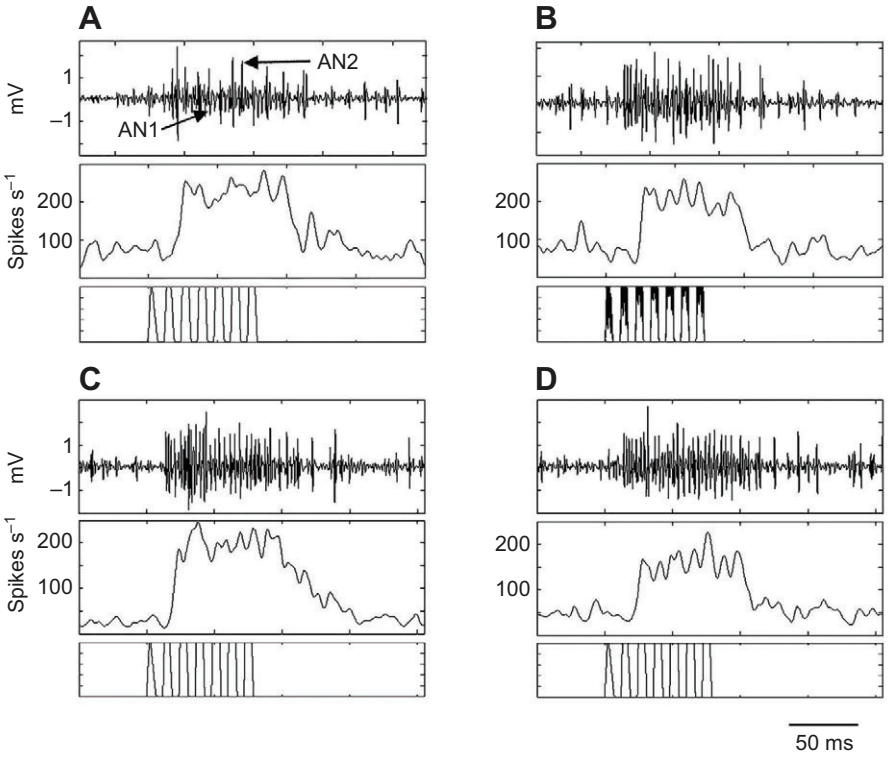

Fig. 9. Responses of the AN1 and AN2 of *Gryllus assimilis* to four types of chirps presented at 80 dB. Each panel shows an example recording (top), firing rate of AN1 averaged over 34 chirps (middle – see Fig. 7 legend for details) and the stimulus marker (bottom). Chirp-pulse frequency: (A) 3.5 kHz, (B) 10.5+14+17.5 kHz, (C) 10.5 kHz and (D) 14 kHz.

been on how the loud, high-frequency ticks are detected. Except for in studies on *T. oceanicus*, which has no ticks in its courtship song, the role of low-frequency pulses and, correspondingly, of AN1, has been neglected. Comparison of our behavioral and electrophysiological results suggests that to be behaviorally effective, chirps must elicit temporally patterned activity of AN1, and possibly also of AN2. Although AN1 is often described as being sharply tuned to the species-typical low frequency, its selectivity varies among species (Schmidt et al., 2011), and in *G. assimilis* tuning is sufficiently broad that even 14.5 kHz sound pulses are effective when presented at behaviorally relevant intensities. Similarly, chirps consisting of only low-frequency pulses can stimulate AN2 as well as AN1. Activity of AN2 alone is not sufficient for chirp recognition, because a song with 17 kHz chirp pulses (stimulus 10), which stimulates only AN2, was completely ineffective. Thus, activation of a 'chirp recognizer' by AN1, possibly with simultaneous input from AN2, might form part of the song recognition mechanism. Nevertheless, this is not a strict requirement for recognition, because a song consisting only of high-frequency ticks with no chirps is only slightly less effective than the complete song.

The high-frequency ticks of natural songs stimulate only AN2, suggesting that this neuron might provide input to a 'tick recognizer'. As for chirps, however, this input is not required for song recognition, because a chirps-only song is quite effective.

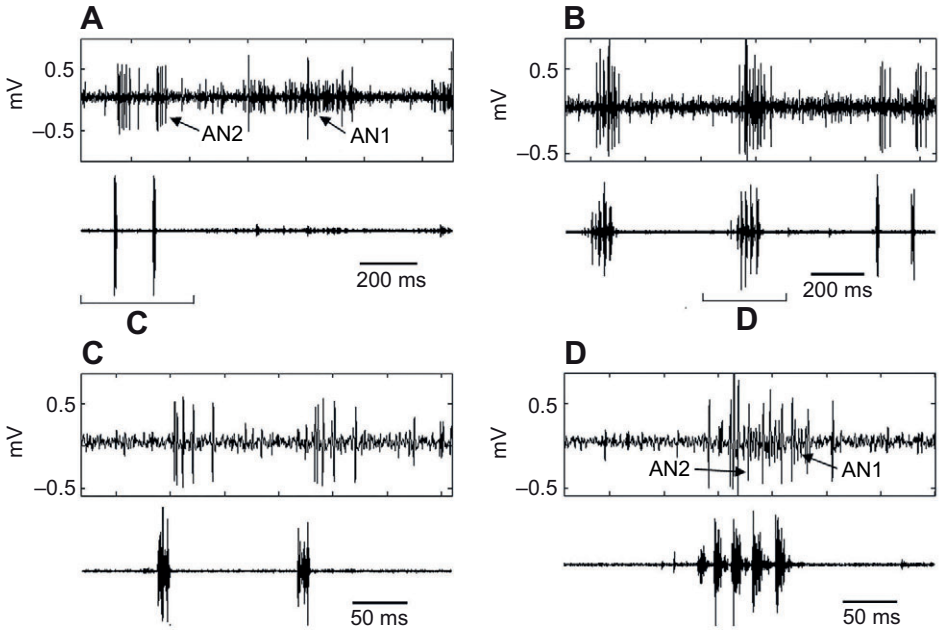

Fig. 10. Representative responses of AN1 (small action potentials) and AN2 (large action potentials) to courtship by two *Gryllus assimilis* males (A,C and B,D, respectively). Recordings are shown at two different scales. C and D show responses to a pair of ticks (C) and a chirp (D).

Our results also suggest that inappropriate activity of AN1 and AN2, rather than simply being ignored by the recognition mechanisms, might in fact be inhibitory. For example, the response frequency to a stimulus that drove AN2 at the chirp-pulse rate, without accompanying activity of AN1 (stimulus 10), was significantly lower than that to a stimulus with no chirps (stimulus 3). We note that the pulse rate of chirps, ca. 70 Hz, is within the range of echolocation calls emitted by hunting bats as they approach their prey (Jones and Holdereid, 2007), raising the possibility that activation of bat-detecting circuits might contribute to the inhibition of responsiveness to courtship.

‘Sensory permissiveness’?

The ‘sensory exploitation hypothesis’ proposes that the characteristics of sensory systems may drive the evolution of signal structure (Ryan et al., 1990; Ryan, 1998). A signaler’s trait may be favored by selection because it fits preexisting features of the receiver’s sensory system. The classical example is the preference for the male call in the female tungara frog *Physalaemus pustulosus*. Female basilar papilla tuning is biased toward lower-than-average frequencies in one of the two elements of the male’s call, the ‘chuck’, explaining female preference for the lower-frequency chucks produced by larger males (Ryan et al., 1990).

We showed that the spectral characteristics of chirp pulses of *G. assimilis* are surprisingly variable, with some pulses entirely lacking the low fundamental frequency that characterizes non-tick pulses of courtship songs of other *Gryllus* species. Females respond to song models mimicking these pulses as readily as to models with only low-frequency chirp pulses. Our electrophysiological experiments suggest that this can be understood in terms of the tuning of AN1. In contrast to the sensory exploitation hypothesis, we suggest that courtship communication in *G. assimilis* may be an example of ‘sensory permissiveness’, in which broad tuning of the sensory system allowed a relaxation of selection pressure for precise control of sound frequency.

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