

bisphosphate carboxylase small-subunit promoter and the nopaline synthase promoter in Ti-plasmid transformants.

These results demonstrate that an RNA virus can be both engineered and used as a convenient and efficient gene expression vector at the level of single cells. Direct infectivity of poliovirus cDNA (19) and the likelihood that in vitro transcription will allow expression of cloned cDNA to other RNA viruses (3) suggest that other plant, animal, and bacterial RNA viruses should be amenable to similar manipulation. The stability of nonselected sequences in RNA genomes has been questioned (20) because of high estimated mutation rates. Such effects were not apparent in this study, but future experiments to test RNA virus expression of heterologous genes in whole plants over many replication cycles will allow further assessment of this question.

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## Bilateral Syringeal Interaction in Vocal Production of an Oscine Bird Sound

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**The vocal organ, or syrinx, of oscine birds has two parts, each of which has generally been presumed to operate independently of the other. A significant counter-example is now demonstrated in the production of a common vocalization by the black-capped chickadee (*Parus atricapillus*), in which the two acoustic sources interact in a nonlinear fashion. This bird produces a sound with multiple frequency components that are heterodyne products resulting from cross-modulation between two signals, thus providing evidence that avian phonation can involve cooperative coupling between the two syringeal sources.**

THE AVIAN SYRINX IS UNIQUE among the sound-generating organs of vertebrates. Most notably, many birds, including members of the suborder oscines or the "true songbirds," possess a two-part syrinx. Situated at the junction of the two primary bronchi, each half of this organ has an independent set of muscles, membranes, and nerves thought to be involved in phonation (1-3). While this morphological peculiarity has been known since the 1800's, it was only recently suggested that the two sides of an oscine syrinx might be functionally independent, thus enabling a bird to sing an "internal duet." Initial support for this hypothesis came from acoustic analyses of birdsongs in which elements presumed to arise from two independent sources were isolated spectrographically or through the use of frequency filters (2, 4). Further evidence arose from the discovery that transection of the hypoglossal motor nerve to either side of the organ leads to a predictable loss of certain acoustic elements

in a complex song; the deleted elements are presumed to have been generated by the disabled side of the syrinx (3). On the basis of this evidence, it is now generally accepted that songbirds are capable of using their two voices simultaneously and independently (5), and oscine phonation thus has become an important model system for studying the lateralization of motor control (3).

While it is likely that the two sides of an oscine syrinx can be functionally decoupled in the production of some sounds, it may be premature to assume that the two halves never interact cooperatively during phonation. Given their close proximity, and the number of structural and aerodynamic connections between them, the acoustic sources could also be coupled. We here report the production of a complex signal by the black-capped chickadee (*Parus atricapillus*) through a previously unreported mechanism of interaction between the two syringeal sides.

The "dee" syllable, the terminal note in

the "chick-a-dee" call from which this species derives its name, is composed of 12 to 20 temporally overlapping frequency components (Fig. 1). These components, evenly spaced at approximately 400-Hz intervals, were originally considered to be a source-generated harmonic series (2), but two facts contradict this hypothesis. (i) Significant energy is only rarely present at the predicted fundamental frequency and at what would be the second and third harmonics. Since the avian vocal tract is presumed to have little effect as an acoustic filter (2), it is unclear how to account for these missing frequencies. (ii) In 32 percent of a sample of "dee" syllables examined, the frequencies of the spectral components were clearly not integral multiples of the spacing between them, yielding an ambiguous value for a possible fundamental frequency (6). The lowest frequency component that consistently displays significant energy invariably occurs at what would be the fourth harmonic, or approximately 1600 Hz (Fig. 1). The next highest frequency component (about 2000 Hz) is also invariably of strong amplitude (7). If these two frequency components are labeled as peaks 1 and 2, there is generally an increase in the energy of the successive peaks up to peaks 5 to 8 (about 3000 to 4000 Hz), followed by a decrease. Most of the spectral energy occurs below 7000 Hz.

If the "dee" syllable is not a harmonic

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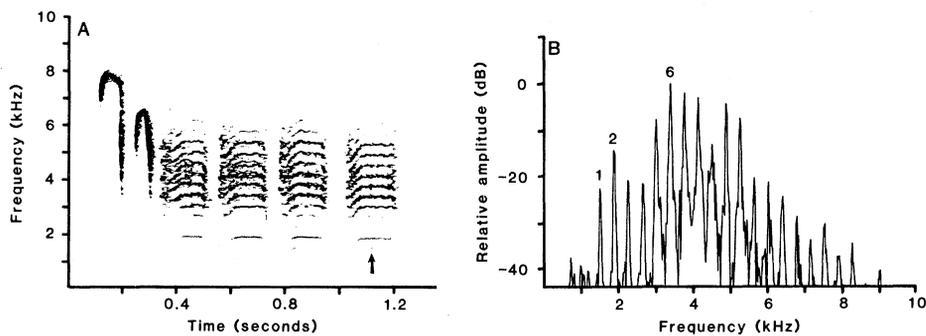


Fig. 1. (A) Narrow-band sonagram (temporal resolution, 22 msec; frequency resolution, 45 Hz) of a normal "chick-a-dee" call with two introductory and four "dee" syllables. (B) Amplitude spectrum for a 40-msec section of a "dee" (arrow in A) from a Nicolet FFT analyzer (frequency resolution, 25 Hz). Spectral component amplitudes are measured in decibels relative to the component of maximum amplitude, which is assigned 0 dB. The first two components with significant energy and the component of maximum amplitude are numbered.

series, what is the origin of its evenly spaced spectral components? We performed unilateral hypoglossal nerve sections on six chickadees. In three cases the right side was sectioned to render it nonfunctional; in the other three cases the left side was sectioned (8). The vocalizations produced by postoperative birds were inconsistent with the hypothesis that the two syrinxal sources operate independently. Neither side acting alone generates the normal "dee" syllable, nor can the output of the two voices be simply added to give the signal of an intact bird. Instead, sectioning either side gives rise to a noisy sound in which is embedded a strong harmonic signal with a fundamental frequency in the range of 1500 to 2000 Hz along with two or three higher harmonics (Fig. 2).

While the results of denervating either side are qualitatively similar, the frequency

of the fundamental in a postoperative signal depends on which side is cut. With the right side sectioned, the fundamental frequency occurs at about 1400 to 1600 Hz (Fig. 2, E and F), whereas if the left side is cut, the fundamental occurs at 1800 to 2000 Hz (Fig. 2, G and H). The fundamental frequency of the postoperative signal corresponds to peak 1 in the intact signal when the right side is sectioned, and to peak 2 in the intact signal when the left side is sectioned (in Fig. 2, compare A and E, B and F, C and G, and D and H) (9).

These results present an intriguing puzzle. The strong harmonic content in postoperative calls presumably arises from the solitary action of the acoustic source on the intact side (contralateral to the nerve section). But a linear summation of the postoperative signals from the left and right sides does not give rise to the spectrum of a normal call.

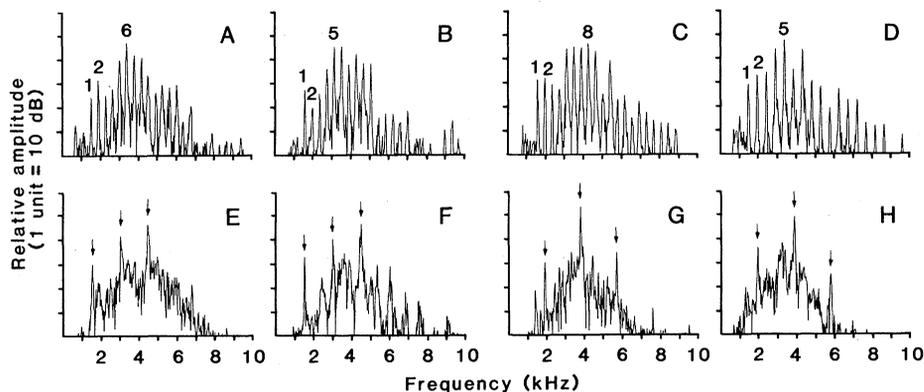


Fig. 2. (A to D) Representative spectra of normal "dee" syllables recorded from four different birds (frequency components are numbered as in Fig. 1B). (E and F) Spectra of "dee" syllables produced by two birds after transection of the right tracheosyrinxal nerve. (G and H) Spectra of "dee" syllables produced by two birds after transection of the left tracheosyrinxal nerve. Arrows indicate harmonic components. Each postoperative signal was generated by the same bird responsible for the preoperative signal above it. Note the correspondence between the fundamental frequencies of the postoperative signals and either component 1 (A and E, B and F) or component 2 (C and G, D and H) of the preoperative signals.

Thus, the chickadee's two syrinxal halves cannot be acting independently, but must be coupled in some fashion. The problem, then, is to account for the production by an intact bird of frequencies that are absent when each syrinxal source acts alone.

The correspondence between spectral components 1 and 2 of the intact signal and the fundamental frequencies of the postoperative signals, and the fact that the frequency interval between all spectral components invariably equals the interval between components 1 and 2, suggest that the additional spectral components in the intact call may arise as sum and difference frequencies, namely heterodyne frequencies, resulting from a nonlinear interaction between the two syrinxal sources. The simplest such interaction is amplitude modulation. In the case of modulation between two sinusoidal signals, the spectral output not only includes the original frequencies  $f_1$  and  $f_2$ , but also their sums and differences,  $(f_2 + f_1)$  and  $(f_2 - f_1)$ . Two harmonic signals, such as those observed in the postoperative chickadee recordings, can be represented by the frequencies  $f_1, 2f_1, 3f_1, \dots$  from the left side and  $f_2, 2f_2, 3f_2, \dots$  from the right. If these signals are cross-modulated, a large number of sum and difference frequencies ( $mf_1 \pm mf_2$ ) result, all spaced at frequency intervals equal to the difference between  $f_2$  and  $f_1$  (10). By substituting observed values of the harmonics in the postoperative signals, namely with fundamentals of  $f_1 = 1600$  Hz and  $f_2 = 2000$  Hz, a series of heterodyne frequencies are generated that correspond precisely to the frequency components observed in a normal "dee" syllable (11).

The spectral structure of the "dee" syllable thus represents, not a harmonic series with a low fundamental frequency, but a series of sum and difference frequencies resulting from cross-modulation between two simpler harmonic series with relatively high fundamental frequencies (12). Amplitude modulation, modeled mathematically as multiplication, accounts for the generation of heterodyne frequencies closely resembling the normal signal. Other models of nonlinearity also generate new frequencies not seen in the independent operation of the two sources alone, but each model has a unique solution. To our knowledge, only the multiplication model presented here results in the frequency pattern observed in the "dee."

Given the present state of uncertainty over how oscillation arises in an oscine syrinx (13), we can only speculate on the physical mechanism responsible for coupling between the two sides. One possibility is that pressure events from each oscillator are transmitted to the contralateral oscillator. Transmission could occur via the lumen

of the vocal tract or via the interclavicular air sac that surrounds the syrinx and ramifies through the space between the two bronchi. The coupling may also be structural, for example, involving the cartilaginous pessulus, to which membranes on both sides of the syrinx are attached. A more thorough understanding of syringeal mechanics is necessary, both to characterize the physical basis of this coupling and to assess the extent to which bilateral interactions may be a common property of syringeal operation.

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5. Acoustic evidence typically adduced in support of the two-voice hypothesis is the simultaneous presence of two (or more) nonharmonically related frequency components in a bird sound. An important caveat apparently overlooked by most authors is that many physical oscillation systems generate non-harmonic overtones. Thus, reports of two "voices" in bird sounds must be treated cautiously if unsupported by physiological experimentation.
6. Based on an examination of 1138 syllables, included in 255 calls recorded from 23 individual birds in the field (range, 2 to 22; median, 10 calls per bird; range, 10 to 119; median, 42 "dees" per bird). A fundamental frequency was considered ambiguous if its calculated value deviated from the predicted value by at least 50 Hz (representing, for example, a 25 percent deviation for a predicted fundamental of 400 Hz).
7. In different individuals, and to a lesser extent in different calls of the same individual, the frequencies of these components may vary from 1300 to 1700 Hz and 1700 to 2100 Hz, respectively. However, the two components are always spaced at roughly 350 to 450 Hz, and the spacing of higher frequency components corresponds to the spacing between these two spectral peaks. The frequency values of 1600 and 2000 Hz are realistic and convenient estimates to use for the sake of argument.
8. Either the left or right tracheosyringeal branch was cut approximately 2 mm below the main trunk of the 12th cranial (hypoglossal) nerve. Sectioning this branch has the same effect on syringeal function as cutting the roots above the anastomosis of the 12th and 10th cranial nerves (3). Postoperative vocalizations were recorded 2 or 3 days after surgery. Surgical histories and further procedural details may be found in S. Nowicki [thesis, Cornell University (1985)].
9. The frequency components (in hertz) marked in Fig. 2 are: (A) 1 = 1500, 2 = 1875; (E) arrow = 1500; (B) 1 = 1525, 2 = 1950; (F) arrow = 1500; (C) 1 = 1525, 2 = 1925; (G) arrow = 1900; (D) 1 = 1425, 2 = 1900; (H) arrow = 1925. Average values and standard deviations of postoperative fundamental frequencies for these four birds ( $n = 10$  "dees" per bird) are: 1498  $\pm$  51 Hz, 1453  $\pm$  32 Hz (right nerve sectioned, corresponding to 2, E and F); 1878  $\pm$  25 Hz, 1943  $\pm$  44 Hz (left nerve sectioned, 2, G and H). Other spectral components, not harmonically related to the fundamental frequency, sometimes emerge from the noise in post-operative signals, but these components are of lower amplitude and vary extensively among different signals produced by the same bird. The overall syllable syntax of calls (for example, Fig. 1A) remains unchanged in postoperative birds.
10. If  $V_1(t) = A_1 \cos f_1 t + A_2 \cos 2f_1 t \dots + A_n \cos n f_1 t$  and  $V_2(t) = B_1 \cos f_2 t + B_2 \cos 2f_2 t \dots + B_m \cos m f_2 t$ , where  $n$  and  $m$  are integers, their multiplication gives rise to the general terms

$$\frac{A_n B_m}{2} \cos(m f_2 + n f_1) t + \frac{A_n B_m}{2} \cos(m f_2 - n f_1) t$$

(The factor  $2\pi$  has been omitted from the foregoing terms for clarity.)

11. The sum and difference frequencies generated in the range of interest are: 400, 1200, 1600, 2000, 2400, 2800, 3200, 3600, 4000, 4400, 4800, 5200, 5600, 6000, 6800, 7200, and 7600 Hz, corresponding to the components observed in a normal "dee" syllable with component 1 = 1600 Hz and component 2 = 2000 Hz. Each modulation product also has associated with it a theoretical value for the amplitude of that component, but these predicted values assume no frequency-dependency in the coupling coefficient and are thus of limited value.
12. Field observations and experimentation suggest that the resulting 400 Hz periodicity of the chickadee's "dee" syllable, as reflected in the frequency interval between these components, is an important carrier of information, modifiable through learning, concerning social affiliation [D. L. Mammen and S.

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## Immunization with an Isolate-Common Surface Protein Protects Cattle Against Anaplasmosis

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Hemoparasitic diseases are endemic in half the world's livestock production areas and are the greatest obstacle to improved meat, milk, and fiber production in the Third World. The most prevalent of these diseases, anaplasmosis, occurs throughout tropical and subtropical regions and is responsible for 50,000 to 100,000 cattle deaths annually in the United States alone. Despite its prevalence and the severity of the losses, an effective immunoprophylaxis for anaplasmosis has not been developed. A neutralization-sensitive epitope on a surface protein with a molecular weight of 105,000 (Am 105) of the causative rickettsia *Anaplasma marginale* was identified by monoclonal antibody inhibition of infectivity. This epitope was determined to be common to eight isolates with antigenic, morphologic, and protein structural differences. Cattle immunized with Am 105 purified by immunoaffinity chromatography were protected against challenge with virulent *Anaplasma marginale*. The identification of Am 105 as bearing isolate-common epitopes capable of inducing protection in immunized cattle provides the basis for the development of an effective subunit vaccine for bovine anaplasmosis.

RICKETTSIAL INFECTIONS FREQUENTLY escape early diagnosis and rapidly progress to cause severe illness and death. Despite the need for immunoprophylaxis to prevent infections, there has been little progress in developing effective rickettsial vaccines. This lack of progress results, in part, from the complexity of the organism's invertebrate vector and vertebrate stages, antigenically variant strains, and their ability to persist in the host. *Anaplasma marginale*, the causative agent of bovine anaplasmosis, demonstrates this complexity and remains without effective immunoprophylaxis. The organism appears as small spherical bodies in the red cells of cattle and is transmitted either directly between cattle by blood-contaminated fomites or via infected ixodid ticks (1, 2). After the initial bodies of *A. marginale* have infected the erythrocytes they replicate intracellularly by binary fission and then emerge by exocytosis to infect additional erythrocytes (3). A severe anemia develops during

acute infection that can result in dramatic weight loss, abortion, and death (4). Cattle recovered from acute infection are resistant to challenge with the homologous isolate; however, they remain susceptible to infection from heterologous isolates (5, 6). Isolates throughout the United States have antigenic (7), morphologic (8), protein structural (9), and virulence (10) differences. Clearly, a vaccine for anaplasmosis would be required to provide protection against all the isolate types in a given livestock region.

Our approach to development of an effective vaccine is to identify one or more isolate-common surface antigens capable of inducing neutralizing antibody and use the isolated immunogen or its cloned, expressed replica as a vaccine. We have identified five surface proteins (molecular weight 105K, 86K, 61K, 36K, and 31K) on the initial

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