

Frequency as a releaser in the courtship song of two crickets, *Gryllus bimaculatus* (de Geer) and *Teleogryllus oceanicus*: a neuroethological analysis

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Abstract. 1. The courtship behavior of male field crickets, *Gryllus bimaculatus* (De Geer) and *Teleogryllus oceanicus*, is a complex, multimodal behavioral act that involves acoustic signals (a courtship song; Fig. 1A,B). The dominant frequency is 4.5 kHz for *T. oceanicus* song (Fig. 1A) and 13.5 kHz for *G. bimaculatus* (Fig. 1B).

2. When courting males are deprived of their courtship song by wing amputation, their courtship success declines markedly but is restored when courting is accompanied by tape-recordings of their courtship songs or a synthetic courtship song with only the dominant frequency of the natural song; other naturally occurring frequency components are ineffective for restoring mating success (Figs. 4, 5).

3. It has been suggested that an identified auditory interneuron, AN2, plays a critical role in courtship success. Chronic recordings of AN2 in an intact, tethered female show that AN2's response to the natural courtship song and synthesized songs at 4.5 and 13.5 kHz is similar in *T. oceanicus*. By contrast, in *G. bimaculatus*, AN2's response to the natural courtship song and synthesized song at 13.5 kHz, but not at 4.5 kHz, is similar (Figs. 2,3).

4. In behavioral experiments, playback of a 30 kHz synthetic courtship song in *G. bimaculatus* does not restore courtship success, yet this same stimulus elicits as strong a response from AN2 as does the normal courtship song (Fig. 6). Thus, contrary to earlier work by others, we conclude AN2 is not, by itself, a critical neural link in the courtship behavior of these two species of crickets.

Key words: Cricket – Courtship – Acoustic interneuron – Neuroethology – Playback

Introduction

Male and female field crickets are solitary, nocturnal insects. Males hold territories that they defend against oth-

er males and from which they emit their calling song. The calling song advertises a male's readiness to mate and is species-specific. Females fly and/or walk to calling males. When a female is attracted and arrives on a male's territory, the male usually initiates courtship behavior. In most field crickets, including *Gryllus bimaculatus* (De Geer) and *Teleogryllus oceanicus*, courtship is multimodal and involves by tactile, chemical, and acoustic signals (von Hörmann-Heck 1957; Loher and Rence 1978). Von Hörmann-Heck (1957) and Burk (1983) demonstrated that courtship song is important for eliciting acceptance behavior - copulatory mounting - by the female in both *G. bimaculatus* and *T. oceanicus* species.

The courtship song of *T. oceanicus* is similar to its calling song in its spectral and some temporal properties, but slightly lower in intensity. The major difference between them is in temporal pattern: the calling song consists of a 5–6 pulse chirp followed by a series of 8–12 doublet pulses, while the courtship song consists of the chirp followed by a long continuous series of softer pulses, the trill (Fig. 1A). Like the calling song, the fundamental frequency, as well as the dominant frequency, of the courtship song is 4.5 kHz, with harmonic frequencies of lower intensity up to at least 32 kHz (Fig. 1A).

By contrast, in *G. bimaculatus*, the courtship song differs markedly from the calling song in both spectral and temporal properties (Rheinlaender et al. 1976). The stereotyped calling song consists of a chirp of about five loud pulses, each separated by an interpulse interval of about 30 ms, producing a spectral peak around 4.5 kHz, with lower intensity harmonics around 9–10 kHz and around 13–15 kHz. The harmonics are approximately 25 dB SPL lower in peak amplitude than the fundamental frequency (unpublished observations). The more variable courtship song is lower in intensity and has a higher proportion of energy above 11 kHz (Fig. 1B). The courtship song consists of two types of sound pulses: lower-intensity, 4.5 kHz pulses, and much higher-intensity pulses with energy at 4–5 kHz and 11–16 kHz. The interpulse interval of the larger-amplitude pulses is ca. 300–400 ms (Fig. 1B).

Abbreviations: AN2, ascending neuron 2; SPL, sound pressure level

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Given the spectral variety within each courtship song of the two species of crickets, as well as the specific differences between them, an opportunity was presented for investigating the role of individual frequency components by using pure tone synthetic songs in playback experiments. While earlier investigators (Burk 1983; Crankshaw 1979) used playback of natural courtship songs to determine their role in mating success, decomposed and simplified electronic songs have not previously been utilized. Clearly, it is important to know whether the entire song is necessary or whether certain critical frequency components can be dissected out, and this was one goal of our investigations.

The neural basis of acoustic behavior of the female during courtship is poorly understood, but some correlations between the frequency selectivity of courtship signals and neural sensitivity can be made. In *G. bimaculatus*, an identified, prothoracic, auditory interneuron, HF1AN (Popov and Markovich 1982), is known to be sensitive to high frequencies, and its best-frequency coincides with the high-frequency peak of the power spectrum of the larger-amplitude, natural courtship song pulses. This interneuron is also known as AN₂ (Wohlers and Huber 1982), and HF₁ (Rheinlaender et al. 1976) in *G. bimaculatus*, and is probably homologous to interneuron-1 in *Teleogryllus oceanicus* (Moiseff and Hoy 1983). Both Rheinlaender et al. (1976) and Popov and Markovich (1982) have previously demonstrated that this interneuron responds strongly to natural courtship song for *G. bimaculatus*. Harrison et al (1988) showed that this same acoustic neuron codes the courtship song in *T. oceanicus* and they refer to the neuron as AN2. For clarity, we will refer to this acoustic neuron as AN2 in this manuscript.

This investigation focused on two objectives. First, we re-investigated the question of whether courtship song was necessary to elicit mounting by the females, and therefore to ascertain its role in reproductive success in these two species of crickets. In particular, can simplified electronic models of the natural courtship song effectively substitute for natural song in eliciting the female's acceptance behavior? Our second objective was to examine the possibility that AN2 is the critical neural link in courtship song recognition and, thus, in the copulatory behavior of female crickets, as proposed by Harrison et al. (1988). To test this, we have performed two kinds of experiments which were designed to draw parallels between the efficacy of a given courtship song to restore mating success to a muted male, and the ability of that same song to elicit an excitatory discharge in AN2. Thus, we recorded chronically the activity of AN2 in tethered females, standing on a platform, that were being courted by a singing male, or by a muted male whose courtship was accompanied by the playback of an electronic song model. Then, we tested the courtship success of muted males with a playback of synthesized song at specific harmonic frequencies of the courtship song. The results of such experiments show that AN2 is not sufficient alone to initiate copulatory acceptance in females.

A preliminary account of this work has been previously reported (Murray and Hoy 1988; Hoy and Libersat 1989).

Materials and methods

Animals. Male and female *Teleogryllus oceanicus* and *Gryllus bimaculatus* were taken from an established laboratory colony. They were raised at 26°C and at high density on a 14L:10D light cycle. Food (Purina Cat Chow) and water were provided ad libitum.

Behavioral procedures. For our baseline normal courtship experiments males and females were separated as last-instar nymphs. One to two weeks before the final molt to adulthood, individual males and females were isolated and reared in separate containers. Two to 3 weeks after the final molt to adulthood, courtship tests were carried out on single pairs of crickets, each individually identified with paint marks. The identity of all males (and females) by their paint markings permitted us to keep track of individuals through the various procedures of muting and playback tests. All behavioral experiments were performed in an anechoic room at 21 ± 1°C.

In control, muted, and playback tests, single male-female pairs were introduced into a 19 cm diameter, screen-bottomed arena. To reduce the role of visual signals, illumination was provided only by a 25 Watt red bulb at 40 cm, which was sufficient to permit the experimenter to observe behavioral interactions. The beginning of courtship was defined as the moment when the male began to stridulate (scissoring movements of his forewings to produce courtship song). Courtship was recorded as a "success" if the female mounted the male within 5 min from the beginning of the emission of courtship song. When courtship was completed, the male was removed from the test chamber and anesthetized by cooling. He was muted by cutting off the forewings just anterior to the stridulatory file. After 1–3 days, the same pair was re-introduced into the arena. For muted males, the beginning of courtship was defined as the moment when the male made scissoring movements of his forewing stubs, medially and laterally, at the approximate courtship song pulse rate. The male was allowed to court the female for up to 5 min when the pair was removed and courtship was recorded as a "failure", or until the female mounted ("success"). One to three days after the muted courtship trial, the same pair was again introduced into the arena. When the male began the wing motions of courtship, a tape-recorded courtship song was played from below the screen-bottom of the arena for 5 min or until the female mounted. The playback was continuous and not stopped before 5 min even if the male stopped moving his wing stubs. The intensity was matched to the approximate sound pressure level (dB SPL) of the natural song pulses or their harmonics (see Results section) by standardizing the intensity of each synthesized stimulus tape to the same SPL at 30 cm. Pairs were divided into 3 groups and each group was played a different courtship song during the playback treatment part of the experiment. Otherwise, the 3 groups were treated similarly. One group of pairs was played the males' own, natural courtship song. A second group was played a 4.5 kHz synthetic courtship song. The third group was played a 13.5 kHz synthetic courtship song. In all three parts of the experiment (normal, muted, playback), the pair was separated and removed after mounting and before transferral of the spermatophore.

Sound analysis of the songs and acoustic stimulation. The songs of *Teleogryllus oceanicus* and *Gryllus bimaculatus* were recorded in the laboratory. A single male-female pair was introduced into a bag-like wire mesh container 20 by 15 cm and placed in a sound proof anechoic cage (1.2 × 0.8 × 0.8 m). The song was recorded with a 0.25 inch condenser microphone (B&K 4135) attached to a Bruel & Kjaer type 2209 SPL meter. The output of the B&K sound level meter was recorded on a Nagra IV-D recorder whose tape speed was 15 inches/s. The frequency response of the tape recorder at this speed is flat up to 30 kHz. Sound spectra were analysed from these tapes with a Kay Elemetrics real time sound analyser. Statistical analysis for the average power spectra of the courtship songs, shown in Fig. 1, was performed only on data expressed in millipascals (linear scale) after conversion from dB (Log scale). The sound pressure level was determined at two distances: (1) as near to the female as possible without touching her, when she was tethered and

being courted by a calling male, and (2) at a distance of 30 cm from the wings of the singing male using the B&K microphone, connected to a Bruel and Kjaer 2209 sound level meter, aimed directly at the male's dorsal surface. In order to calibrate the power spectra of the songs, the sound pressure level was measured both with and without a band octave filter centered around the song's dominant frequency.

Tape recordings of natural courtship song were recorded with a Nagra IV S recorder and played back through a Nagra IV L or S tape recorder. 5 min samples of the courtship song of each male were recorded, beginning from the initiation of courtship singing until the female attempted to mount, at which time the pair was momentarily separated; successive bouts (usually 3 to 6) of courtship song were so recorded until 5 min of sound were acquired. Synthetic courtship songs were generated from a Hewlett-Packard 350D audio oscillator. Pulse trains were generated by a Master 8 stimulator and fed into a custom built pulse shaper and filtered by a Krohn-Hite 3550 band-pass filter. These songs were tape recorded and used as a playback stimulus. The duration and intensity of the synthesized sound pulse and the pulse repetition rate of the pulse train were based on the harmonic and temporal characteristics of natural courtship song (see Results section). All acoustic recordings and playbacks were done at a tape speed of 15 inches/s.

Sound pressure levels are expressed as peak values in dB relative to $2 \times 10^{-5} \text{ N/m}^2$ as measured with a 0.25 inch condenser microphone (Bruel & Kjaer type 4135) attached to the Bruel & Kjaer type 2209 SPL meter. Playback of natural and synthetic courtship songs was made through the Nagra IV S recorder, amplified with a power amplifier (Nikko NA790), and Realistic 8.89 cm piezoelectric tweeters.

Chronic recordings of AN2. Females were first immobilized by chilling and then fixed dorsal side up on a wax platform for the surgery. Then, we gently pulled the head of the animal forward and exposed a large portion of the neck cuticle. The neck cuticle was opened with scissors and the gut gently displaced to the side with fine forceps to expose the neck connectives. In most experiments, the gut was removed by cutting it just posterior to the subesophageal ganglion and pulling it out from a slit made in the abdomen. At this point, the female was fixed with wax to a rod by the pronotum and mounted onto a metal holder. The cricket was placed inside an observation and recording box ($1.2 \times 0.8 \times 0.8 \text{ m}$), that was lined with 9 cm foam wedges to reduce echoes. A large platform ($15 \times 10 \text{ cm}$) provided a "walking" surface for the tethered female. The platform was then adjusted such that the animal was maintained in a normal walking posture. One of the two cervical connectives was gently split longitudinally by means of a sharpened tungsten wire in order to isolate auditory axons in the lateral half of the cervical connective. Hook electrodes were positioned around this bundle of axons and vaseline was applied to improve the signal to noise ratio, as well as to reduce damage to the preparation by desiccation. The auditory fibers were identified by their frequency sensitivities, measured in 5 kHz steps from 5 to 30 kHz at 80 dB. When the physiological characterization indicated that the pair of AN2/AN1 or AN2 alone had been isolated with a good signal to noise ratio, we then introduced a male on the platform, alongside the female. After antennal contact with the female, the male usually initiated a courtship song. In such experiments we recorded both the response of an acoustic neuron in the female, and the male's courtship song. The extracellular response and the temporal parameters of the song (natural or playback) were recorded on a Vetter model D FM instrumentation tape recorder set at a tape speed of 15 inches/s for subsequent off-line analysis.

Playback of synthetic courtship songs was conducted through the Nagra IV S recorder with a Realistic SA-10 stereo amplifier through a Realistic 8.89 cm piezoelectric tweeter placed above the arena and via a Hewlett-Packard 350D attenuator. Sound pressure levels were adjusted with the B&K sound level meter.

In both the behavioral tests and the chronic recordings of AN2, the sound pressure level of all playbacks (natural song, 4.5, 13.5 and

30 kHz songs) was adjusted relative to the *peak* sound pressure level in dB relative to $20 \mu\text{Pa}$ (dB SPL) at the female. The significance level criterion is a $P = 0.05$ for all statistical tests unless indicated otherwise.

Results

Behavior

Typically, after a pair of crickets was introduced into the arena, both the male and the female explored the periphery of the arena, with much antennation. After this exploratory phase, the pair approached each other and if the female antennated the male, she usually chased the male away unless he produced the courtship song. After the male initiated the courtship song the female usually followed him for about a minute as he walked slowly around the arena courting. If the female stopped following him, the male sometimes changed from courtship song to bouts of calling song. If the female appeared to be responsive, the male stopped both his walking and his singing, backed up under the female, and attempted to attach a spermatophore to her genitalia. Failures usually occurred when the female did not follow the male while he was emitting courtship song. We observed no obvious differences in the vigor and quality of courtship among the courting males of our three situations: (1) normal, intact males, (2) males muted by wing amputation, and (3) playback-added-to-courtship males, other than the absence of sound from males whose wings had been amputated.

Courtship songs

In order to make synthetic models of courtship song, we recorded the courtship songs of each of the two species of crickets and characterized the temporal properties and the power spectrum of each song.

For *Teleogryllus oceanicus*, the courtship song (Fig. 1A; right panel) consists of a chirp of 5–10 sound pulses each $33 \pm 4 \text{ ms}$ (mean \pm SD) in duration with a pulse interval of $72 \pm 3 \text{ ms}$ that lasts for roughly $700 \pm 170 \text{ ms}$, followed by a trill of briefer sound pulses ($19 \pm 12 \text{ ms}$ in duration with a pulse interval of $24 \pm 2 \text{ ms}$) that lasts for roughly 3 s. The chirp phase of the song ($104 \pm 2 \text{ dB}$, measured at female) is louder than the trill phase of the song ($95 \pm 1.8 \text{ dB}$, at female). The trill phase of the courtship song appears important for courtship success for the following reasons: (a) during a courtship interaction, the duration of the trill phase of the courtship song would often increase in duration to such an extent that the male might attempt to copulate during his trill, and before producing another chirp; (b) Burk (1983) showed that males producing only the chirp part of the song were not accepted by females. Therefore, we analysed the frequency content only of the trill part of the song, and in the synthesized song, temporal and frequency parameters were based on those of the trill phase of the natural song. The power spectrum of the trill is rich in harmonics which extend at least up to 28 kHz, but is

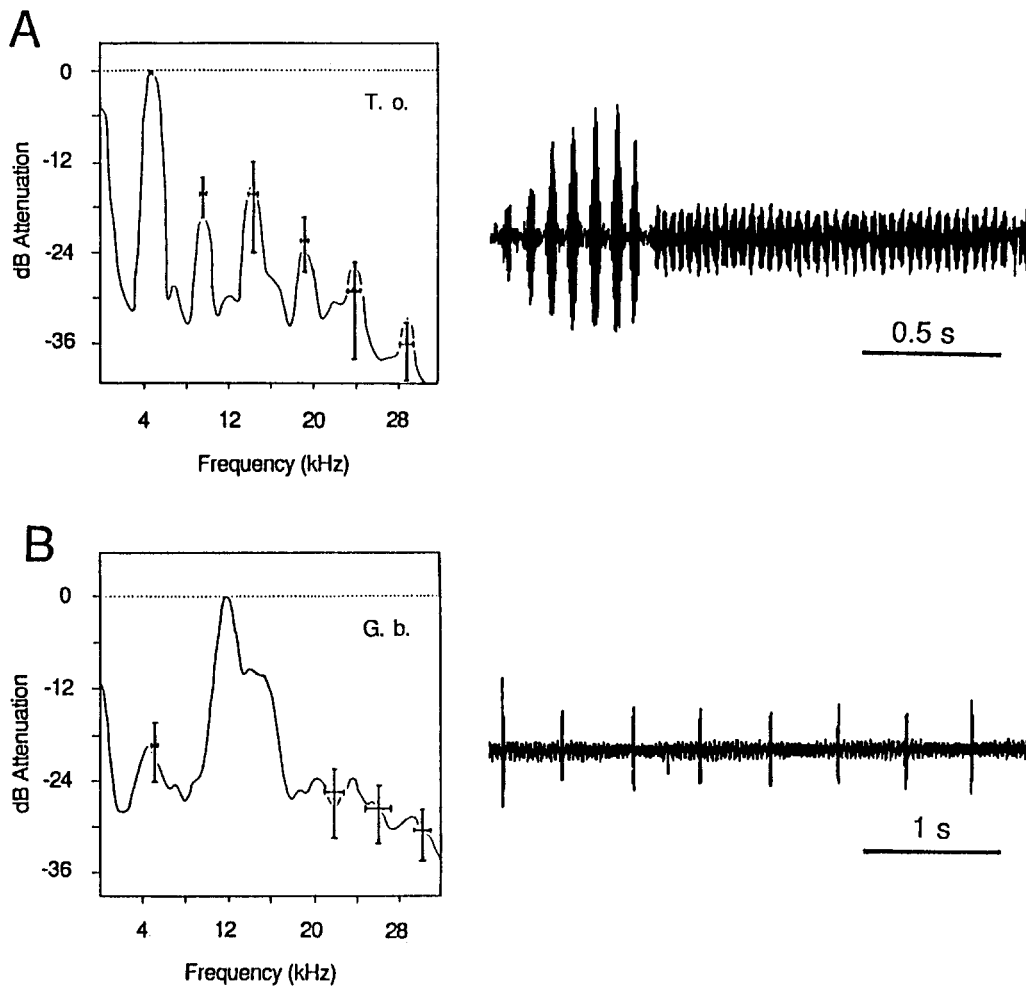


Fig. 1. Courtship songs of *T. oceanicus* (A) and *G. bimaculatus* (B). **A Right panel:** Oscillogram of a courtship song bout from a male *T. oceanicus*. The courtship song consists of a chirp of large amplitude sound pulses followed by a trill of lower amplitude sound pulses. **Left panel:** Spectral properties of courtship song (average power spectrum in dB SPL relative to the peak of the power spectrum). The curve is a power spectrum of a single representative courtship pulse taken from the trill phase of the courtship song shown on the oscillogram (right panel). Each *point* represents the average of 28 trill pulses analysed from four individuals. The *error bars* indicate the standard deviation of the frequency and the amplitude of the peaks in the power spectrum. The energy peak occurs at the first harmonic of the song (around 4.8 kHz) with a second (9.6 kHz) and third harmonic (14.3 kHz) of roughly the same amplitude. Harmonics in the ultrasonic domain (19.1 kHz; 23.8 kHz; 28.6 kHz) are clearly visible at up to at least 30 kHz. **B Right panel:** Oscillogram

of a courtship song bout from a male *G. bimaculatus*. The courtship song contains larger-amplitude pulses interspersed with many smaller-amplitude pulses. The larger-amplitude pulses have energy concentrated around 11–16 kHz and around 4–5 kHz (*left panel*). As in A, the left panel shows a power spectrum of a representative large courtship pulse taken from the courtship song shown in the right panel. Each *point* represents the average of 28 courtship pulses analysed in 4 individuals. The energy peak occurs around the third harmonic of the song (around 11–16 kHz) with a second clear peak at the first harmonic (around 4.9 kHz). The power spectrum of the larger-amplitude courtship pulses was variable but on average, the third harmonic was 20 dB higher than the 4.9 kHz component. In *G. bimaculatus*, harmonics in the ultrasonic domain (21.5 kHz; 25.9 kHz; 30 kHz) are not clearly defined. In left panels of A and B, the spectrum is truncated beyond 32 kHz due to the limitations of the tape recorder

dominated by the fundamental frequency (Fig. 1A; left panel). The amplitude of the fundamental frequency, or first harmonic, of the trill phase (4.7 kHz) was measured at 95 dB SPL at the female while the second (9.6 kHz; 83 dB), third (14.3 kHz; 82 dB) and fourth (19 kHz; 75 dB) harmonics are lower in intensity.

For *Gryllus bimaculatus*, the courtship song is simpler. It contains larger-amplitude pulses, 97 ± 1.8 dB measured at the female. These pulses are emitted at a rate of approximately 3/s, interspersed with many (5–25) smaller-amplitude pulses (Fig. 1B; right panel). The larger-amplitude pulses are 5–10 ms long and have energy concentrated around 11–16 kHz (dominant frequency) and

around 4–5 kHz (fundamental frequency), as shown in Fig. 1B (left panel). The smaller-amplitude pulses are 4–6 ms long and have a spectral maximum at 4–5 kHz, which was also noted earlier by Popov and Markovich (1982). The power spectrum of the larger-amplitude courtship pulses was variable but the 11–16 kHz frequency component was on average 20 dB higher than the 4.5 kHz component.

Neurophysiological correlates

Identification of the recorded neuron. The physiology of AN2 has been extensively described (Casaday and Hoy

1977; Popov and Markovich 1982; Wohlers and Huber 1982; Moiseff and Hoy 1983; Nolen and Hoy 1987; Harrison et al. 1988). AN2 has the following physiological properties: it is excited by high frequency sound (30 kHz) and inhibited by the lower frequencies of the calling song (4–5 kHz). In *T. oceanicus*, a 5 kHz sound pulse elicits a short latency inhibitory synaptic potential followed by a weak excitatory synaptic potential (Nolen and Hoy 1987). Although neural inhibition of AN2 in *G. bimaculatus* has not been rigorously shown as in *T. oceanicus*, the long latency of AN2's response to 5 kHz sounds in *G. bimaculatus* almost certainly implies it. As a result, the initial firing rate and the number of action potentials produced by AN2 is on average two to three times larger for 30 kHz sounds than for 4.5 kHz sounds (Nolen and Hoy 1987; Rheinlaender et al. 1976, see Fig. 2A). The response latency to a 30 kHz, 70 dB sound pulse is roughly 12 ms compared to 30 ms for 4.5 kHz sounds at the same intensity (Nolen and Hoy 1987; see Fig. 2A). Based on these criteria, we could unequivocally identify the activity of AN2 in our extracellular recording of the connectives (Fig. 2A).

Chronic recording of AN2 during courtship behavior. *Teleogryllus oceanicus*. We were successful in recording AN2's response to various song playbacks in the presence of a courting male, in four chronically prepared female crickets. A representative recording of AN2 in one of these animals is shown in Fig. 2. Figure 2A shows that the number of action potentials and the initial firing rate of AN2 is at least 3 times higher in response to 30 kHz than to 4.5 kHz sounds. Also, the response latency to a 30 kHz sound pulse is much shorter than to a 4.5 kHz sound pulse (Fig. 2A; bottom panels). In some recordings, the smaller spikes of AN1 could be seen in the trace when we stimulated with a 4.5 kHz tone. Chronic recording of AN2 during courtship showed that the neuron could follow the temporal patterns of both the chirp and the trill phases of the song (Fig. 2B). In the same preparations, playback of synthesized songs at 13.5 and 4.5 kHz were delivered to the chronically prepared female as soon as a muted, courting male started moving its wing stumps rhythmically. Such playbacks showed that the neuron would follow the pulse repetition rate of the synthesized trill quite accurately, just as for the trill of the natural song (compare Fig. 2B right panel with Fig. 2C).

***Gryllus bimaculatus*.** Chronic recordings were obtained from 4 individuals and a representative example is shown in Fig. 3. The differences between the acoustic neuron's response to 4.5 and 30 kHz tones as measured by the number of action potentials, initial firing rate and response latency were similar to those found for *T. oceanicus* as already shown in Fig. 2 and consistent with previously reported auditory properties of AN2. Chronic recording of the female's AN2 during courtship showed that the neuron produced a short, dense burst of spikes in response to each courtship sound pulse of the natural song, the 4.5 kHz and the 13.5 kHz synthesized songs (Fig. 3A). Viewing one of these bursts for each playback at a much higher sweep speed permits one to see that the AN2 response was qualitatively comparable for the natural and the 13.5 kHz sound pulse (Fig. 3B). By contrast,

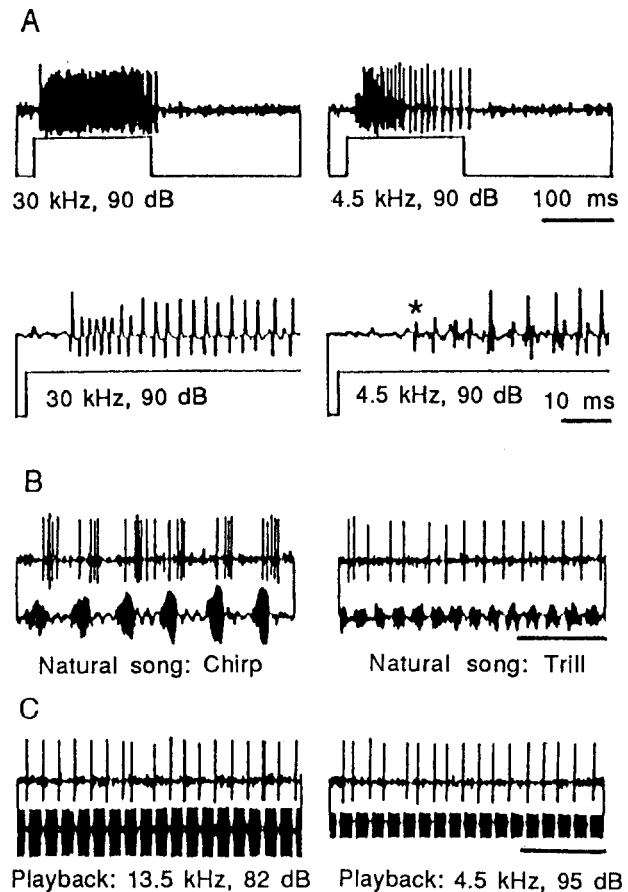


Fig. 2A–C. Representative extracellular responses of AN2 to sound pulses and to various songs in *T. oceanicus*. **A** AN2 responds more strongly in number of action potentials to a 30 kHz sound pulse (left top panel) than to a 4.5 kHz sound pulse (right top panel). In addition, viewing the onset of the AN2 response at a higher sweep speed (bottom panels) shows that: 1) the response latency taken as the onset of the sound pulse to the response of AN2 is much shorter for the 30 kHz sound pulse (left bottom panel) than for the 4.5 kHz sound pulse (right bottom panel); 2) the initial firing rate of AN2 is higher in response to 30 kHz compared to 4.5 kHz sound. On the extracellular recording shown on the left panel (30 kHz sound), AN2 fires at such a high rate that the absolute size of the extracellular action potential is smaller in the initial part of the burst owing to the spike refractory period. At lower sound intensities of 30 kHz sounds, the spike size remained constant within the burst. In the extracellular recording shown on the right panel (4.5 kHz sound), AN1 could also be identified on the traces as a smaller spike (asterisk) with the shortest latency and a different shape. **B** The response of AN2 (top trace) to the natural courtship song playback (bottom trace); left panel shows the chirp phase, right panel shows the trill phase. **C** Left panel: The response of AN2 (top trace) to a synthesized trill with a sound frequency of the third harmonic (13.5 kHz) of the natural courtship song (bottom trace). Right panel: The response of AN2 (top trace) to a synthesized trill with a sound frequency of the first harmonic (4.5 kHz) of the natural courtship song (bottom trace) (A,B,C from the same animal). Calibration for B and C: 100 ms

such response was very different when comparing the response to the 4.5 kHz with that to either 13.5 kHz or the natural sound pulse, specifically, the spike rate was much higher in the latter than in the former (Fig. 3B). To summarize, our neurophysiological results permit the hypothesis that activity in AN2 alone might be critical for courtship success in both *T. oceanicus* and *G. bimaculatus*,

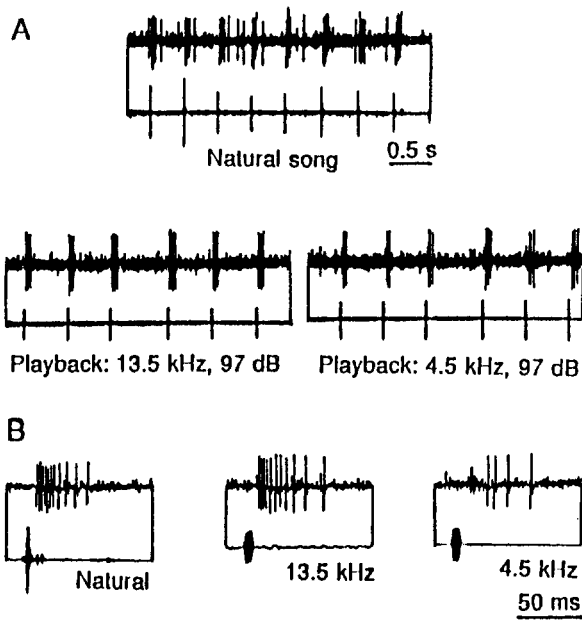


Fig. 3A–C. Representative extracellular responses of AN2 to various songs in *G. bimaculatus*. **A** The response of AN2 (top trace) to the natural courtship song playback, a synthesized song with a carrier frequency of the third harmonic (13.5 kHz) or the first harmonic (4.5 kHz) of the natural courtship song. **B** One of the AN2 bursts of spikes from the recordings shown in **A** occurring in response to each playback, expanded in time, showing the pattern of AN2 response (notice the similarity in AN2 responses to a natural courtship sound pulse and to a 13.5 kHz sound pulse)

as previously suggested by Harrison et al. (1988) in *T. oceanicus*.

Courtship success rates

Based on our chronic recordings of AN2 in *T. oceanicus*, we can predict that if activity in AN2 is, by itself, sufficient for courtship success, any song playback (4.5 kHz, 13.5 kHz or natural) should restore courtship success of muted males in *T. oceanicus*. By contrast, for *G. bimaculatus*, if AN2 alone is sufficient for courtship success, the 13.5 kHz is the most likely playback to be effective in restoring courtship success of muted males. This is because AN2's response to 13.5 kHz playback in this species closely resembles that to the natural song playback and, in contrast, its response to 4.5 kHz playback is rather weak (Fig. 3B). Figures 4 and 5 summarize the results of three muting/playback experiments on three samples of male-female pairs of *T. oceanicus* and *G. bimaculatus*. For each of the three samples the proportion of successful courtship events was recorded under the following conditions: (1) no treatment of the male (*normal group*), (2) male muted by wing amputation (*muted group*), and (3) male muted but courtship accompanied by a playback of either a recorded natural or a synthetic courtship song (*playback group*). Although one would not expect differences among the three groups with respect to the normal and the muted groups, the data are not pooled so that we can compare the responses to the

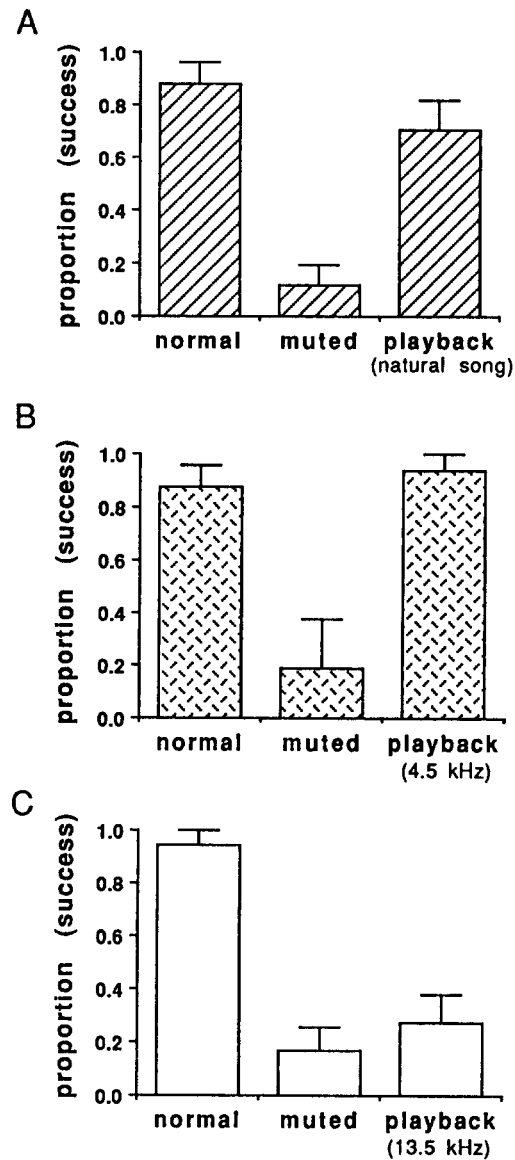


Fig. 4A–C. The proportion of successful courtship for 3 groups of pairs under 3 male treatments in *T. oceanicus*. **A** The first group of pairs ($n = 17$) was exposed to a recording of their own, natural song playback. **B** The second group ($n = 16$) was exposed to 4.5 kHz, 95 dB synthetic courtship song playback. **C** The third group ($n = 18$) was exposed to 13.5 kHz, 82 dB synthetic courtship song playback. The error bars are the standard deviations of the proportions

various altered courtship conditions, within each sample. We made the assumption that muting can only decrease mating and that playback could only increase mating success, so one-sided sign tests were applied between normal and muted male success rates and between muted and playback success rates. In accord with this expectation, the pooled proportion of success for all of the muted males is significantly lower than that of all normal males in each species (χ^2 -test; $P < 0.005$). In addition, in all six test situations, the proportion of success of muted males' courtship is significantly lower than that of normal, untreated males' courtship in each of the six samples (one-sided sign test; paired samples; $P = 0.003$) (Conover

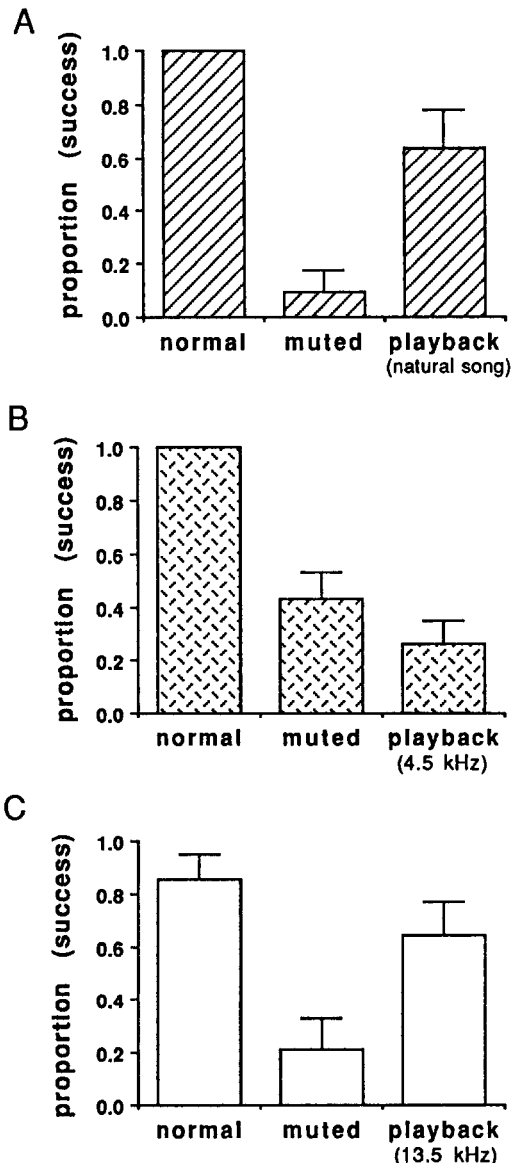


Fig. 5A–C. The proportion of successful courtship for 3 groups of pairs under 3 male treatments in *G. bimaculatus*. **A** The first group of pairs ($n = 11$ pairs) was exposed to a recording of their own, natural song playback. **B** The second group ($n = 23$ pairs) was exposed to 4.5 kHz, 97 dB synthetic courtship song playback. **C** The third group ($n = 14$ pairs) was exposed to 13.5 kHz, 97 dB synthetic courtship song playback. The error bars are the standard deviations of the proportions

1971) (Fig. 4A,B,C). In 4 of the 6 experiments, the success in playback males' courtship was significantly higher than that of muted courtship (one-sided sign test; paired samples): $P = 0.003$ for the natural song group (Fig. 4A) and $P = 0.001$ for the 4.5 kHz (Fig. 4B) group in *T. oceanicus*; $P = 0.036$ for the natural song group (Fig. 5A) and $P = 0.016$ for the 13.5 kHz group in *G. bimaculatus* (Fig. 5C). By contrast, the proportion of success of muted males was comparable to the proportion of success of the same males when the 13.5 kHz in *T. oceanicus* (Fig. 4C; $P = 0.31$) and 4.5 kHz in *G. bimaculatus* synthetic courtship songs were played back (Fig. 5B; $P = 0.96$).

The proportion of courtship success of normal, untreated males was not significantly different from that of the same males which had been muted, as long as they courted with the acoustic accompaniment of taped playback of their own courtship song for both species (two-sided sign test; paired samples; $P = 0.12$ for *T. oceanicus* and $P = 0.06$ for *G. bimaculatus*). Neither was there a significant difference in the proportions of courtship success between normal courtship and playback courtship when the playback was a 4.5 kHz for *T. oceanicus* ($P = 0.5$) or 13.5 kHz synthetic song for *G. bimaculatus* ($P = 0.18$).

There was no significant difference between the proportions of success of the 3 groups of normal, untreated males of each species (χ^2 -test; $P > 0.05$) and there was no significant difference between the proportions of success of the three groups of muted males for each species ($P > 0.15$). However, there was a significant difference between the proportions of success of the three groups of males for which different courtship songs were played back ($P < 0.001$ for *T. oceanicus* and $P = 0.03$ for *G. bimaculatus*).

Courtship success and activity in AN2

Teleogryllus oceanicus. Although AN2 is responsive to the natural courtship song, the 4.5 kHz artificial courtship song and the 13.5 kHz artificial courtship song (Fig. 2B, C), only the natural song and the 4.5 kHz playbacks restores courtship success in muted males (Fig. 4). Thus, these results rule out the possibility that activity in AN2 is by itself sufficient to initiate courtship acceptance in females of *T. oceanicus*.

Gryllus bimaculatus. By contrast, in *G. bimaculatus*, the courtship success rates with natural song and playback of 13.5 kHz artificial song correlate well with strong responses elicited by both natural and 13.5 kHz songs in AN2. This correlation allows the possibility that AN2 alone could mediate courtship success in *G. bimaculatus*. In order to test this possibility, we took advantage of the fact that AN2 responds equally well to a range of higher frequency sounds, from 11 kHz to over 50 kHz. We applied the same experimental procedures as presented above (courtship test and neurophysiological recordings of AN2) but synthesized our courtship song using pure 30 kHz, instead of 4.5 kHz or 13.5 kHz pure tone frequencies (Fig. 6). When the 30 kHz song was played back to a tethered female, which was being courted by a muted male, the response of the female's AN2 was no different than that observed in response to the natural song playback under the same experimental conditions (Fig. 6A). In fact, viewing one of these bursts at a much higher sweep speed permits one to see that the AN2 response was not qualitatively different, for either the natural or for the 30 kHz sound pulse (Fig. 6B). Given this result, we could directly test whether AN2 is critical for courtship success by playing back a 30 kHz song to the female during her courtship by a muted male. To our surprise, the 30 kHz playback *did not* restore courtship success;

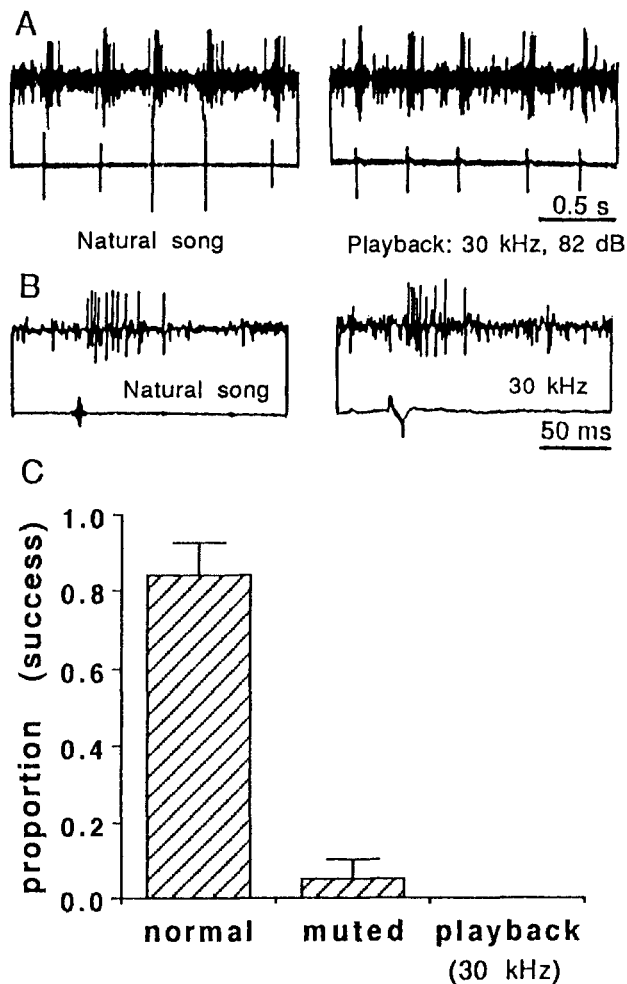


Fig. 6A–D. Ultrasound and courtship success in *G. bimaculatus*. **A** Playback of the natural song (left panel) or the 30 kHz synthesized song (right panel) evoke similar bursting responses in AN2 (calibration: 0.4 s). **B** Viewing one of the bursts elicited by a natural song pulse (left panel) or a 30 kHz pulse (right panel) shows that the AN2 response pattern (number of action potentials, latency, and the initial firing rate) is very similar for both stimuli. The envelope of the 30 kHz pulse, 82 dB, 10 ms is not trapezoidal because of the frequency response limitations (20 kHz) of the instrumentation tape recorder and only the onset and the end of the pulse appear on the recording. **C** A 30 kHz synthesized song playback does not restore courtship success, as the success rate of the muted males exposed to that synthesized song playback was as low as the success rate of the muted males ($n = 19$ pairs). In fact, none of the pairs in the 30 kHz playback was successful. The error bars are the standard deviations of the proportions

there was no significant difference in courtship success between muted males and that of these muted males accompanied with the 30 kHz playback (Fig. 6C; $P = 0.5$). We can conclude that activity in AN2, by itself, is not sufficient to initiate mating behavior in females of either *G. bimaculatus* or *T. oceanicus*.

Discussion

The role of courtship songs in the mating success of insects had been shown in earlier work. In the cockroach,

Gromphadorhina portentosa, muted males were much less likely to mate than normal intact males, or than muted males that courted to the accompaniment of tape recorded courtship hisses (Nelson and Fraser 1980). In cricket courtship, muted males are also less likely to be accepted by females of *Acheta domesticus* (Crankshaw 1979) or *Teleogryllus* (Burk 1983; Loher and Rence 1978). In our experiments, we used not only normal courtship songs, we played back “simplified”, electronically-synthesized courtship songs, consisting of pure tones based on the dominant frequency and harmonics of the natural song. Our results show that the female need not necessarily be exposed to the full-blown, harmonically-complex, natural courtship song for mating to occur, but that she need only hear a courtship song consisting of the dominant frequency.

Courtship success rates

Playback of tape recordings of natural courtship songs restored the mating success of muted males during their courtship. However, the fact that some muted males of *G. bimaculatus* as well as of *T. oceanicus* successfully mated with females, shows that courtship song is not absolutely necessary for successful courtship, although the success rate of courting males is certainly lower without courtship song than with it. This contradicts an earlier report on *G. bimaculatus* (von Hörmann-Heck 1957) that stated that courtship song is strictly necessary to elicit mounting by the female.

The success rate of muted *T. oceanicus* males courting to the accompaniment of a pure-tone 4.5 kHz synthetic courtship song closely approximated that of normal males. However, the success of males courting to the accompaniment of a pure-tone 13.5 kHz synthetic song more nearly approximated the low success rate of the wing-amputated males courting silently (Fig. 4). This result, in *Teleogryllus*, demonstrates that the low-frequency (4.5 kHz) component of the natural courtship song is the critical frequency component of a successful song and that the 13.5 kHz component is neither necessary nor sufficient for eliciting mounting behavior in the female. Apparently, then, the female did not distinguish between a 4.5 kHz synthetic song and the natural song, which consists of a 4.5 kHz fundamental plus a series of harmonic frequencies; in *Teleogryllus*, the 4.5 kHz fundamental is also the dominant (most energetic) component. Moreover, the 4.5 kHz synthetic courtship song consisted only of pulses of a simple temporal structure, as they also occur in the trill phase of natural courtship song. Therefore, neither the chirp phase of the courtship song nor the higher harmonics of the trill pulses found in natural courtship song (Fig. 1A) are necessary to elicit mating behavior in the female.

The courtship song of *G. bimaculatus* differs both spectrally and temporally from that of *T. oceanicus*. The dominant frequency of *bimaculatus* song is not its 4.5 kHz fundamental frequency, but the third harmonic, 13.5 kHz. The temporal pattern of *bimaculatus* song is not as regular or complex as the *oceanicus* song, and

consists of a more-or-less regular series of “tick-like” pulses. Thus, in *G. bimaculatus*, the success rate of muted males courting to the accompaniment of the 13.5 kHz synthetic song restores mating success to normal rates, whereas the success rate of muted males courting with a 4.5 kHz synthetic song does not restore mating success (Fig. 5). This result demonstrates that it is the dominant, high-frequency (11–16 kHz) component of the natural courtship song that is the critical frequency component of a successful song and that the less intense, low-frequency component is neither necessary nor sufficient for eliciting mounting. Moreover, neither the small-amplitude pulses, nor the complex temporal structure of the large-amplitude pulses found in natural courtship song (Fig. 1B) are necessary to elicit a normal proportion of mounting.

Neurophysiological correlates

In *T. oceanicus*, a second-order neuron, AN2, which is called int-1 in this cricket species, is sensitive to high frequency sounds (Moiseff and Hoy 1983; Nolen and Hoy 1987; Harrison et al. 1988). From intracellular recordings of AN2, Harrison et al. (1988) found that it responded faithfully to the temporal pattern of the trill phase of courtship song, but not to the temporal pattern of calling or aggression songs. Their intracellular recordings were made in an acute physiological preparation. By contrast, we made extracellular, chronic recordings in AN2, in a behaviorally-intact female that was being courted by a singing male. In this preparation, we found that AN2 can, in fact, respond to the chirp part of the courtship song (Fig. 2B), which implies that it almost certainly responds to the calling song. In this respect, our results are similar to those obtained earlier by Hutchings and Lewis (1984). We also found that AN2 responds to 4.5 kHz tone pulses, 13.5 kHz tone pulses, as well as to playbacks of the trill phase of natural courtship songs. Recall, however, that in terms of the female’s behavior, only the 4.5 kHz playback restores the courtship success in courting, muted males. This means that the high frequency component of the courtship song is neither necessary nor sufficient for courtship success, and consequently, activity in AN2 is not sufficient for recognition of the courtship song in *T. oceanicus*, contrary to the assertion of Harrison et al. (1988). While we cannot exclude some role for AN2 in courtship recognition, it is not a sufficient one.

In the case of *G. bimaculatus* also, the identified, ascending auditory interneuron, AN2, is sensitive to high frequency sounds (Rheinlaender et al. 1976; Popov and Markovich 1982). Consistent with these earlier reports, we find that AN2 is not only more responsive to 13.5 kHz than it is to 4.5 kHz, based on spikes per tone burst, but it also discharges at a significantly higher instantaneous rate to 13.5 kHz than to 4.5 kHz tones. This robust response to the dominant frequency of the courtship song might suggest that AN2, in *G. bimaculatus*, does play a role in courtship song recognition, as suggested by Rheinlaender et al. (1976). In principle, during courtship behavior, it would be possible that females base their decision to mount the male solely on the maximal rate of

firing of AN2, because the dominant frequency peak of the courtship song (11–16 kHz) is highly excitatory to AN2 (Fig. 3). However, AN2 in *G. bimaculatus*, has an extended excitatory bandwidth, ranging from 11 kHz to over 50 kHz (Nolen and Hoy, unpublished); a response to a 13.5 kHz tone burst is matched by an equally vigorous spike burst to a 30 kHz tone burst (30 ms tone, 5 ms rise/fall time; 10 dB above threshold; see also Fig. 6). Thus, AN2 responds identically to the natural song and to the 30 kHz synthesized song. This is the basis for a simple behavioral test of the hypothesis that the excitation of AN2 triggers the recognition of courtship song in the female. Will an artificial courtship song consisting of pure 30 kHz sound pulses restore mating success to mute males if it is played back during courtship? Clearly, the 30 kHz playback did not restore courtship success (Fig. 6). Thus, in *G. bimaculatus*, activity in AN2 is not sufficient to trigger mating behavior in the female.

However, AN2 is not the only ascending acoustic neuron in both species. Wohlers and Huber (1982) have described at least 3 prothoracic interneurons which send an axon toward the brain. It is then possible that delicately balanced activity in these 3 neurons is necessary for courtship recognition. Because only the dominant frequency of the courtship song of each species is effective in restoring courtship success in muted crickets, the specific level of activity of each neuron would be different for each species.

In *T. oceanicus*, int-1 (= AN2) has been demonstrated to be necessary and sufficient to trigger negative-phonotactic steering behavior in flying crickets (Nolen and Hoy 1984). This steering presumably helps the animal to avoid predacious bats that hunt with ultrasonic biosonar signals. We note that in *G. bimaculatus*, the instantaneous firing rate of AN2 to 13.5 kHz exceeds the 180 spikes/s threshold that, in *T. oceanicus*, triggers escape steering in the flying insect. As shown by Nolen and Hoy (1986), *G. bimaculatus* also exhibits a negative-phonotactic steering response to a source of high-frequency ultrasound. If this response is also triggered by AN2 (as in *T. oceanicus*), and at a similar threshold firing rate, then a female *G. bimaculatus* would receive ambiguous information encoded in AN2. How could the female distinguish between the search phase of a predacious bat (ca. 3/s) and the courtship song (also ca. 3/s) of a potential mate? Why don’t females exhibit negative-phonotactic steering from courting males? This could be explained in terms of behavioral context: when in the air and flying, high-frequency sound would indicate the presence of a bat and would elicit negative-phonotactic steering, but when walking, high-frequency sound would imply the presence of a potential mate and could elicit mounting behavior. This context dependent behavior could be mediated by many potential neural mechanisms.

We have demonstrated that there is a special relevance of the third harmonic frequency component of the male’s courtship song (11–16 kHz) in the elicitation of courtship behavior from the female cricket in *G. bimaculatus*. By contrast, in *T. oceanicus*, the first harmonic frequency component of the male’s courtship song (4.5 kHz) is important in the elicitation of courtship behavior from the

female cricket. For both species, it turns out to be not the fundamental frequency but the dominant, or most energetic component, frequency of the courtship call which carries the essential "message." We also present evidence that an identified auditory interneuron called AN2, known to play a role in predator avoidance, and which had been hypothesized to also play a key role in courtship song recognition, does not play a sufficient role in priming courtship behavior, as previously thought.

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