Hormonal Acceleration of Song Development Illuminates Motor Control Mechanism in Canaries

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ABSTRACT: **In songbirds, the ontogeny of singing behavior shows strong parallels with human speech learning. As in humans, development of learned vocal behavior requires exposure to an acoustic model of species-typical vocalizations, and, subsequently, a sensorimotor practice period after which the vocalization is produced in a stereotyped manner. This requires mastering motor instructions driving the vocal organ and the respiratory system. Recently, it was shown that, in the case of canaries (*Serinus canaria*), the diverse syllables, constituting the song, are generated with air sac pressure patterns with characteristic shapes, remarkably, those belonging to a very specific mathematical family. Here, we treated juvenile canaries with testosterone at the onset of the sensorimotor practice period. This hormone exposure accelerated the development of song into stereotyped adultlike song. After 20 days of testosterone treatment, subsyringeal air sac pressure patterns of song resembled those produced by adults, while those of untreated control birds of the same age did not. Detailed temporal structure and modulation patterns emerged rapidly with testosterone treatment, and all previously identified categories of adult song were observed. This research shows that the known effect of testosterone on the neural circuits gives rise to the stereotyped categories of respiratory motor gestures. Extensive practice of these motor patterns during the sensorimotor phase is not required for their expression.** © 2010 Wiley Periodicals, Inc. Develop Neurobiol 00: 000–000, 2010

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INTRODUCTION

In oscine songbirds, song develops in a well-defined sequence of ontogenetic stages. The first period of song learning can be characterized as the acquisition of information about conspecific song (sensory period) as the juvenile listens to song and forms a memory of song features (song template) (Brainard and Doupe, 2002; Adret, 2008). The second stage in song development is a sensorimotor period, in which the juvenile bird produces vocalizations that are adjusted over time (plastic song) until they resemble song features of the template (Hultsch and Todt, 2004). This stage is thought to be important for practicing the motor skills of singing. The final stage of song development (crystallization) is a rapid reduction in acoustic variability resulting in highly stereotyped song.

There is considerable variability in the timing of song ontogeny between species. Although zebra finches (*Taeniopygia guttata*) develop adult song within 90–100 posthatching days (PHD), this process takes 8–12 months in canaries (*Serinus canaria*) and

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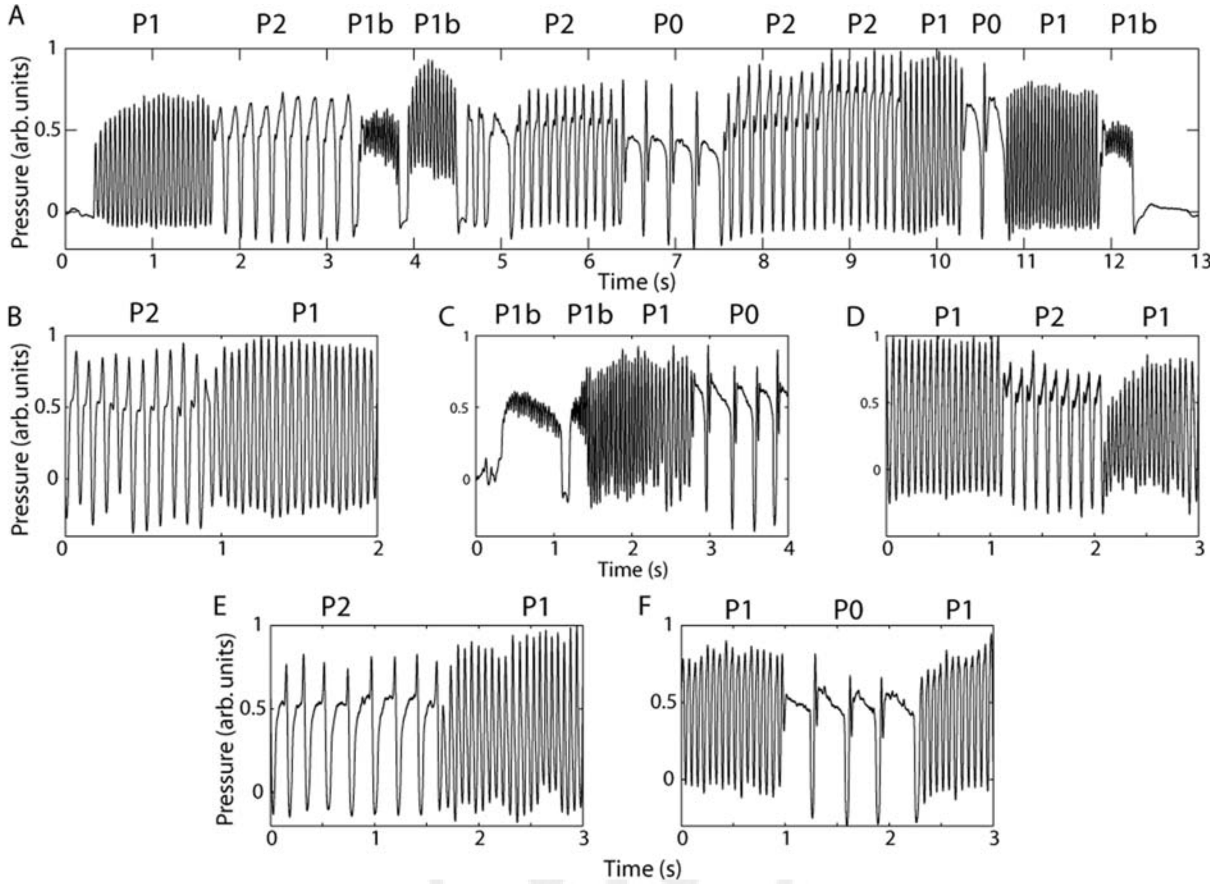

Figure 1 Characteristic air sac pressure patterns in adult canaries. In the top panel, we display the air sac pressure time series data (i.e., air sac pressure vs. time) recorded during song production of a nontestosterone-treated adult canary (A). Following panels (B–F) display segments of air sac pressure time series data during song production of five distinct canaries. The segments are identified with P1, P1b, P2, and P0 indicating patterns of the same class, which were clustered to be similar by the SVD method (see Methods section). The individual whose pressure time series is displayed in (A) presents the four pressure pattern classes, which are found in every bird we have analyzed (see Methods section); therefore, we call the set of these four patterns a "base" for our posterior analysis of pressure patterns used in the song of testosterone-treated juveniles.

many other species with annual breeding cycles (e.g., Tramontin and Brenowitz, 2000; Brainard and Doupe, 2002). In canaries, the sensory stage lasts approximately to PHD 60, whereas the sensorimotor period lasts for another 6 months. At the beginning of the first breeding season, song crystallizes into stereotyped, adult song. Typical adult canary song consists of a sequence of repeated syllables (phrases) that differ in duration and frequency modulation [(Lehongre et al., 2008) and Fig. 1(A)].

Sex hormones have been shown to be important for the song-learning process and for song production because of their capacity for reshaping neural circuits and peripheral systems [(Zeigler and Marler, 2008, Part V)]. For example, it was shown that sex steroids were able to cause a size increase of several of the areas of the song circuit brain comparable to the seasonal increase (Tramontin et al., 2003). In particular, circulating testosterone plays an important role in song development (Ball et al., 2003; Harding, 2004). Hormone receptors have been found in the main areas of the song circuit. Androgen receptors were found in all the brain areas of the motor circuit and of the anterior forebrain pathway (e.g., Perlman et al., 2003; Brenowitz, 2008), whereas the occurrence of estrogen receptors is species specific and in the motor circuit they were only found in HVC (Gahr et al., 1987; Gahr, 1990; Gahr et al., 1993; Schlinger and Brenowitz, 2002). Furthermore, androgen receptors were also found in areas of the midbrain and hindbrain involved in the premotor control of the respiratory and syringeal systems (Gahr and Wild, 1997), and androgen-binding proteins are present in the syringeal tissue (Lieberburg and Nottebohm, 1979). It is clear from all this work that the role of steroids is of fundamental importance for the song circuit to understand the way they change behavior by changing the neural circuitry, but the details of these processes are yet to be understood.

Castrated male songbirds (swamp and song sparrows) go through the learning stages despite low levels of circulating testosterone but without crystallization of their song (Marler et al., 1998). Testosterone implants lead to the crystallization of the song, suggesting that sex steroids play a more critical role in the motor production than in the learning process. During the plastic stages of song development, testosterone levels are low, whereas an increase coincides with song crystallization (e.g., Brenowitz, 2008). In canaries, seasonally fluctuating levels of testosterone are associated with the alternation between crystallized song during the breeding season, and a more plastic phase during the rest of the year during which new syllables can be added to a male's repertoire. Furthermore, adultlike, stereotyped song can be rapidly induced in juvenile songbirds by administration of testosterone (e.g., Heid et al., 1985; Whaling et al., 1995; Gardner et al., 2005). This rapid induction of stereotyped song abbreviates the sensorimotor phase of song ontogeny, thus influencing the detailed processes of plasticity in motor control of song (Nordeen and Nordeen, 2004). The interplay between a shortened practice phase and the biophysical effects of testosterone on the neural circuitry is thought to result in apparent deficits in crystallized song in the zebra finch (Korsia and Bottjer, 1991), whereas the underlying motor gestures of accelerated, testosterone-induced song stereotypy, have not been investigated in any songbird species.

The generation of adult birdsong requires coordinated activity of respiratory, syringeal, and upper vocal tract motor systems (Suthers and Zollinger, 2004). For song production, oscine birds generate air sac pressure pulses, which enable energy transfer to the sound-generating labia (Mindlin and Laje, 2005). Active regulation of frequency and labial opening requires precise motor control of the syringeal muscles, which needs to be coordinated with motor instructions to the respiratory muscles generating the pressure patterns (Goller and Suthers, 1996). Canary song is remarkable for its long duration and high syllable repetition rates. Despite high-syllable repetition rates, canaries replenish the air expelled for the production of a syllable during rapid minibreaths that correspond to the silent intersyllable intervals (Hartley and Suthers, 1990). This alternation between expiration and inspiration can give rise to phrases with syllable repetition rates of $\sim 30.\mathrm{s}^{-1}$. Phrases with higher syllable repetition rates are generated by modulation of a sustained expiratory pressure pulse (pulsatile phrase).

Each syllable type is generated by a distinct, stereotyped air sac pressure pattern. Different patterns vary in the degree of modulation of expiratory pressure, ranging from simple unmodulated pulses (most rapid minibreath phrases) to repeatedly modulated pulses (pulsatile phrases). These respiratory patterns of canary song have been studied in detail in regard to a putative neural substrate that can give rise to the diversity of motor gestures found in the different phrase types of song (Trevisan et al., 2006). This work showed that the diversity of pressure patterns of song phrases does not constitute an unpredictable set of motor gestures that might arise from an equally unpredictable set of motor instructions. All observed pressure patterns of adult canary song corresponded to the different subharmonic solutions of a simple nonlinear driven system (Alonso et al., 2009; see Methods section), suggesting a robust, underlying mathematical structure. This interpretation of the diversity of the respiratory gestures as subharmonic solutions of a simple nonlinear system accounts simultaneously for the morphological features of pressure patterns as well as for the syllabic timing of phrases.

Here, we want to test the hypothesis that testosterone-treated juveniles also present the delicate morphologies predicted by the mathematical model. The rationale behind this hypothesis is that elevated levels of circulating testosterone could lead to a maturation of the neural circuitry for song production. In this way, the respiratory motor gestures that fit the model should emerge prematurely. In contrast, we do not expect untreated juveniles to produce the full spectrum of pressure patterns.

MATERIALS AND METHODS

Animals, Recordings, Hormonal Treatment, and Surgery

Thirteen male, juvenile domestic canaries (*Serinus canaria*) were used in this study. They were housed in individual cages (30 × 25 × 30 cm), given seed and water *ad libitum*, and kept at a 14:10-h light–dark cycle. Eleven of these birds were bought from local breeders who breed them for their color, shape, and conformation, and two were bred in our laboratory colony. The birds were divided into two groups, five birds constituted the experimental group (group I), and the other eight animals were used as the control group (group II). For comparison, song and subsyringeal air sac pressure were recorded from five untreated adults.

The recording setup consisted of a microphone (TAKSTAR SGC 568) placed in front of each cage. Sound was recorded using a microphone preamplifier (PR4V SM pro audio) and a multichannel sound card (MAYA 1010, 44.1-kHz sample rate) directly onto a computer.

Birds in group I were topically treated with 300-mg testosterone gel (1%; androlone, Laboratorios BETA), equivalent to 3 mg of testosterone, starting at PHD 60 once per day for 20 days. Although we did not measure the levels of circulating testosterone in these birds, in a previous study, these testosterone gel doses were successful in eliciting malelike song in adult female canaries (Mendez et al., 2006). Sound recordings started on the first day of treatment for birds in both groups.

After 20 days of testosterone treatment, simultaneous recordings of sound and subsyringeal air sac pressure were made in the members of groups I and II. Air sac pressure was monitored through a flexible cannula (silastic tubing, o.d. 1.65 mm), which was inserted through the abdominal wall into the anterior–thoracic air sac under ketamine/xylazine anesthesia. The free end of the cannula was connected to a miniature piezoresistive pressure transducer (Fujikura model FPM-02PG), which was mounted on the bird's back [for a more detailed description, see Goller and Suthers (1996)]. The voltage signal from the transducer was amplified and modulated to make it suitable for recording with a sound card (MAYA 1010). Typically, birds started singing reliably 1 or 2 days after the surgery. Songs and the pressure transducer signal were recorded continuously. The modulated pressure recording was then demodulated to get a relative voltage trace of air sac pressure.

All animal procedures were in accordance of NIH guidelines and approved by the Animal Care and Use Committee.

Clusters of Adult, No Treated Pressure Patterns

Canaries utter songs that consist of repetitions of ∼10 acoustically different syllables. The production of a syllable requires the generation and coordination of many motor commands (the activities of syringeal and respiratory muscles). Therefore, a parsimonious hypothesis would indicate that, to generate a diversity of acoustically different syllables, these motor patterns should be just as diverse. Yet, the inspection of the air sac pressure used to generate the uttered syllables during song suggests that a smaller number of qualitatively different pressure patterns is necessary. To quantify this observation, we proceeded to perform a singular value decomposition (SVD) of the time traces of air sac pressure for different birds. This method provides a basis for the modal decomposition of an ensemble of functions. Its most striking property, being its optimality, it provides the most efficient way of capturing the dominant components of an infinite dimensional process with a finite (and often just a few) number of modes (Holmes et al., 1996).

We chose an adult bird singing particularly rich songs and selected one of them, consisting 10 acoustically different syllables. We defined a set of scalar fields $u_k(t)$, each of them as the fragment of the air sac pressure time series $P(t)$ during the kth vocalization. For each of the 10 different syllable types in the selected song, we built four vectors $U_k(t)$, $k = 1,\ldots,4$, with the pressure patterns used in four consecutive repetitions of the syllable. To build vectors of the same size, we computed the times between consecutive minima of the pressure patterns and divided that time interval into 100 equally spaced incremental times. Then, we defined $u_k(t_i) = P(t_i^*)$, where t_i^* *is* the closest sampled time to t_i. For each vector, we computed the correlation matrix $U(t_i, t_j) = u(t_i)*u(t_j)$ and built an average of the correlation matrices over the whole song $<U(t_i, t_j)>$, averaging over the four times 10 selected syllables (four examples of pressure for each of the 10 acoustically different syllables). With this representative of the correlation matrix, we looked for an empirical base to decompose the different pressure patterns. Using the subroutine svdcmp (Press et al., 1988), we performed a SVD of the matrix $<U(t_i, t_j)>$. The inspection of the nonzero eigenvalues indicated that truncating the base in four modes would capture most of the energy of the measured patterns (Holmes et al., 1996). The four eigenvectors obtained through SVD can be used to classify pressure patterns. Projecting a song from one individual onto this base, we identified four clusters of projecting coefficients. We randomly chose one pressure pattern as representative of all the patterns whose projection coefficients would fall within a cluster and repeated the procedure for each cluster. In this way, we selected four pressure patterns and built with it a "base" of pressure patterns.

F2 The result is displayed in Figure 2(A), where each point corresponds to a syllable. The coordinates of each point are the coefficients of the corresponding pressure pattern when expanded in the first two eigenvectors of the base computed earlier. The different point types correspond to syllables uttered by different birds. The "□" points, for example, correspond to the bird whose song was used to define the base [Fig. 1(A)]. The time traces of the pressure projected into the base were different from those used to compute the eigenvectors of the SVD. Notice that the "□" signs form four different clusters. To test the hypothesis that other birds would also produce their diversity of syllables with a small set of pressure patterns (moreover, qualitatively similar across individuals), we projected the pressure patterns used during their song, in the base previously computed. Five different additional birds were measured, and the pressure patterns were projected in our original set of eigenvectors. Notice that the coefficients of the SVD expansion in the base computed with the first bird cluster in the same four groups. In Figure 2(B), pressure patterns are expanded in the first eigenvector and a linear combination of the remaining eigenvectors of the base previously mentioned. Analogously, in Figure 2(C), pressure patterns are expanded in the second eigenvector and a linear combination of the first and third eigenvectors. Notice that the structure of four clusters can be distinguished in both panels.

Classification of Respiratory Patterns

Unlike linear systems (as for example in a simple harmonic oscillator), which under forcing respond simply following

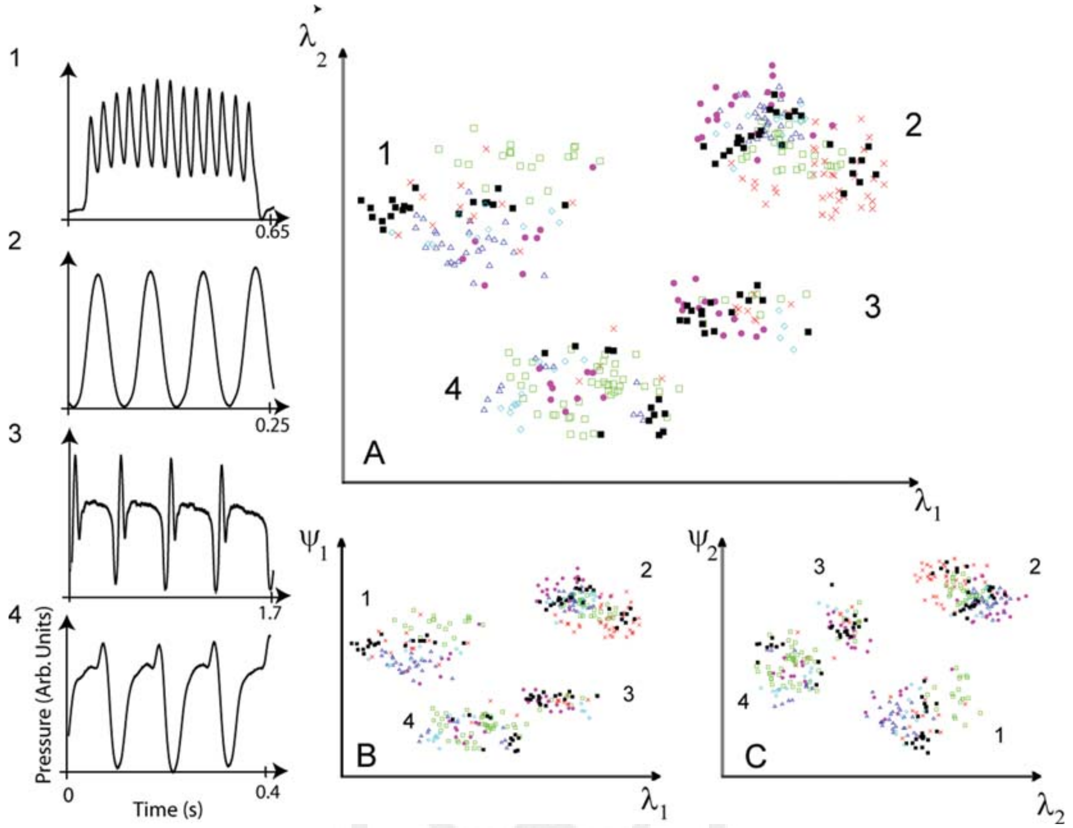

Figure 2 Pressure patterns in adults cluster in a small number of classes. Coefficients of the corresponding pressure patterns of six adult canaries when projected in the first two eigenvectors of the base computed by the SDV method are located at coordinates (λ_1 and λ_2), different point types are used for different birds, and each point represents a pressure gesture (A). Coefficients when projected in the first eigenvector and a linear combination of the remaining eigenvectors of the base are located at coordinates (λ_1 and ψ_1) (B), and when projected in the second eigenvector and a linear combination of the first and third eigenvectors are located at coordinates (λ_2 and ψ_2) (C). Despite the acoustic diversity of syllables, pressure patterns arrange in four clusters; therefore, four basic pressure patterns were used in the songs of the adults in our study. Shapes of the pressure patterns within each cluster are identified by the numbers one to four.

the driver, nonlinear systems (as for example certain neural populations) respond to a driver in different ways, depending on parameters such as the frequency and amplitude of the driver. Typically, a driven nonlinear system will respond similarly for a range of the parameters, but qualitatively different solutions can be found as these are further changed. The system can respond presenting solutions with the same period of the driver or displaying periodic solutions with a period that is a multiple of the driving period. These solutions are known as subharmonics. The families of solutions of nonlinear driven systems have specific shapes, and, in fact, nonlinear systems can be classified according to the family of shapes that they present (Mindlin et al., 1990; Gilmore, 1998; Gilmore and Lefranc, 2002). Remarkably, to generate diverse syllables, canaries use a set of air sac pressure patterns with the characteristic shapes of a simple nonlinear system; that is, there is an underlying mathematical structure behind this diversity, probably linked to the neural mechanisms that give rise to these patterns. Alonso et al. (2009) showed that the different pressure patterns corresponding to different syllables in the songs of adult canaries could be fitted as subharmonic solutions of the following system of two equations:

$$\frac{dx}{dt} = y$$

$$\frac{dy}{dt} = \alpha_0 + A\cos(\theta)\cos(\omega t) + (\beta_0 + A\sin(\theta)\cos(\omega t))x + x^2 - xy - x^3 - x^2 y, \quad (1)$$

where the parameters (A, θ, and ω) were fitted for each syllable type while α_0 and β_0 had fixed values, one and three, respectively. Parameter A represents the amplitude of the forcing, ω is the frequency, and θ gives a relative weight of the two terms of the forcing [seeAlonso et al. (2009)]. Wide regions of the parameter space exist for different solutions [Fig. 3]. It is not possible to fit an arbitrary set of time series

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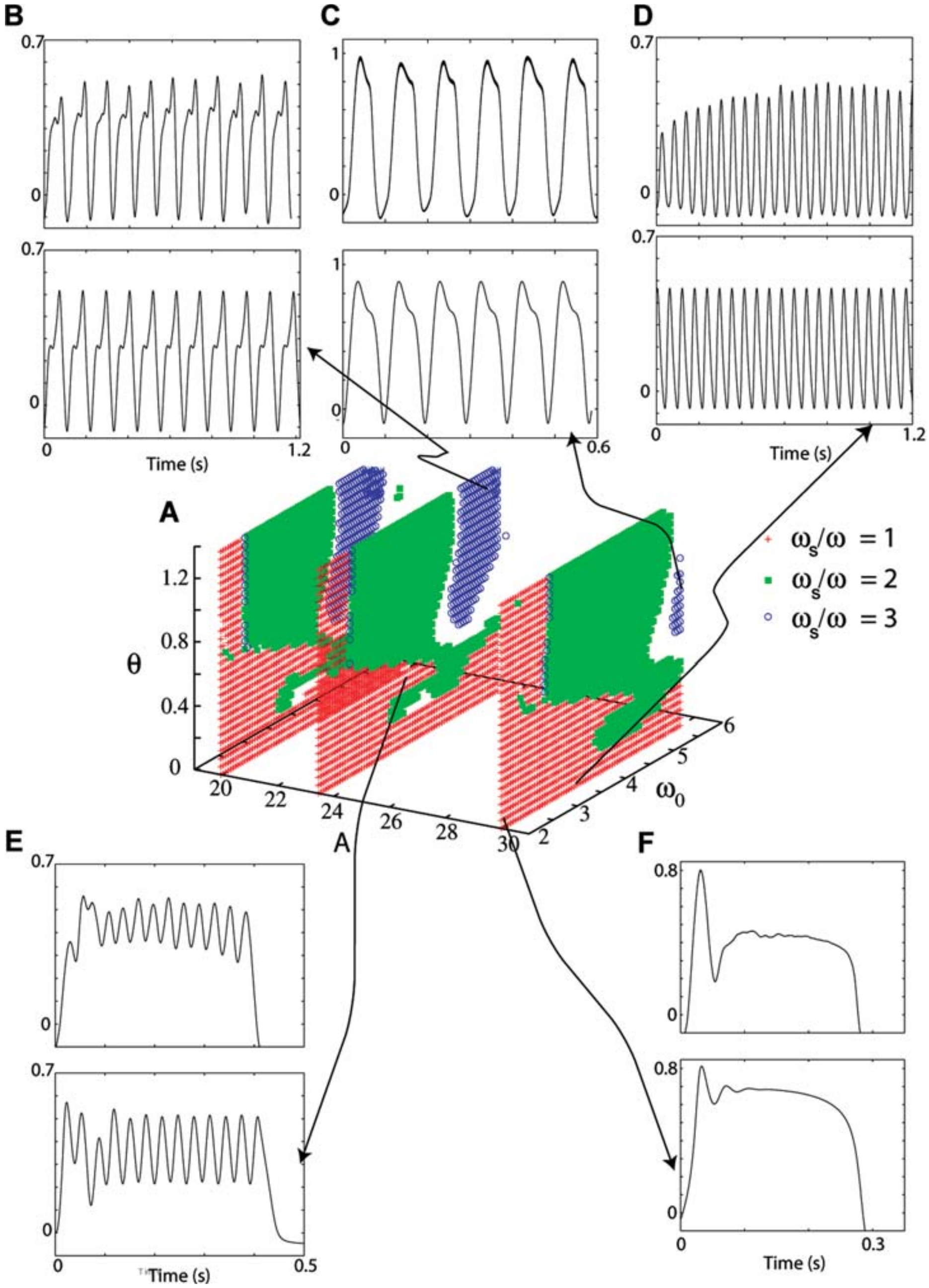

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Figure 3 Parameter space of a low-dimensional model for pressure patterns. The solutions of the dynamical model used to fit the pressure patterns used by nontreated adult canaries in Alonso et al. (2009). Three cuts of the (A, ω, and θ) parameter space, for three values of A are shown (A). Parameter A represents the amplitude of the forcing, ω is the frequency, and θ is gives a relative weight of the two terms of the forcing. Regions of the parameter space where different subharmonic solutions exist are displayed with different point types (ω_s stands for the frequency of the solution). From these regions, five sets of parameters (A, ω, and θ) were selected, and the simulations of the model for these values are displayed in the bottom insets. On top of these, we show experimental segments of the pressure patterns used by the adult whose patterns were used as a base for our study. The (A, ω, and θ) values used in the simulations are (23.45, 4.83, and 0.91) (B), (30, 4.95, and 0.39) (C); (30, 0.28, and 3.08) (D), (42.98, 5.66, and 0.16) (E), and (40, 0.3, and 0.325) (F). Within the parameter region where solutions with two maxima exist, we distinguish solutions as the ones in panels B and C according to whether the first maximum is smaller (bigger) than the second one. Both realizations occur in the birds of our study.

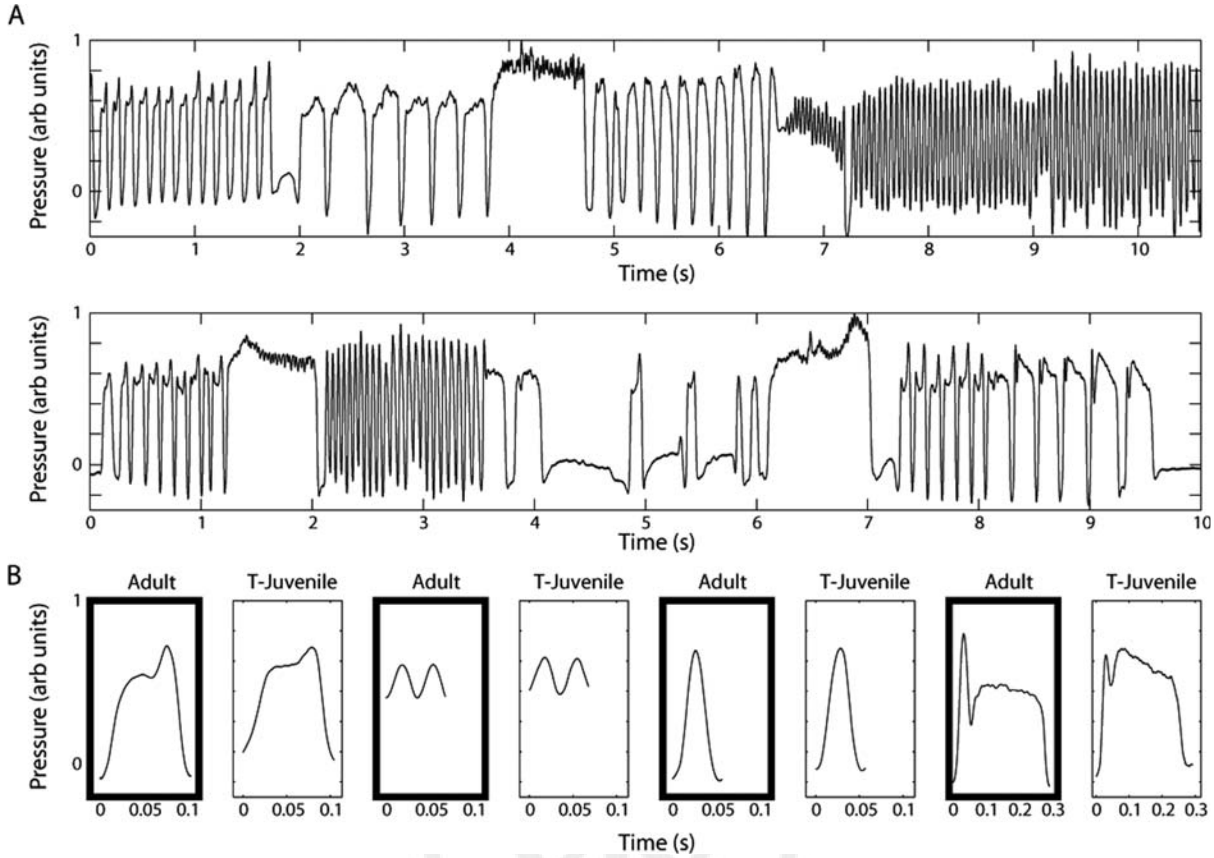

Figure 4 Pressure patterns of a testosterone treated juvenile after 14 days of treatment. Two segments of time pressure data corresponding from a testosterone-treated juvenile, after 20 days of treatment (A). They are built out of segments, which are good approximations of the shapes identified as a "base" in the non treated adults. Each respiratory gesture is compared to the respective element of the "base," adult gestures and the similar gesture produced by testosterone treated by is displayed. Similar patterns must present a cross correlation coefficient bigger than 0.99 (B).

shapes as solutions of a given nonlinear model, because differential equations can be classified in terms of the morphological features of their sets of solutions. In Figure 3, we show different solutions obtained numerically by integrating these equations. This model is used to test the hypothesis that testosterone-induced air sac pressure patterns of juvenile canaries fit within the finite set of characteristic shapes within the parameter region that was explored to fit adult pressure patterns in Alonso et al. (2009). Similarity, in this context, is defined in a profound and restrictive sense: belonging to a family of curves, which can be solutions of the dynamical model that is capable of synthesizing normal adult pressure patterns. The dynamical system used in Alonso et al. (2009) was obtained as the simplest nonlinear system capable of displaying the measured patterns as its solutions and not as the computational implementation of a particular neural model.

To quantify the similarity between pressure patterns of juveniles and adults, we proceeded as follows. First, we defined two vectors where we saved the pressure values of the respiratory gestures to be compared. We called these vectors $x[i]$, $i = 1 \ldots n$ and $y[i]$, $i = 1 \ldots n$, where n divided by the sample rate equals the duration of the pressure gesture. Then, we plotted x against y and computed a linear regression. The respiratory gestures were considered similar if two criteria were met. First, the slope, m, obtained in the regression must differ from one in less than a threshold value ε, that is, $|m - 1| < \varepsilon$. Second, the Euclidean distance between x and y must be less than another threshold value δ, that is, $D = \sqrt{\sum (x[\mathrm{i}] - y[\mathrm{i}])^2}\delta$. In this way, the respiratory gestures of each bird were grouped in classes, each class containing similar patterns. In this work, we call two patterns p_1 and p_2 "similar" if their cross correlation $r \equiv \max \frac{1}{N} \int p_1(t) p_2(t - \tau) dt > 0.99$, where N is a normalization constant.

From each of these classes, a representative pattern was selected. We refer to this set of pressure patterns as the "base" of adult pressure gestures. The four air sac pressure patterns of the base are (1) a harmonic oscillation [Fig. 4(B), fifth panel]; (2) patterns with two maxima per period [Fig. 4(B), first panel]; (3) a pulse with an initial peak fol-

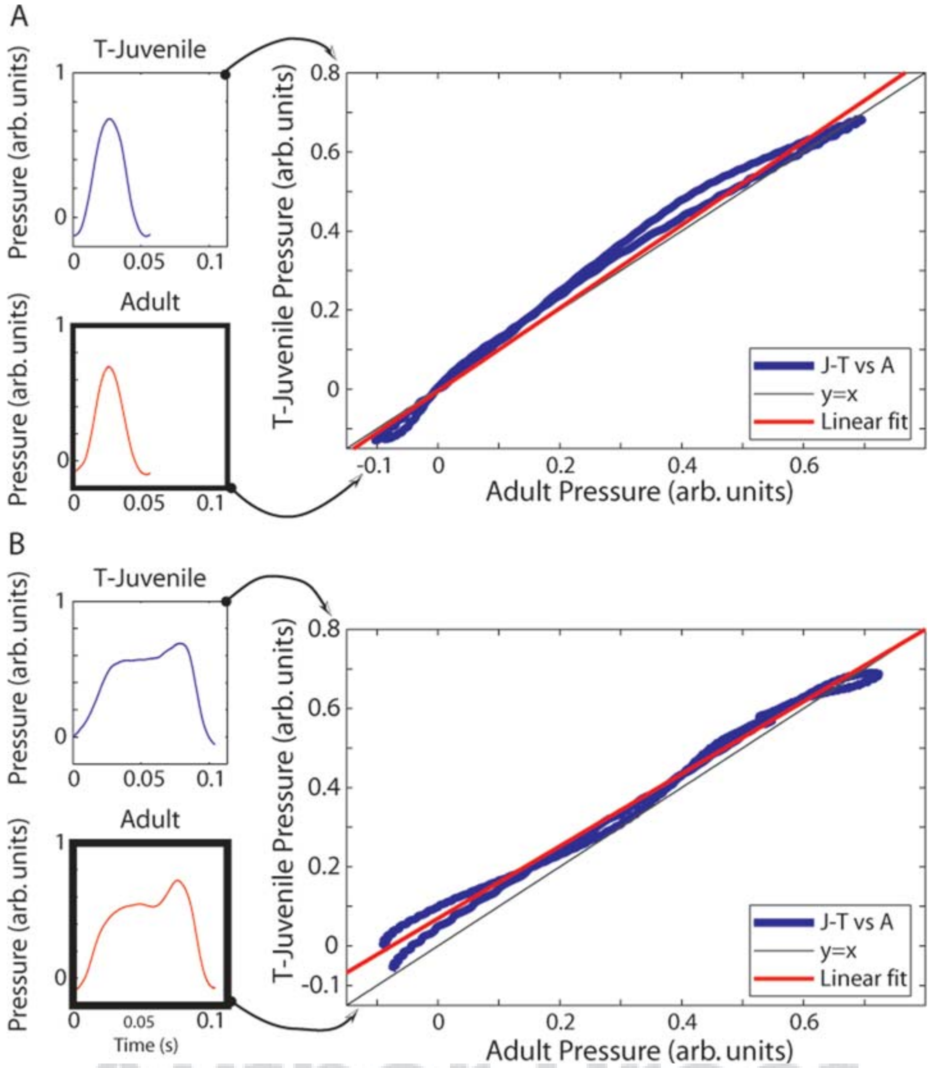

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Figure 5 Method for identification of patterns in testosterone-treated juveniles. Respiratory gestures produced by testosterone treated juvenile are compared to elements of the base (see Methods section). A testosterone-treated juvenile pressure pattern is recognized to be similar to a P1 pressure pattern, The slope, m, the coefficient of determination, R^2, and chi-square, χ^2 are $m = 1.05$, $R^2 = 0.987$, and $\chi^2 = 0.001$ (A). A testosterone-treated juvenile pressure pattern is recognized similar to a P2 pressure pattern. The slope, m, the coefficient of determination, R^2, and chi-square, χ^2 are $m = 0.92$, $R^2 = 0.957$, and $\chi^2 = 0.002$ (B).

lowed by a slow decay of its pressure value [Fig. 4(B), seventh panel]; and (4) a sustained pulse with repetitive pressure oscillations [Fig. 4(B), third panel]. These pressure patterns are called P1, P2, P0, and P1b. Air sac pressure patterns produced by testosterone-treated juveniles were compared to the patterns of the base. We used the same procedure stated earlier with $x[i]$ representing one element of the base. In Figure 5(A), a pressure pattern produced by a testosterone-treated juvenile is compared to one element of our base of pressure patterns. Plotting one against the other, similar patterns give rise to an almost linear plot. This means the two criteria are met: first, the slope of a linear regression differs from one less than the threshold value ε. Second, that the Euclidean distance between the patterns is less than the threshold value δ. To define the threshold values, we proceeded as follows. First, we randomly selected one song for each of the birds in our study. By inspection, we classified the pressure patterns in each of the songs, according to its similarity to the elements of the base, which were then used to extract patterns Then, we chose values of ε and δ, such that the algorithm, applied to each song, would allow us to identify, at least one of the patterns in it, as similar to the elements of the base. This provided us with the values $(\varepsilon, \delta) = (0.06, 3.5)$ for all the songs. In other words, with this choice of ε and δ, two patterns within each of the clusters obtained through SVD are also similar with the correlation criterium.

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The patterns selected using these procedures are displayed as the bottom panels of the insets in Figure 3 next to representatives of the solutions of the model that was used in Alonso et al. (2009) to fit air sac pressure patterns during song of adult male canaries.

Sound Envelope

To obtain the sound envelope of the song, the sound files were filtered with a digital low-pass filter. The cut-off frequency was set at 60 Hz. The digital filter was implemented in custom-written software (MATLAB, The MathWorks, Natick, MA) based on an algorithm emulating an RC integrator circuit. To have a zero-phase distortion between the sound envelope and the sound, we filtered the data for a first time. A way to get rid of the phase distortion introduced by this filtering process is to perform it backwards. The simplest way to do it is performing a three-step procedure consisting of (1) inversion of the filtered file ($x^i \mapsto x^{\text{final}-i}$), (2) application the filter, and (3) inversion of the output $x^j_{\text{filtered}} \mapsto x^{\text{final}-j}_{\text{filtered}}$. This allows us to obtain a sound envelope without phase distortion from the actual sound time series (Oppenheim and Schafer, 1989). This sound envelope was stored in a vector $se[i]$, $i = 1 \ldots m$, where m divided by the sample rate equals the song's duration.

Close Returns

To find syllable repetitions within song sequences, we analyzed the sound envelope, $se[i]$ $i = 1 \ldots m$, using the method of close returns (Gilmore, 1998). This method relies on the observation that if a time series presents an interval in which it behaves almost periodically with period p, then the difference between the values at $se[i]$ and $se[i+p]$ will be small. The difference between the values stored at $se[i]$ and $se[i+p]$ was computed as $d(i,p) = |se[i] - se[i+p]|$ for pairs of integer values (i,p). Whenever the difference $d(i,p)$ was smaller than threshold value ρ, a point is displayed at coordinates (i,p). For example, an almost periodic signal will appear as a straight line in the close return plot, and the length of the line corresponds to the duration of the periodic segment in the sound signal. Alternatively, in the close return plot in terms of time and period (measured in seconds), a point was displayed at (i/SR,p/SR), where SR is the value of the sample rate in hertz. As long as the signal remains almost periodic, the distance between points at an integer number of periods will satisfy the condition to be close, that is, $d(i, np) < \rho$ with n an integer. In this way, when a signal is periodic with period p, we do not only have a straight line at p but also at $n*p$, for $n = 2, \ldots, N$, where N will depend on the length of the interval where the signal is periodic.

Development of Syllabic Rates

Sound recordings from the first 14 days of treatment for animals in groups I and II were analyzed in the following way. The sound envelope and a close return plot were computed for five song bouts from each bird for each day of treatment. To see the repetition rate of pressure gestures, that is, the syllabic rate, a histogram was made for the points of the close return plot. Specifically, we take an interval $[t_1, t_2]$ in the close return plot and within that interval take bins in the period axes. Then, we count the number of points that are found in each bin. If that interval is one where the signal is almost periodic with period p, we obtain a distribution with maximal counts at p, $2p$, etc. If the signal is not periodic, peaks will be absent. From the first maximum, we can obtain the period of the signal at that interval. The syllabic rate is calculated as the reciprocal of the period. A syllabic rate was computed whenever a pattern repeated itself at least four times for every pattern except the very slow P0 pattern type, for which syllabic rates were computed if the pattern would repeat itself once. This procedure is repeated for the whole length of a song, for several songs per day per bird. For each day, all syllabic rates are computed and then plotted.

RESULTS

Respiratory Patterns in Testosterone-Treated Juveniles

After 20 days of testosterone treatment, air sac pressure patterns of song in juvenile canaries resembled those of adults, including all types of the "base." At this point, respiratory gestures were organized in phrases, which are formed by the repetition of a stereotyped respiratory pattern and resemble the respiratory gestures produced by adult canaries [Figs. 1 and 4]. Air sac pressure patterns of different syllable types were recorded in four testosterone treated juveniles while the fifth bird did not sing after implantation of the pressure cannula. The pulses were compared to those produced by adult canaries (base types) and the similarity calculated [Fig. 6 and Table 1]. The patterns that can be described as a harmonic oscillation [P1-like patterns; Fig. 6(A)] are present in the songs of all four juveniles, whereas the respiratory pulse, which consists of a first peak followed by a slow decay [P0-like patterns, Fig. 6(B)], was produced by three individuals. Although the first peak is smaller for juveniles 9 and 11, and for juvenile 04, it is barely suggested that the criteria we used for recognition were still achieved. Also, the amplitude of this first peak was fairly variable in syllables within and between adult individuals [see Fig. 1, panels (A), (C), and (F)]. A pulsatile pattern (P1b-like patterns) was generated by three of the four individuals [Fig. 6(C)], whereas the fourth pattern [P2 like patterns, Fig. 6(D)] of the base was produced by all individuals. It is therefore clear that, after 20 days of testosterone

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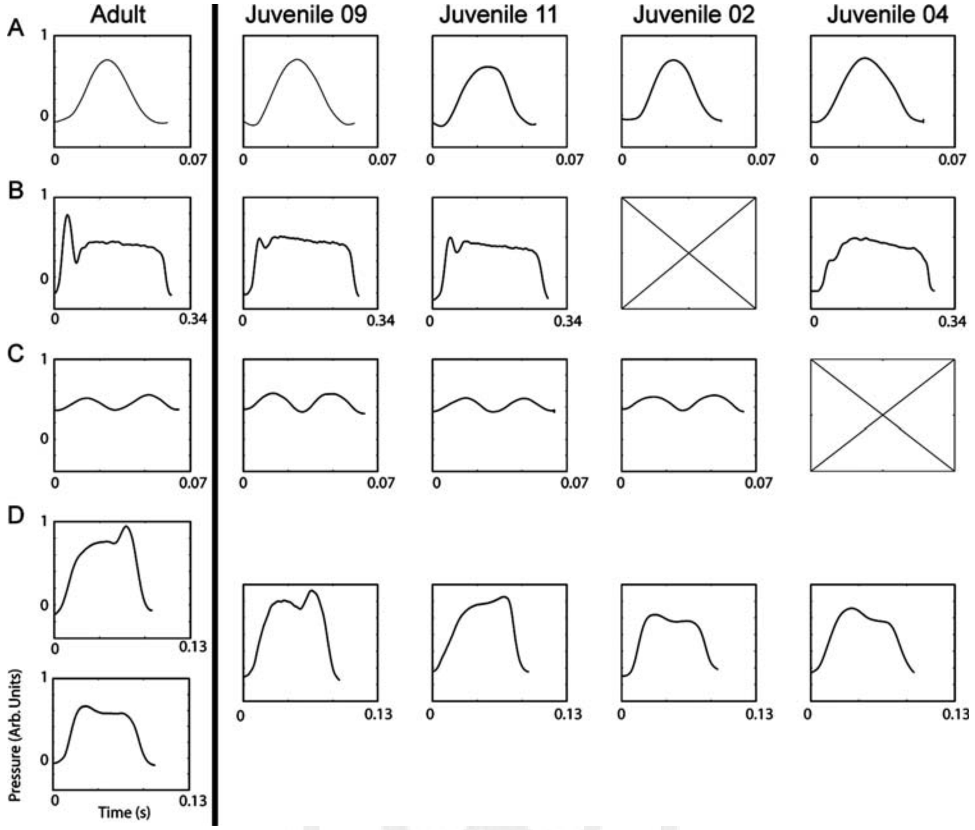

Figure 6 Pressure patterns found in different testosterone-treated juveniles. The basic shapes of pressure patterns chosen as "base" from one adult (leftmost column) were produced by testosterone treated juveniles after 20 days of treatment. Two of the treated birds presented all the shapes of the base, while two of the birds presented a subset of them.

treatment, all air sac pressure categories of adult song were generated at least by three of four individuals.

Respiratory Patterns in Untreated Juveniles

The respiratory patterns of untreated juveniles were substantially different from those of T-treated juveniles. Examples of pressure patterns of song from a treated juvenile, adult, and untreated juvenile [Fig. 7] illustrate the different song organization in untreated juveniles. Song in the untreated juvenile lacked repetition of individual pulses into phrases, and the individual pressure pulses are difficult to associate with those of the adult base. To analyze the stereotypy of pressure patterns, we compared each pressure pulse with all the pressure patterns produced in a song. We plot a point in the position (*i*,*j*) when the pressure pulses (*i*,*j*) are recognized as similar (see Methods section). In Figure 8(A), we analyzed the pressure gestures from Figure 1(A). The stereotypy of pressure patterns is reflected in the clusters of points around the diagonal. The same analysis for a group of seven

Table 1 Quantification of Similarity of Recognized Patterns

	T-Juvenile 09	T-Juvenile 11	T-Juvenile 02	T-Juvenile 04
P1	$D = 1.99$ $m = 1.05$ $R^2 = 0.99$ $\chi^2 = 0.002$	$D = 2.32$ $m = 0.99$ $R^2 = 0.99$ $\chi^2 = 0.002$	$D = 1.06$ $m = 1.02$ $R^2 = 0.99$ $\chi^2 = 0.0005$	$D = 3.05$ $m = 1.02$ $R^2 = 0.99$ $\chi^2 = 0.004$
P0	$D = 3.38$ $m = 1.02$ $R^2 = 0.98$ $\chi^2 = 0.001$	$D = 2.81$ $m = 1.02$ $R^2 = 0.98$ $\chi^2 = 0.001$		$D = 2.82$ $m = 0.99$ $R^2 = 0.98$ $\chi^2 = 0.001$
P1b	$D = 0.60$ $m = 0.99$ $R^2 = 0.96$ $\chi^2 = 0.001$	$D = 1.3$ $m = 0.91$ $R^2 = 0.86$ $\chi^2 = 0.0006$	$D = 1.17$ $m = 1.01$ $R^2 = 0.89$ $\chi^2 = 0.0005$	
P2	$D = 3.33$ $m = 1.05$ $R^2 = 0.99$ $\chi^2 = 0.003$	$D = 2.92$ $m = 0.94$ $R^2 = 0.99$ $\chi^2 = 0.002$	$D = 3.30$ $m = 0.99$ $R^2 = 0.98$ $\chi^2 = 0.002$	$D = 0.82$ $m = 1.03$ $R^2 = 0.99$ $\chi^2 = 0.0002$

For each recognized patterns in Figure 5, the Euclidian distance *D*, the linear regression slope *m*, the coefficient of determination R^2, and χ^2. Patterns were recognized if $D < 3.5$, $|m - 1| < 0.06$, $R^2 > 0.85$, and $\chi^2 < 0.005$.

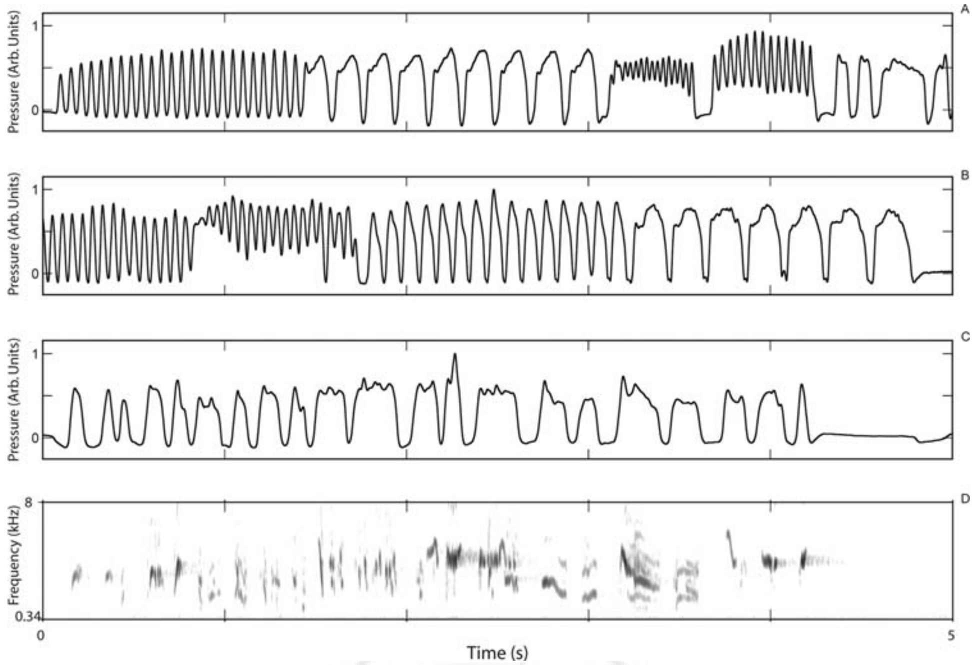

Figure 7 Nontreated juvenile pressure pattern. Air sac pressure time series data for a nontreated adult (A), a testosterone-treated juvenile (B), and a nontreated juvenile (C). Both treated and nontreated juvenile are aged 80 days; at the initiation of the sensory motor period. Both adult and testosterone-treated juvenile present repetitive and stereotyped patterns conversely nontreated juvenile patterns present a lack of repetitions and stereotypy. In panel (D), we show the spectrogram corresponding to the song produced by the nontreated juvenile.

bouts produced by a nontreated juvenile is shown in Figure 8(B). The absence of clusters is the signature of the lack of stereotypy and repetitions.

To compare the shapes of pressure pulses, we compared two elements of the base [Fig. 8(D,E) filled lines] with the pressure patterns from the untreated juveniles. The untreated juveniles produced simple gestures corresponding to the P1 type in our base. However, the expiratory pulses were significantly longer for nontreated juveniles P1 type gestures last, mean ± SD: 0.11% ± 0.36% while P1 type patterns of the base last mean ± SD: 0.06% ± 0.01%, performing a two sample *t*-test: $t_{12} = -10.50$, $p < 0.0001$, ($N = 15$), at the 0.05 level the two means are significantly different.

Solutions with two local maxima can be observed although none of them satisfy the similarity criterion; the distance between the pattern and the element of the base is $\delta = 6.2 > 3.5$, the value used to define similarity (see Methods section). The pulsatile respiratory pattern was absent in the gestures produced by the nontreated juveniles.

Ontogeny of Syllabic Rates

In addition to the emergence of adultlike pressure patterns, the temporal arrangement of syllables into phrases also rapidly emerged (as was previously observed by Gardner et al., 2005). The ontogeny of syllabic rates was measured for three T-treated juveniles from sound recordings, which were started on the first day of hormonal treatment. At the first day of treatment, 60 PHD, song production of T-treated and untreated juveniles was highly variable and of low amplitude, and syllable repetition into phrases was completely absent [Fig. 9(A,B)]. The absence of straight lines in the close return plots for the sound envelope of both treatment groups [Fig. 9(A,B), bottom panels] illustrates that repetitive production of the same syllable does not occur at this stage. In T-treated juveniles, however, song structure changes over the course of the first 5 days of T-administration and at the 14th day of treatment the temporal structure of song resembles that of adult birds [Fig. 9(D)]. The close return plot [Fig. 9(D), bottom panel] illustrates phrase structure as indicated by nearly straight lines and the occurrence of different syllable repetition rates in the different placement of lines along the vertical axis. At this same age, untreated control birds still sang plastic song without syllable repetitions [Fig. 9(C)]. The comparison with the song of an adult male canary in breeding condition [Fig. 9(E)] indicates clearly that the main song structure is present after 14 days of T-treatment of juveniles (at PHD 74).

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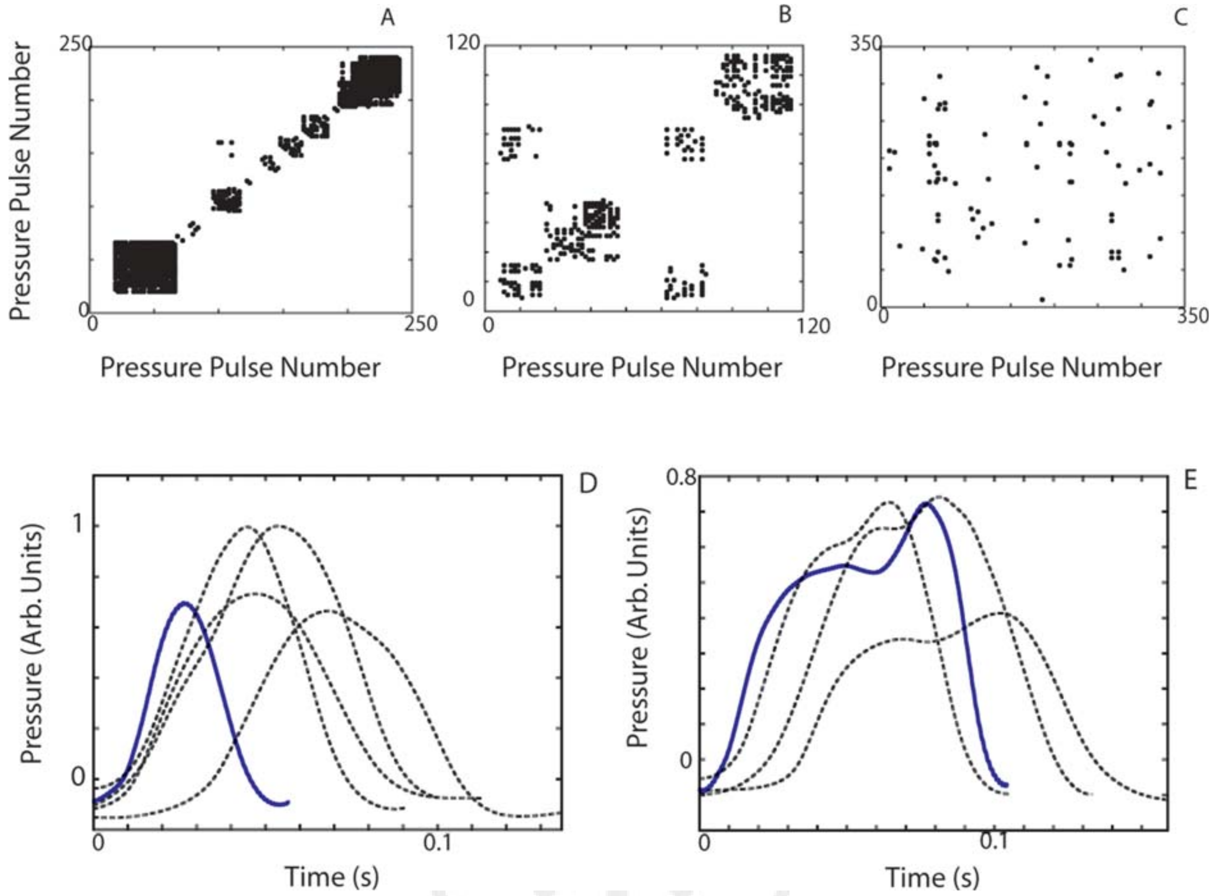

Figure 8 Absence of stereotypy in the nontreated juvenile pressure gestures. Points around the diagonal in (A) show the stereotypy and the repetitive structure of pressure patterns displayed in Figure 1(A). Each pressure pulse produced during a song is compared to every pulse produced in the song, and a point is plotted in the position (*i*,*j*) when two pressure patterns are recognized as similar (see Methods section) The same analysis is performed for a testosterone treated juvenile pressure patterns (B) and a nontreated juvenile (C). The absence of point clusters indicates the absence of stereotypy and repetitive patterns. A comparison of pressure patterns produced by a nontreated juvenile (dashed line) with the elements of the base (solid line) is shown in (D) and (E).

Even after only 3–5 days of T-treatment, song started to become organized into phrases, but different syllables, corresponding to different types of respiratory gestures, emerged at different times. Silent respiration of T-treated juveniles ranges from 1 to 2 Hz, being only comparable with the production of long syllables as the ones associated to P0 type pressure patterns and much slower than the rest of respiratory patterns used to produce song. Silent pressure patterns were not harmonically related to simplest respiratory patterns produced by T-treated juveniles. Syllables with a syllable repetition rate of 14–17.s^{-1} were the first to be organized into phrases, followed by syllables with lower repetition rate, and pulsatile syllables with the highest rate emerged last, between 4 and 8 days after onset of T-treatment [Fig. 10]. Syllable repetition rates change somewhat during the recording period for each category. The classification of syllables by repetition rate corresponds to the categorization based on air sac pressure patterns of song after 3 weeks of T-treatment and adult song [Figs. 2 and 4]. Syllabic repetition rates ranging between 13 and 25 Hz correspond to P1-like pressure patterns; between 8 and 12 Hz, they are P2-like patterns; and at low-syllabic rates (2–5 Hz), they are P0-like patterns. Finally, syllabic rates >27 Hz correspond to P1b-like patterns (pulsatile syllables). This categorization accounts for all repeating segments of the time series found in the crystallized songs of T-treated and adult birds.

DISCUSSION

Here, we show that after a few days of testosterone treatment juvenile canaries develop adultlike song

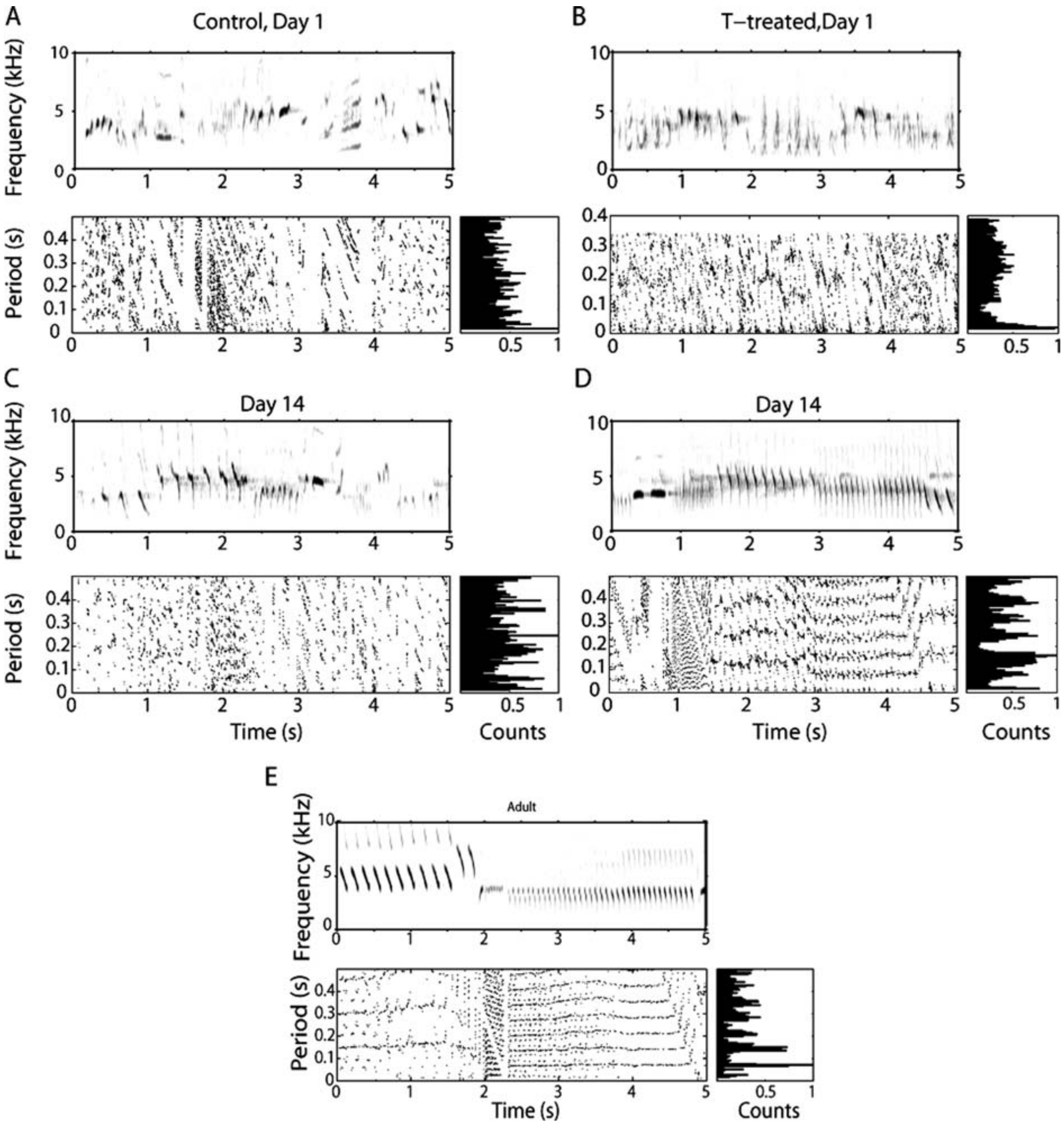

Figure 9 The emergence of structure in testosterone-treated juveniles during the treatment. Sonograms (top panels) and close return plots (bottom panels) for a control juvenile (A), a testosterone treated juvenile on the first day of treatment (B), the same control individual as in (A) after 14 days (C), and the same testosterone treated juvenile as in (B), after 14 days of treatment (D). The top panels display sonograms of their vocalization, that is, a frequency versus time representation of the uttered sounds. The bottom panels display close return plots, which is a graphical representation of the recurrences present in the uttered sound (see Methods section). The small insets at the right of the close return plots display the number of points plotted for each value of p. In this way, a set of well-defined peaks reveals the existence of recurrences. Although no clear structures are found in the control birds, the *t*-treated juveniles showed clear recurrences after 14 days of treatment [cf. (C) with (D)].

structure and that, after 20 days, respiratory patterns of song are stereotyped. Respiratory gestures to produce different syllables at that point match all types identified in adult song. T-treated juveniles in our experiments had a slightly smaller repertoire size than normal adults (mean of 16 ± 2 syllables against 20 ± 1) (Lehongre et al., 2008). The syllables produced by T-treated juveniles presented characteristics of adult syllables. The emergence of adultlike pressure patterns provides some validation for a model suggesting a neural substrate of a periodically forced nonlinear circuit for the generation of different respiratory gestures of song. The results show that extensive vocal practice during the sensorimotor phase is not required for the emergence of adultlike song structure and respiratory gestures. In the following, we will discuss these results in light of the known effects of testosterone on the neural substrates of song production, and how the results of this study may elucidate the nature of the respiratory motor program for song production and the stages of song learning.

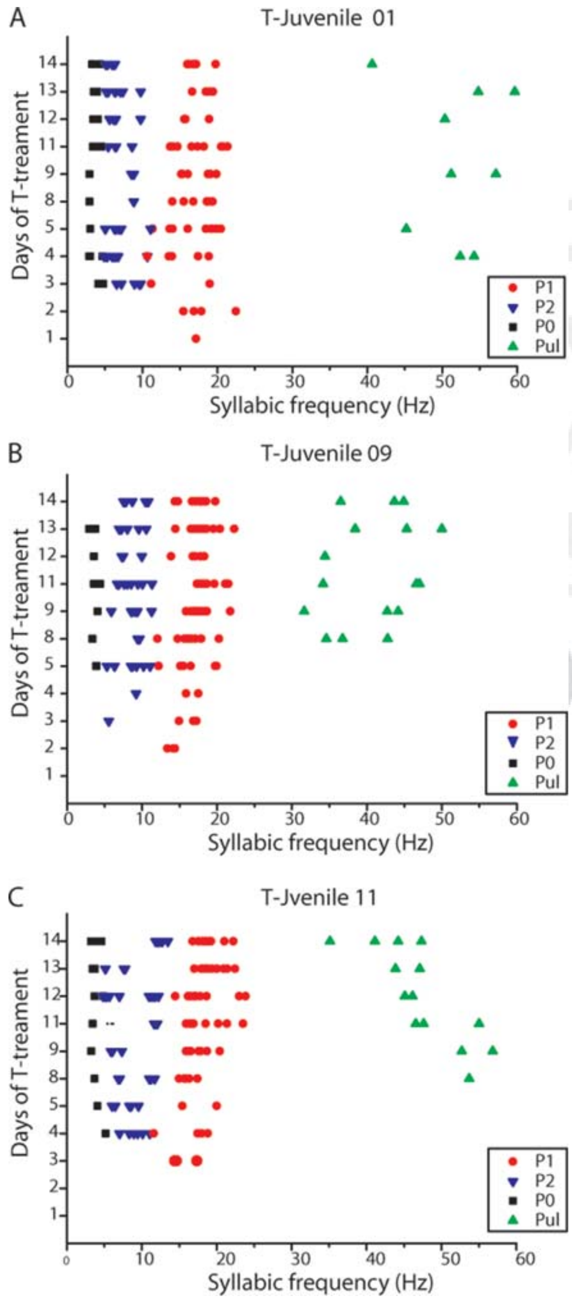

Figure 10 Time evolution of the syllabic rate during treatment. For three different testosterone treated juveniles (A–C), we display the evolution of the syllabic frequencies (horizontal axis) of different sound patterns as a function of time (vertical axis). The different point types denote different sound envelope shapes, which resemble the pressure patterns measured for the birds after 14 days of treatment. For the three birds treated with testosterone, the first sound envelope pattern, which is clearly identified in the close return plots, is a P1 solution (see Methods section), between 12 and 25 Hz, which appear as early as in the second day of treatment. By the fourth day of treatment, the P2 solutions and the P0 solutions are clearly seen in the close return plots. The pulsatile solutions are unambiguously found by the eighth day of treatment. By the 14th day of treatment, well-defined patterns clearly resembling the air sac pressure patterns are seen in the *t*-treated birds. The different sound envelope shapes are found in ranges of syllabic rates, which are the same ones of the pressure patterns for nontreated adults. After 14 days of treatment, no structure as this one has been found for the sounds uttered by control birds.

The putative model for neural control of respiratory motor gestures of song proposes a nonlinear circuit, which is driven by a simple periodic input (Trevisan et al., 2006; Arneodo et al., 2008). It is unclear where this putative circuit may be located within the song control pathway. A likely location is within the respiratory premotor nuclei (nucleus retroambigualis, Ram, and nucleus parambigualis, PAm) (Trevisan et al., 2006), but a more distributed manifestation, including the robust nucleus of the arcopallium (RA), is also possible. With this model, simple changes in the forcing (e.g., an increase or decrease of the frequency) are sufficient to produce qualitatively different outputs, corresponding to the categories of air sac pressure pulses described earlier. The shapes that we recognize and use as a base are not an arbitrary set: they present the morphological features of the solution of a *low dimensional* nonlinear model (Alonso et al., 2009). The synthetic solutions of this mathematical model do not only account for the shapes of the pressure patterns but also the relative timing of the modeled syllables and thus the temporal structure of song.

Using this model, the results of this study lead to conclusions in the context of the effects of testosterone on the neural control mechanisms. Changes in the song circuit during vocal development and seasonal cycles coincide with changes in circulating testosterone (e.g. De Voogd, 1991). In the brain, circulating testosterone is converted to estradiol through aromatase, and many of the neural changes are affected by the latter. As circulating testosterone levels rise, the number of connections between HVC and RA increases and electrophysiological properties of RA neurons as well as their firing properties change drastically (Meitzen et al., 2007a,b, 2009). Increased spontaneous firing rates may be the result of altered cellular properties, changing connectivity within this nucleus, and changing input from HVC and LMAN. Testosterone also affects the peripheral structures for song production (Luine et al., 1980; Bleisch et al., 1984; Lohmann and Gahr, 2000). In birds, the best-documented effect is a proliferation of syringeal musculature. Effects on other tissues are known in mammals and are likely in birds (Abitbol et al., 1999; King et al., 2001; Cynx et al., 2005), but currently no data exist on testosterone effects on the vibrating tissues, the labia.

HVC, RA, respiratory areas in the brain, nXIIts, and even the syringeal muscles express steroid receptors (Lieberburg and Nottebohm, 1979; Gahr and Wild, 1997; Schlinger and Brenowitz, 2002; Perlman et al., 2003). The presence of receptors suggests that all these central and peripheral sites of song control are affected by changing testosterone levels and may therefore play a role in the observed changes. Although the role of testosterone in effecting changes in the peripheral systems is not sufficiently understood, the emergence of adultlike syllable morphology and phrase organization is most likely caused by changes in the motor planning and execution stage, thus involving the telencephalic nuclei. Generation of song syllables and phrases requires a high degree of coordination between respiratory, syringeal, and upper vocal tract motor systems, which makes it highly unlikely that peripheral changes alone could explain the observed changes.

The effects of testosterone on neural song motor control structures could account for the observed changes in respiratory motor gestures and behavioral effects within the framework of the putative neural model for respiratory control of song production. One way to explain the emergence of the adultlike categories is by a change in the forcing of the nonlinear circuit from an otherwise similar neural circuit. This change in forcing could be implemented by a switch in input to RA from the anterior forebrain pathway to a strengthened connection from HVC (Mooney and Rao, 1994) or from RA to the respiratory network. In particular, there is evidence that the connections between HVC and RA strengthen during the vocal learning process (e.g., Alvarez-Buylla et al., 1994; Bottjer and Arnold, 1997). Furthermore, there is a switch in the influence of the anterior forebrain pathway from being the driver of subsong without the need of HVC involvement (Aronov et al., 2008) to no longer being essential for song production in adult birds (e.g., Scharff and Nottebohm, 1991). Testosterone treatment may accelerate this switch and judging by our results it can happen within 5–8 days of the onset of T-administration. Interestingly, the emergence of particular pressure shapes and the restructuring of the song sequence into phrases happen simultaneously.

Another, not necessarily exclusive possibility, is that the emergence of the patterns is caused by testosterone induced changes of the neural network. For example, electrophysiological recordings from RA neurons provide indirect support that such network changes do occur in this nucleus during vocal ontogeny. Exposure to song and subsequent song production coincides with a change in the spontaneous firing rate of RA neurons (Shank and Margoliash, 2009). Seasonal changes in the neurophysiological properties and firing rate of RA neurons support the notion that elevated testosterone levels can mediate network changes within RA (Meitzen et al., 2007a). Increased connectivity within RA (Spiro et al., 1999) favors more synchronized action compatible with low-dimensional, nontrivial dynamics, as the one which is present in the full set of pressure pulse categories in the T-treated juveniles.

The biological changes that occur during vocal ontogeny or follow seasonal fluctuations in testosterone levels argue strongly for the involvement of both mechanisms in the rapid emergence of adultlike respiratory motor gestures for song in the T-treated juvenile canaries. The rapid change in song structure suggests that the switch in forcing and the restructuring of the network occurs quickly. It is plausible to postulate such a rapid time course, because changes in RA neurons and the RA circuitry do occur rapidly (Adret and Margoliash, 2002; Shank and Margoliash, 2009).

Testosterone administration to female canaries induces regular singing, whereas it occurs only sporadically in untreated females (Leonard, 1939; Hartley et al., 1997). In contrast to male song, however, female song only contains pressure pulses of the P1-category, minibreath, and pulsatile, but does not include P2 and P0 types (Mendez et al., 2006). This absence of the more complex pressure pulses with modulations could be the result of lower amplitude of the forcing signal. P1 type pressure pulses are possible with a forcing signal of low amplitude, whereas P2 and P0 only arise at higher amplitude. The administration of exogenous testosterone to female canaries does not result in a complete masculinization of the song control circuit. For example, the volumes of HVC and RA for testosterone-treated female canaries are significantly smaller than the corresponding volumes for adult males (Appeltants et al., 2003). It is therefore plausible that a smaller number of neurons results in less connectivity between HVC and RA, which is consistent with lower amplitude of the forcing signal. Similarly, all air sac pressure patterns of untreated juveniles are of the P1-type of fairly low frequency, and they are not organized into phrases. These respiratory characteristics are consistent with an output from the putative model that is driven by slow, irregular forcing. Absence of a clear periodic nature of the forcing signal could explain the highly variable pressure shapes and the lack of organization of syllables into phrases.

The observation that young canaries develop all adult categories of respiratory motor gestures after a few days of testosterone treatment raises the question of what role a prolonged sensorimotor period plays in song ontogeny. This period is regarded as a motor practice phase during which song gradually improves toward more adultlike acoustic and temporal structure (e.g., Brainard and Doupe, 2002). The effects on adult song that follow experimental manipulation of the motor practice phase do however not unequivocally support this interpretation. Testosterone-induced "crystallization" at early stages in development caused deficits in adult song in some experiments (Korsia and Bottjer, 1991; Whaling et al., 1995; Titus et al., 1997), whereas reversible alteration of auditory feedback during long periods of the sensorimotor phase did not cause lasting song deficits in the zebra finch (Pytte and Suthers, 2000). Perhaps, early administration of testosterone induces changes in the neural circuitry that affect the integration of template acquisition with sensorimotor processes. It is therefore possible that the observed deficits in song do not result from reduced motor practice. The emergence of all categories of respiratory motor gestures in 70-day-old canaries is consistent with this latter possibility. However, we did not test how similar stereotypy of motor gestures and song repertoires of our T-treated juveniles were to those of normal adult song. Nevertheless, a normal, extended sensorimotor practice period is not required for emergence of adult motor patterns per se. It may be required, however, for fine scale refinement of motor gestures for individual syllables and for formation of large syllable repertoires.

In conclusion, the observed emergence of adultlike respiratory motor gestures in T-treated young canaries is consistent with the putative model that the diversity of respiratory motor gestures emerges from a periodically driven nonlinear neural network (Arneodo et al., 2008). Although the location of this network is still putative, increasing evidence from behavioral, physiological, and anatomical studies points toward the existence of a simple neural mechanism for generating diverse vocal patterns.

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