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Singing with reduced air sac volume causes uniform decrease in airflow and sound amplitude in the zebra finch

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SUMMARY

Song of the zebra finch (*Taeniopygia guttata*) is a complex temporal sequence generated by a drastic change to the regular oscillations of the normal respiratory pattern. It is not known how respiratory functions, such as supply of air volume and gas exchange, are controlled during song. To understand the integration between respiration and song, we manipulated respiration during song by injecting inert dental medium into the air sacs. Increased respiratory rate after injections indicates that the reduction of air affected quiet respiration and that birds compensated for the reduced air volume. During song, air sac pressure, tracheal airflow and sound amplitude decreased substantially with each injection. This decrease was consistently present during each expiratory pulse of the song motif irrespective of the air volume used. Few changes to the temporal pattern of song were noted, such as the increased duration of a minibreath in one bird and the decrease in duration of a long syllable in another bird. Despite the drastic reduction in air sac pressure, airflow and sound amplitude, no increase in abdominal muscle activity was seen. This suggests that during song, birds do not compensate for the reduced physiological or acoustic parameters. Neither somatosensory nor auditory feedback mechanisms appear to effect a correction in expiratory effort to compensate for reduced air sac pressure and sound amplitude.

Key words: birdsong, respiration, sensory feedback, motor control, *Taeniopygia guttata*.

INTRODUCTION

Birdsong is an interesting behavior in regard to respiratory dynamics because its production requires drastic changes in ventilation patterns. How singing and respiration are integrated is largely unknown. In songbirds, song is a learned vocal behavior and respiratory patterns are established during vocal ontogeny. During song development, temporal patterns of song must be assembled such that they do not conflict with physiological and physical constraints. For example, respiratory needs must be met, and flow patterns have to be consistent with available air volume. Once song ontogeny is completed, the respiratory pattern of song is highly stereotyped and presumably represents a compromise solution between maximizing acoustic and temporal parameters of song and the above-mentioned constraints.

Songs of many bird species are complex temporal sequences of alternating sounds and silent periods, which are generated by elaborate respiratory patterns (Suthers et al., 1999). Quiet respiration is characterized by its rhythmic alternation between expiration and inspiration, each driven by similar absolute changes in air sac pressure. Sound is normally produced during expiration and, during song, the expiratory pressure increases at least 10–20-fold over that of quiet respiration. In addition, the duration of respiratory phases becomes more variable (e.g. Suthers and Goller, 1997; Suthers et al., 1999; Goller and Cooper, 2004; Goller and Daley, 2001). Silent periods during song are used to take short inspirations (minibreaths), which are drawn with increased inspiratory pressure relative to that of quiet breathing. In the zebra finch (*Taeniopygia guttata*), song consists of a series of 4–8 different expiratory pulses, corresponding to the song syllables, which alternate with minibreaths (Fig. 1) (Franz and Goller, 2002; Goller and Cooper, 2004). This sequence of stereotyped syllables (motif) is repeated a variable number of times and forms a song bout.

The drastic change in respiratory pattern from quiet breathing to song poses the question of whether or not respiratory functions are maintained during song. First, expiratory airflow during song production cannot exceed the volume of available air in the air sac reservoirs. The timing of expiration and inspiration during the song pattern is dependent on the air volume that is required for sound production. In each species, the temporal pattern of vocal and silent periods during song is therefore determined by the required volume of air, storage volume of air and the need to replenish air during silent periods. Minibreaths are typically much shorter and are driven by higher inspiratory air sac pressure than quiet inspirations. In two species, canary (*Serinus canaria*) and zebra finch, calibrated airflow recordings confirm that minibreaths replenish the air volume that was expelled during the phonatory expirations (Hartley and Suthers, 1989; Goller and Daley, 2001). However, it is unknown how close to the limits of air supply birds operate during song (Goller and Cooper, 2004).

Second, it is largely unknown whether gas exchange is maintained during the drastically altered ventilation patterns of song. Indirect evidence from measurements of oxygen consumption indicates that gas exchange is not compromised because birds do not incur an oxygen debt during song (Oberweger and Goller, 2001; Franz and Goller, 2003). Decreased respiration after song in zebra finches and canaries suggests that some individuals even hyperventilate during song, although hyperventilation in the zebra finch is uncommon (F.G., unpublished observation). The ventilatory pattern of song may therefore increase excretion of carbon dioxide (Hartley and Suthers, 1989; Franz and Goller, 2003). Together, these findings suggest that during song, airflow through the lungs is sufficient for gas exchange and may even be increased compared with ventilation during quiet respiration. However, this may not be true for all

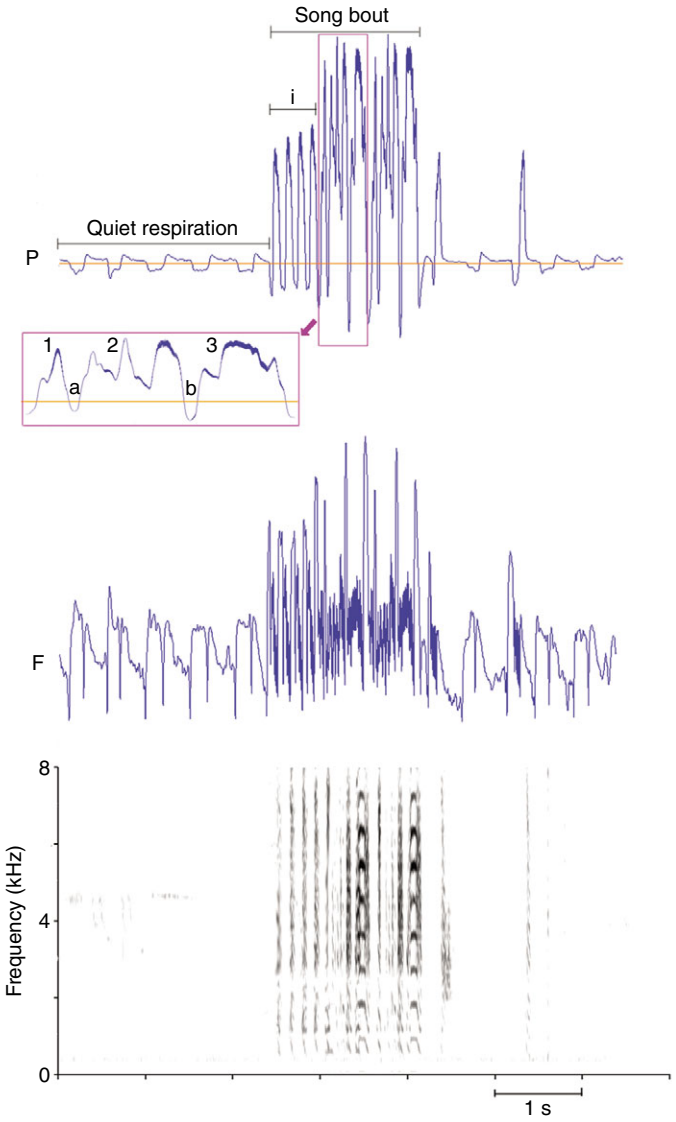

Fig. 1. Example of zebra finch song illustrating physiological and acoustic data. Terminology used for description of different temporal units of song is explained using the air sac pressure trace (P): i, introductory notes. Inset shows the three different syllables corresponding to expiratory pulses of the song motif (1, 2 and 3), interrupted by minibreaths (a and b). Air sac pressure patterns differ markedly between quiet respiration and song. Expiratory pressure is defined as pressure values above and inspiratory pressure as values below the ambient pressure line (orange horizontal line), respectively. Tracheal airflow (F) shows a regular flow pattern during quiet respiration and the altered temporal and amplitude pattern during song. The flow data do not indicate direction of airflow, but the direction can be inferred from air sac pressure. The sound is shown spectrographically (bottom panel).

syllable types, because the volume exchanged during a phonation–minibreath cycle may vary substantially. In canaries, airflow during minibreath-syllables varies at least 6-fold, and during some syllables the exchanged air volume is less than the tracheal deadspace (Hartley and Suthers, 1989).

The avian respiratory system is highly efficient in exchanging oxygen and carbon dioxide (e.g. Maina, 2000; Powell and Scheid, 1989), and this efficiency is in part attributed to the unidirectional flow of air through the lung during both phases of the respiratory cycle. This flow pattern is possible through the intricate morphological design (Fig. 2), where air sacs function as bellows and air reservoirs (e.g. Scheid and Piiper, 1989) and flow through the rigid lungs is primarily directed by aerodynamic valves (e.g. Jones et al., 1981; Banzett et al., 1987; Brown et al., 1995). The anterior and posterior thoracic sets of air sacs both contribute air to expiratory flow. Air from the posterior air sacs perfuses the lungs and then enters the primary bronchi, whereas air from the anterior air sacs is directly routed into the primary bronchi. The vocal organ, the syrinx, is situated where the primary bronchi merge into the trachea. Thus, the air stream for phonation presumably originates in roughly equal parts from both the anterior and posterior reservoirs. This model assumes that airflow patterns during song are similar to those established in anesthetized, quietly breathing birds (Bretz and Schmidt-Nielsen, 1972). Airflow patterns within the respiratory system have not been directly studied during dynamic behaviors such as singing.

In the adult zebra finch, respiratory patterns of song are highly stereotyped (Franz and Goller, 2002). Only slight modification of song tempo occurs in different social contexts (e.g. Cooper and Goller, 2006). The temporal pattern of song varies between males, possibly causing variation in lung ventilation (Franz and Goller, 2002). In addition, the duration of different syllables within a song differs, leading to a variation in the volume of air that is needed to complete each syllable. One experiment showed that individual expiratory syllables require up to 0.3 ml of air (Goller and Daley, 2001).

Manipulations to the respiratory system could give insight into how dynamic behaviors integrate with respiratory needs. Total occlusion of the thoracic air sacs of chickens (*Gallus domesticus*) elicited no changes in quiet respiration during exercise (Brackenbury et al., 1989). Few studies have attempted to experimentally manipulate respiratory dynamics during song. Small injections of air into an anterior thoracic air sac during song in the northern cardinal (*Cardinalis cardinalis*) elicited a compensatory decrease in expiratory effort in the abdominal muscles. This indicates that cardinals use an on-line feedback mechanism to monitor air sac pressure and airflow characteristics during song (Suthers et al., 2002). The small injections were unlikely to affect gas exchange or flow patterns, and these experiments therefore do not address questions about respiratory functions during song.

In an attempt to study the integration between respiratory needs and song, we here describe a chronic manipulation of respiratory functions. We manipulated respiration during song in zebra finches by eliminating part or all of the air volume contained in the posterior thoracic air sacs. We then recorded changes in respiratory and airflow patterns as well as acoustic output during singing.

MATERIALS AND METHODS

Casting of respiratory system

Casts of the respiratory system were made to estimate the volume of individual air sacs and their relative contribution to air supply in the zebra finch. The procedure followed closely the methods developed by Duncker (Duncker, 1971). Birds were euthanized with an overdose of isoflurane. Flexible tubing (Silastic laboratory tubing with inner diameter of 0.76 mm and outer diameter of 1.65 mm; Dow Corning, Midland, MI, USA) was inserted into the glottis of the bird and secured with tissue adhesive, so that there was no leaking around the insertion. The tubing was attached to a reservoir, which was later filled with MICROFIL CP-101 (Flowtech Inc., Carver, MA, USA), a liquid silicone compound for microvascular injection, which is cured by

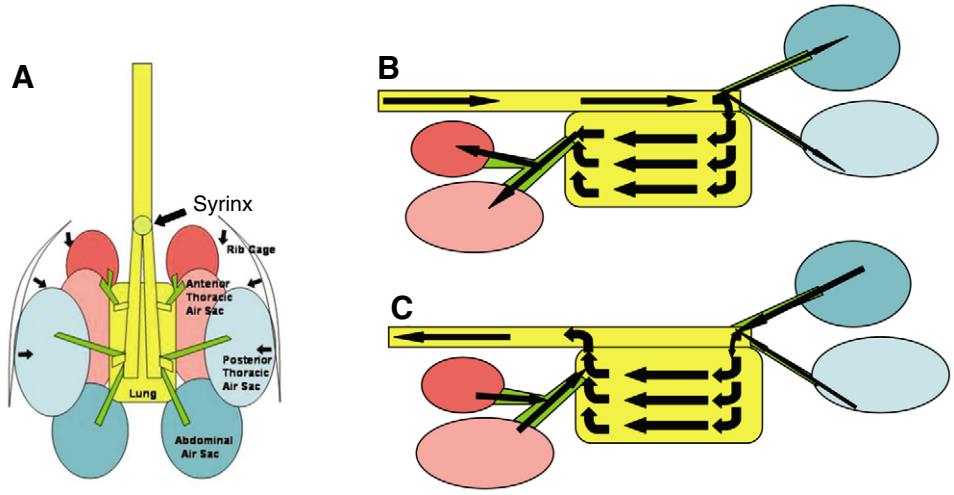

Fig. 2. A schematic of the avian respiratory system, illustrating the major air sacs and their connections to the lung. (A) The lateral and dorsal direction of motion of the rib cage during exhalation is indicated by arrows. (B) The direction of airflow during inspiration. (C) The direction of flow during expiration.

stannous octoate and a crosslink agent, ethyl silicate. The bird was placed in an airtight glass chamber and the chamber was attached through another tube to a vacuum source. The chamber and the zebra finch were evacuated to 1.2–5.0 psi (8.3–34.5 kPa) below the outside air pressure. When the pressure is at its lowest, the CP-101 is released through the glottal tube into the respiratory system of the bird, driven by the higher pressure outside the chamber. The rate of repressurization was kept low *via* a regulatory valve. To avoid rupture of membranes inside the bird, the chamber was also vented slowly as the bird was filling with casting material. After all pressures were equalized, the casting material was left to cure for several minutes. The bird was then placed into a refrigerator to allow full curing of the CP-101 overnight. The body of the bird was then placed into a container with KOH for 3–4 days to expose the cast of the respiratory system. To estimate volumes of different air sacs, we first determined the density of the casting material. We inserted samples into a 1 ml syringe and determined the volume of displaced fluid. These samples were then weighed and the relationship between mass and volume calculated. The volume of air sacs was estimated by weighing the casts.

Injection experiment

Male zebra finches (*Taeniopygia guttata* Gould) were isolated in a small cage, which was placed into a wooden box lined with 5 cm-thick acoustic foam. After the bird resumed singing in the new environment, it was fitted with an elastic belt around its thorax with a Velcro™ tab situated on the back. Birds were tethered with a wire leash attached to the Velcro™ tab. The other end of the leash was led through the top of the wire cage and was attached to a tether arm, which allowed free movement of the bird and was counterbalanced to any additional weight attached to the backpack. After the bird sang again, surgical implantation of a pressure cannula and airflow probe followed.

Timeline

For 1–3 days, the bird was placed in a cage alone and allowed to acclimate to his environment. Once he began to sing, he was belted and leashed and allowed to acclimate again. Once song began again (1–3 more days), surgery was performed, during which a cannula and/or flow probes or wire electrodes for recording of electromyograms (EMG), where applicable, were implanted. This was day 1 of the experiment. Birds usually took 24 h until they resumed singing. Once sufficient data were collected (at least 20 song bouts), which was typically finished on day 2, the left posterior thoracic air sac of the bird was injected late on day 2. On day 3, 20 more song bouts were collected. The bird then received a second injection. Day 4 and beyond, we collected as much song as we could until signal quality deteriorated. The bird was then euthanized and placement and size of the injections were determined.

Measurements of air sac pressure

After food and water deprivation for one hour, birds were anesthetized with isoflurane. A cannula (silastic tubing with 0.76 mm inner and 1.65 mm outer diameter) was inserted below the last rib into the left anterior thoracic air sac and sutured to the rib cage. Tissue adhesive was applied to seal the insertion site. The free end of the cannula was connected to a piezoresistive pressure transducer (FPM-02PG; Fujikura, Tokyo, Japan), which was mounted on the Velcro™ tab on the backpack. A more detailed explanation can be found in Franz and Goller (Franz and Goller, 2002).

Airflow measurements

Tracheal airflow was measured in four birds. Flow probes were custom-built by attaching microbead thermistors (0.13 mm; BB05JA202; Thermometrics, Edison, NJ, USA) to small wires with conductive epoxy and then insulating the contacts with non-conductive epoxy. The skin was opened above the furcula at the midline, exposing the trachea. Just above the membrane of the interclavicular air sac, a small hole was made into the connective tissue between two cartilages of the trachea. The tip of the flow probe was inserted into this hole and then secured in place by a suture around the cartilage just cranial to the flow probe. The wires were routed subcutaneously to microconnectors on the backpack. Airflow was determined by a feedback circuit (Hector Engineering, Ellettsville, IN, USA) and is proportional to the current required to maintain the thermistor at a constant temperature. A more detailed description can be found in Goller and Daley (Goller and Daley, 2001).

Electromyograms from abdominal expiratory muscles

In six birds, EMG were recorded from the abdominal muscle sheet. No effort was made to record specifically from only one of the muscles. Electrodes were placed into the muscle sheet so that they most likely recorded from all three main muscles: m. obliquus abdominis externus, m. obliquus abdominis internus and m. transversus abdominis. Bipolar EMG electrodes were implanted after a small area of the muscle sheet was exposed by opening the skin and connective tissue. Electrode tips were pushed into the muscle sheet, the wires were looped to provide slack and then routed subcutaneously to the back. The incision in the skin was then closed with suture and tissue adhesive. EMG signals were amplified (1 K gain) and band-pass filtered (100–3000 Hz) with a DAGAN EX4-400 amplifier (Minneapolis, MN, USA).

Injecting

In order to reduce the volume of available air within air sacs, we injected dental impression medium (Reprosil Type 1 Hydrophilic Vinyl Polysiloxane Impression Material; Milford, DE, USA). After food deprivation, the bird was again anesthetized with isoflurane. Impression material was filled into a syringe and injected through a hypodermic needle (size 18 G) into a posterior thoracic air sac. Within minutes, the injected material cures into a block but retains some flexibility. Although every effort was made to inject the same amount of material each time, the volume injected into the air sac ranged from 0.08 to 0.23 ml of the impression material. This variation was caused by differing amounts of leakage from the injection site. The puncture in the body wall was closed with surgical suture and tissue adhesive as needed. Typically, we injected first the left air sac and, in a subsequent injection, the right air sac. Once enough song was collected following the second injection, the bird was euthanized. We first determined the placement of the injection *in situ* and then extracted the impression material for determining its volume.

Recording physiological measurements and song

During each stage of the experiment (preinjection, after one injection and after two injections), song was recorded together with physiological data. A female zebra finch was placed in front of the cage at a constant distance to the perch to induce the male to sing. All song used for analysis was directed song to assure that the singing bird faced forward towards the microphone.

Song was recorded with an Audiotechnica AT8356 microphone (Stow, OH, USA) and amplified with a Brownlee amplifier (Model 410; San Jose, CA, USA). The voltage output of the pressure transducer was recorded simultaneously with the sound either on a TEAC 135T multi-channel digital recorder at a sample rate of 24 kHz or directly onto computer (Avisoft recorder, 26–32 kHz sample rate; National Instruments PCI-6220 M, Austin, TX, USA) using Avisoft Recorder software (Avisoft Bioacoustics, Berlin, Germany). Flow and EMGs were recorded simultaneously on separate channels [see also Franz and Goller (Franz and Goller, 2002) for more detailed description].

Analysis

We digitized the TEAC recordings (Data translation 2821G AD converter at a 40 kHz sample rate; Marlboro, MA, USA). We then analyzed the data using SIGNAL 3.1 software (Engineering Design, Berkeley, CA, USA). We looked for temporal and amplitude differences in pressure patterns between the pre-injection and post-injection song bouts.

The respiratory pattern was analyzed during quiet respiration and during song for the various stages of the experiment. For quiet respiration and for each expiratory pulse of the song motif, we measured the duration of the expiratory phase as well as respiratory rate. In addition, we used integrated voltage of each expiration as a measure of total expiratory effort, assuming that syringeal resistance did not change between treatments. Airflow was quantified in a similar fashion, using uncalibrated voltage output. Calibration was not possible for the various stages of the experiment. In order to assess whether the voltage response of the flow probe deteriorated over the course of the experiment, we compared changes in quiet respiration to changes in song syllables.

In order to compare EMG recordings across days, we plotted EMG activity for individual expiratory pulses against air sac pressure during quiet respiration and song. We assumed that changes in quiet respiration reflect a deterioration in the EMG signal over the course of the experiment and used this information to interpret changes during song.

In addition, we quantified various temporal parameters of song. The duration of song bouts, the duration of syllables, and other temporal changes were measured using the air sac pressure pattern. Onset and offset of respiratory pulses can be determined more accurately using air sac pressure than is possible from sound recordings.

Sound amplitude was determined for each syllable by rectifying the voltage signal (i.e. absolute values calculated with 0.1 ms window) and integrating voltage (time window 2 ms) for each syllable. Spectrograms were calculated at each stage of the experiment, and visual comparison of acoustic features was used to identify potential changes in acoustic structure.

Statistical analyses were performed using SPSS and SigmaPlot 8.0 software (SYSTAT, San Jose, CA, USA). Because syllables and corresponding air sac pressure patterns are individually characteristic, we initially tested all syllables for changes with injection treatments using two-tailed Student's *t*-tests on the original measurements. Subsequently, to test for overall effects, we compared percentage change values between treatment groups with two-tailed unpaired or, where appropriate, paired *t*-tests. If multiple tests were performed using the same data set, degrees of freedom were adjusted. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah.

RESULTS

Casting

The volume of air sacs was estimated from silicon casts of the respiratory system to determine what proportion of the available volume our injections removed from the air supply. The mean volume of one anterior thoracic air sac is 0.16±0.02 ml (N=4) and that of one posterior thoracic air is 0.22±0.031 ml (N=9). The volume of the abdominal air sacs is approximately 0.23±0.03 ml (N=6), thus resulting in a total volume of air supply in the posterior air sacs of 0.90 ml. Lung volume is approximately 0.21 ml.

Injection into the posterior thoracic air sacs

In 11 vigorously singing birds, we injected dental impression medium initially into the left posterior thoracic air sac. Birds sang readily after the injection, and song and air sac pressure were recorded again. We then injected dental impression medium into the right posterior thoracic air sac and recorded again. Injections ranged from 0.102 to 0.23 ml, thus taking up between 46 and 100% of one posterior thoracic air sac (Table 1). Smaller injections sometimes resulted in the apparent occlusion of the connection from the air sac to the lung. In

Table 1. Effects of injections on pressure, sound amplitude and airflow during song

	Injection		Pressure (% of pre-injection)			Sound amplitude (% of pre-injection)				Flow (% of pre-injection)	
	(ml)	O/M*	Mean	Range	% Sig.†	Mean	Range	dB	% Sig†	Mean	Range
W42	0.139	O	92.5	90.1–93.8	80	59.9	29.2–76.4	5.2	60	–	–
	0.1055		59.9	48.6–79.9	100	22.3	22.8–75.7	13.3	80	–	–
V74	0.1322	O	72.7	62.2–78.5	100	59.1	42.5–81.7	4.8	80	–	–
	0.102	O	59.6	49.3–74.4	100	32.4	23.2–102.0	10.7	100	–	–
B4	0.111		91.1	86.2–98.1	75	–	–	–	50	95.7	89.3–101.0
	0.131		75.3	63.2–82.4	100	79.1	–	2.15	75	86.2	73.0–98.5
V52	0.135	O	74.4	70.1–81.7	100	59.3	47.4–74.5	4.67	100	63.9	37.3–74.0
	0.136		72.6	66.2–80.2	100	57.5	43.6–65.9	4.88	100	–	–
Y34	0.129	O	87.5	83.4–91.5	100	75.3	68.1–82.4	2.5	50	62.6	47.0–78.2
	0.127		79.3	76.9–81.5	100	63.6	47.4–79.6	4.23	50	43.7	38.4–48.8
B10	0.174	O	78.1	73.3–82.7	100	79.6	55.8–94.2	2.1	20	–	–
	0.224		46.9	26.1–66.9	100	56.4	22.3–73.3	7.0	80	–	–
R9	0.173		87.0	83.5–90.3	100	94.8	83.6–102.5	0.5	25	–	–
	0.215	O	76.2	66.3–82.2	100	56.4	38.6–69.7	5.1	100	–	–
Experiments with one missed injection											
V76	0.08	M	95.1	88.4–99.2	20	95.8	88.4–103.6	0.4	20	–	–
	0.1474	O	88.4	81.4–91.2	60	74.3	59.6–98.6	3.1	60	–	–
P71	0.112	M	91.9	89.2–95.3	100	88.7	79.4–95.3	1.06	75	101	100–101
	0.129		82.3	77.1–85.5	100	79.1	67.4–91.0	2.1	75	91.7	88.9–94.0
W19	0.221	M	93.4	91.2–95.9	86	94.4	86.7–108.1	0.3	29	–	–
	0.231	O	81.6	78.2–84.5	100	67.0	44.1–94.1	3.6	71	–	–
P65	0.173	M	97.2	95.3–98.7	60	100.4	89.2–116.1	0.01	20	–	–
	0.225		86.3	82.4–91.2	100	69.6	53.9–79.3	3.2	100	–	–

*O indicates that the ostium appeared to have been blocked by the injection. M indicates a missed injection.
†Percentage of syllables for which the change was significant at $P<0.05$ in a two-tailed *t*-test. Tests (control *vs* one injection and control *vs* two injections) were calculated for each syllable separately, using 20–40 measurements for each treatment.

eight birds, it appeared that one or both injections had protruded into the ostium (Table 1), presumably removing the entire volume of the occluded air sac from the air reservoir. However, it cannot be assessed whether or not the ostium was completely occluded under the pressurized conditions of song. The four birds in which the first injection missed the air sac serve as a control (Table 1) (see below).

Table 2. Effects of injections on quiet respiration

	% Change in pressure amplitude	% Change in respiratory rate
Y34	79.3***	20.7***
	87.3***	21.0***
V74	17.5**	27.5***
	–16.7***	25.5***
B4	8.2	13.5**
	3.99	31.7***
V52	–14.2***	6.5*
	–13.8**	14.4***
B10	3.15	17.7***
	29.2***	72.4***
R9	–11.8*	–4.4
	–2.3	75.7***
Missed first injection		
P65	–19.3***	–8.35*
	4.03	2.99
W19	–10.0	–2.96
	–11.2	19.6*

Significance at *$P<0.05$, **$P<0.01$ and ***$P<0.001$ in a two-tailed *t*-test on the individual measurements (N=35–55 for each treatment) for respiratory rate and mean expiratory air sac pressure values for 3 s segments of quiet respiration.

Injections affect quiet respiration

Amplitude of air sac pressure pulses and respiratory rate are highly variable during quiet respiration, making comparisons between treatments difficult. We selected 3 s-long segments of quiet respiration (N=35–55; at least 10 s before or after song and not including any calls) for each treatment and calculated respiratory rate and mean expiratory air sac pressure amplitude. Despite substantial variability, respiratory rate showed a highly significant increase after two injections in all birds and a significant increase after one injection into an air sac in all but one bird (Table 2). Missed injections did not have the same effect. Changes to the amplitude of expiratory air sac pressure pulses were much less consistent. One bird showed a drastic increase in air sac pressure, while others ranged from a small increase to a decrease (Table 2). Whereas birds responded to the injections with timing changes during quiet respiration, the respiratory pattern of song remained remarkably unchanged (see below).

One bird, B4, was apneic following almost all preoperational song bouts, indicating hyperventilation during song (Franz and Goller, 2003). The duration of apnea was positively correlated with song bout duration before injections of dental impression medium. With each injection, however, apnea length decreased for bouts of similar length. After the second injection, most song bouts were no longer followed by apnea, although air sac pressure amplitude was still reduced for the first few breaths (Fig. 3).

Injections cause reduced air sac pressure amplitude during song

During song, air sac pressure amplitude decreased substantially in all birds with injections into the left posterior thoracic air sac, and the second injection caused a further decrease. Table 1 shows

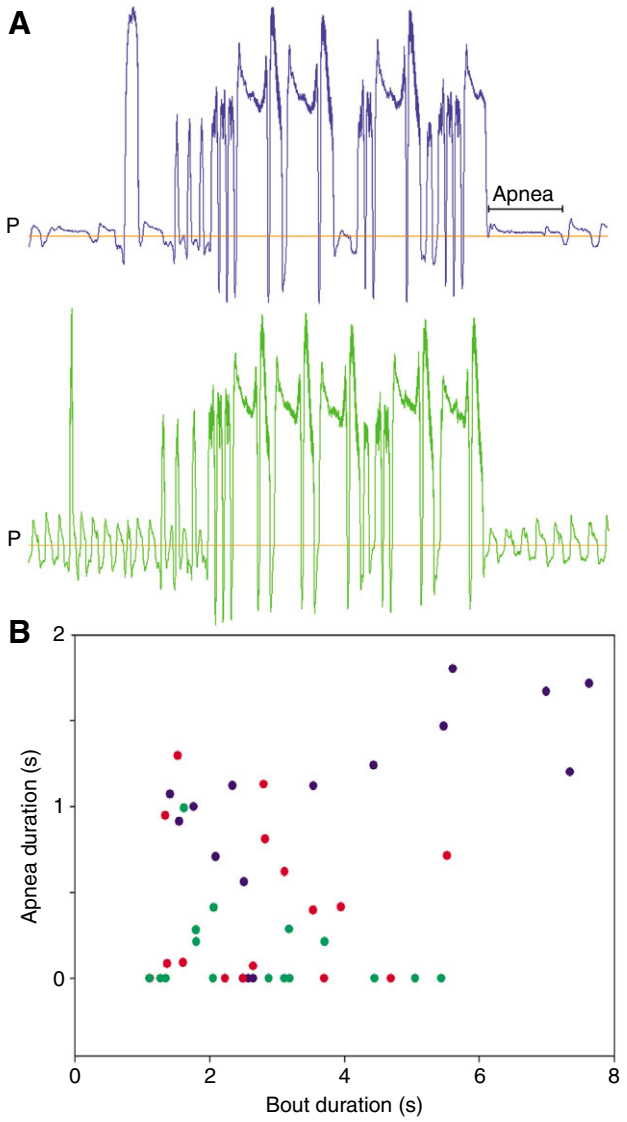

Fig. 3. One bird displayed distinct periods of apnea after song bouts. These periods shortened after the first injection and were shortened or no longer present after the second injection. (A) Air sac pressure traces of song bout before injection (blue) and after two injections (green). (B) Apnea duration before injections (blue circles) was positively correlated with bout duration (linear regression, r=0.65, P=0.0059), but injections reduced or eliminated periods of apnea (red, one injection; green, two injections).

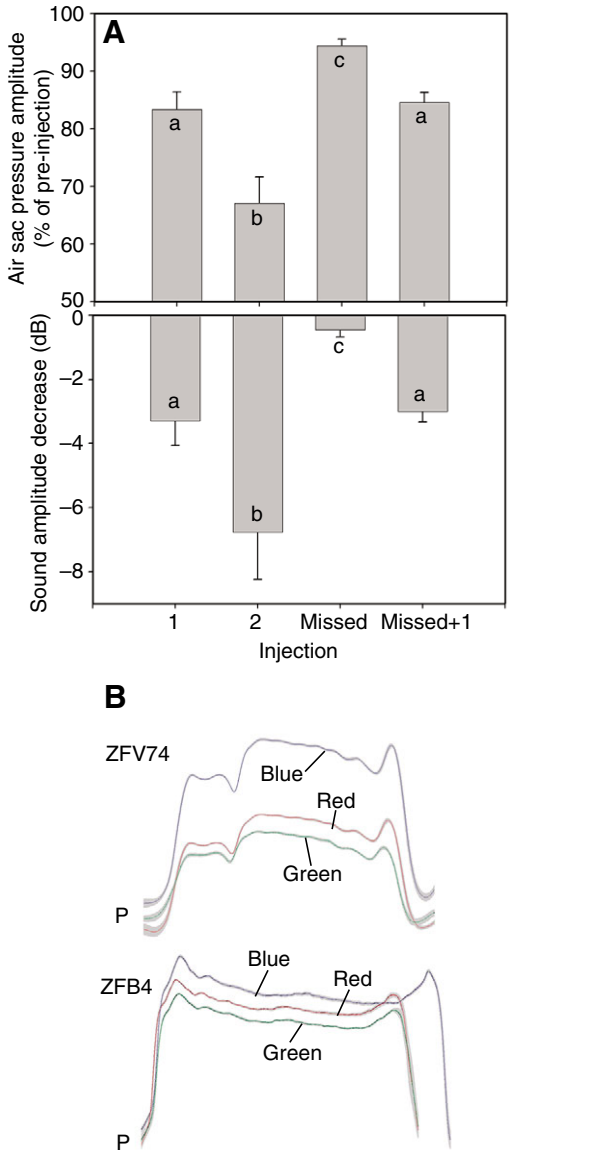

Fig. 4. (A) Mean percent (±1 s.e.m.) of pre-injection air sac pressure (top), and reduction in sound amplitude (bottom) for the one, two, missed and missed-plus-one-injection groups. Different letters indicate significant differences in t-tests (where different birds) and paired t-tests (same bird different treatments). For all significant comparisons, P<0.028. (B) The variation of air sac pressure amplitude was not increased after injection into one (red) or two (green) posterior thoracic air sacs compared with that of pre-injection values (blue). Each trace represents the mean air sac pressure ± 1 s.e.m. (grey area around mean) for 10 renditions of the song syllable in each treatment. Increased grey area at the beginning and end of traces results from slight differences in duration of the expiratory air sac pressure pulse between renditions of the song, which leads to imperfect alignment.

the range of values for individual syllables and indicates that this decrease was significant for almost all of the syllables. The two injections resulted in an average decrease in air sac pressure amplitude to 67.2% of pre-injection song (Fig. 4A), and the reduction from the first to the second injection was significant (Fig. 4A). The reduced air sac pressure amplitude was produced with similar stereotypy as that found in pre-injection air sac pressure patterns (Fig. 4B). The changes in air sac pressure amplitude were therefore not caused by an increase in variability.

Tracheal airflow was monitored in three birds with injections into both posterior thoracic air sacs and in one bird with one missed injection (Table 1). Expiratory airflow decreased with the first injection, and decreased even further with the second (Fig. 5). In two birds, airflow decreased proportionally to the decrease in air sac pressure. In the other two birds, airflow decreased substantially more than would be expected from reduced air sac pressure. Degradation of the voltage response of flow probes over the course of the experiment may have contributed to this disproportionate decline in airflow. Overall, airflow decreased by 8.3% to 56.4%, with an average of 35.1% after the second injection.

The pressure amplitude of individual pulses, corresponding to syllables, typically was decreased uniformly throughout the duration of the syllable (Fig. 6). However, two birds showed a

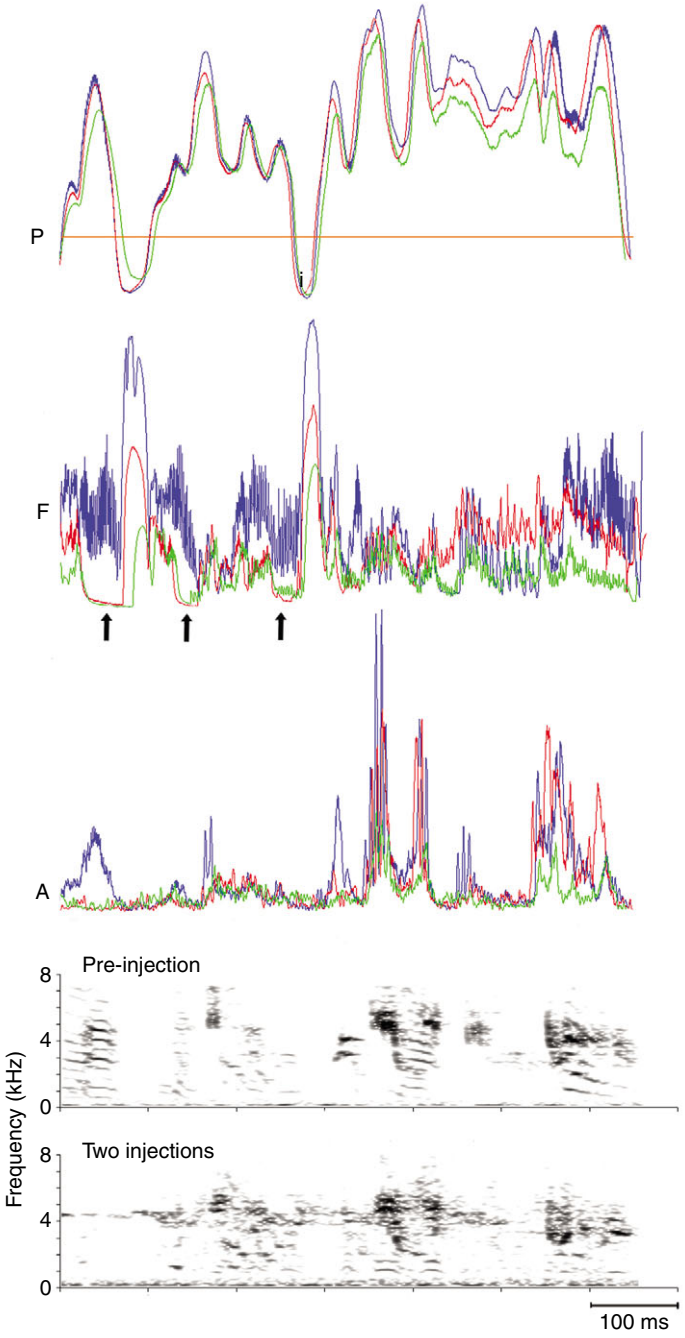

Fig. 5. Air sac pressure (P), tracheal airflow (F) and sound amplitude (A) during song were increasingly reduced after one (red) and two (green) injections into the posterior thoracic air sacs, as compared with pre-injection (blue). A representative song is also shown spectrographically before and after two injections. During the first and second expiratory pulse, the bird closed its syrinx (as indicated by zero tracheal flow; arrows), resulting in elimination of these sound segments. Quiet respiration changed after injections into the posterior thoracic air sacs. The orange horizontal line depicts ambient pressure.

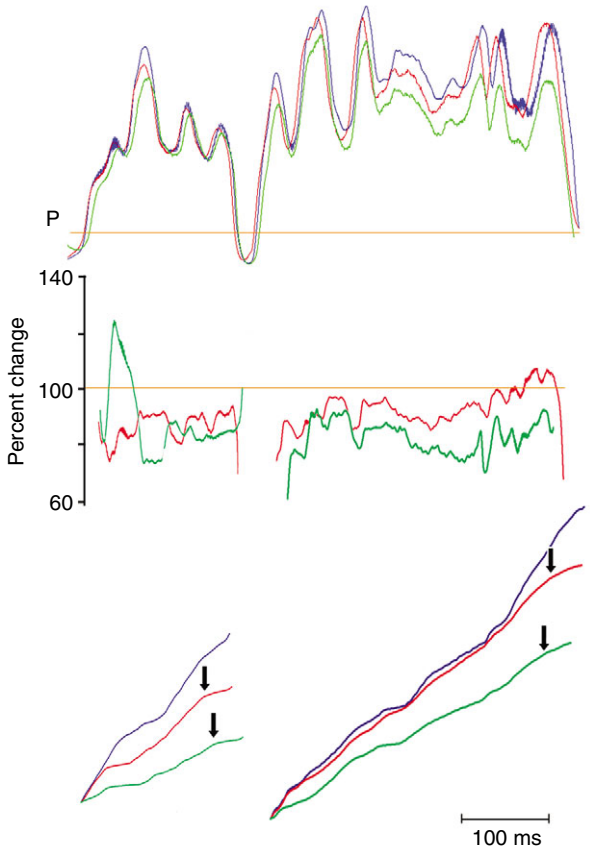

Fig. 6. In zebra finch Y34, air sac pressure was reduced uniformly throughout expiratory pulses, but airflow reduction increased at the end of pulses. The top panel shows the air sac pressure traces for the two syllables (P) of the motif for pre-injection (blue), one injection (red) and two injections (green), with the percent reduction from pre-injection for each injection trace indicating a fairly uniform reduction. Cumulative airflow for each syllable (bottom panel; shown as cumulative flow) indicates not only the overall decrease in flow after injections but also that, at the end of syllables, increases in airflow present in pre-injection song cannot be sustained by the injected bird (change in slope marked by arrows).

differential effect over the duration of syllables. For example, in one syllable of W42, air sac pressure after two injections was 70% of pre-injection values at the onset of the syllable but declined to 40% at the end (Fig. 7). All four syllables in this bird's motif showed a similar decrease over the course of the pressure pulse, regardless of their varying durations ranging from 103 to 144 ms. In the other bird (R9) the air sac pressure declined more towards the end of one long syllable (245 ms), descending from 80% of pre-injection value at the onset to 30% at the end. Changes to shorter syllables (~100 ms) were not as pronounced.

In one bird, whose air sac pressure decreased uniformly over the course of syllables, airflow also decreased uniformly. The slopes of cumulative airflow plots run parallel to each other for the different treatments (Fig. 8). However, in another bird with uniform decrease in air sac pressure, airflow did not decrease uniformly. After injections, the slope of cumulative airflow plots changed from that observed during pre-injection song. For example, the slope decreased near the end of each syllable, indicating a greater reduction in flow than was observed during the early portions of the pulses (Fig. 6, indicated by arrows).

Surprisingly, the temporal pattern of the motif and associated air sac pressure pattern remained intact in most birds. Syllable duration typically changed by only 1–8%, but the change was not significant in many cases (Table 3). Motif duration did not change in many of the birds, except if syllables were omitted. However, specific small changes were noted in some birds. One individual occasionally

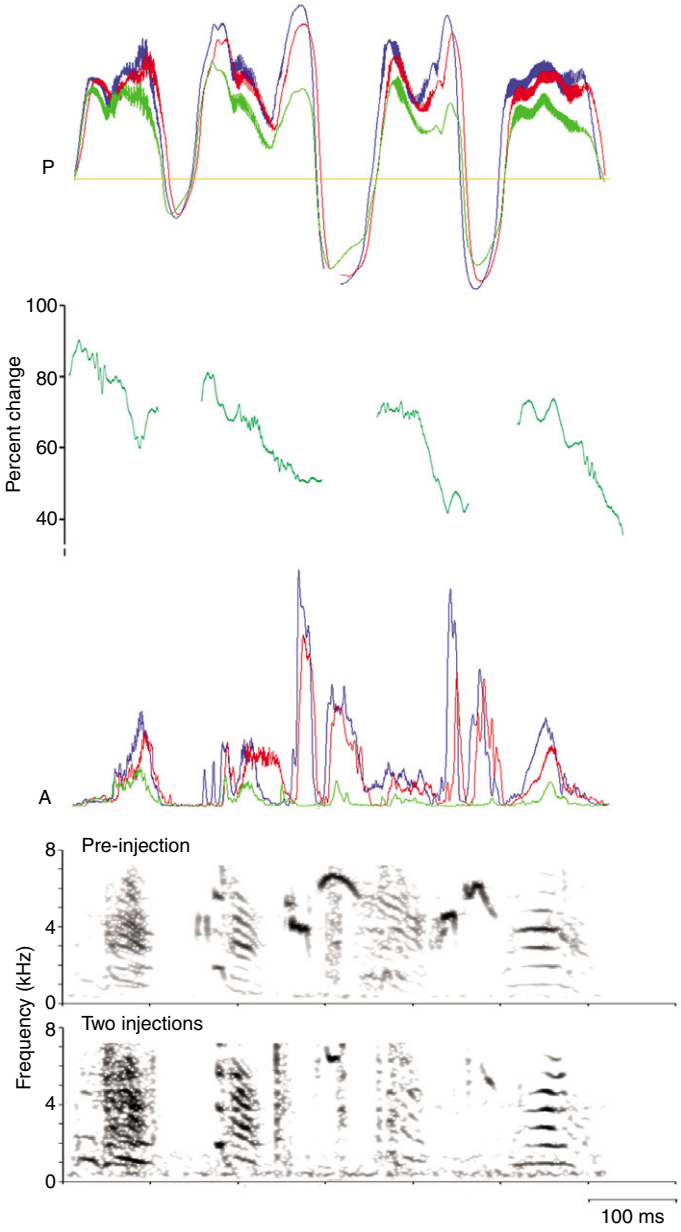

Fig. 7. Zebra finch W42 showed a consistently increasing difference in air sac pressure (shown in direct comparison, P, and below as percent decrease from pre-injection values; colors as in Fig. 5) amplitude throughout each expiratory pulse. Song output during all three treatments is shown as amplitude traces (A; rectified and integrated) and as spectrograms (pre-injection and two injections) and illustrates how particularly high-frequency syllables are reduced in amplitude after the injections. Each of the two sets of high-frequency syllables is a combination of one element produced at the end of an expiratory pulse and the second during the following inspiration (phonatory minibreath). In both sets, the expiratory element disappeared (almost zero amplitude) whereas the inspiratory part was strongly reduced in amplitude.

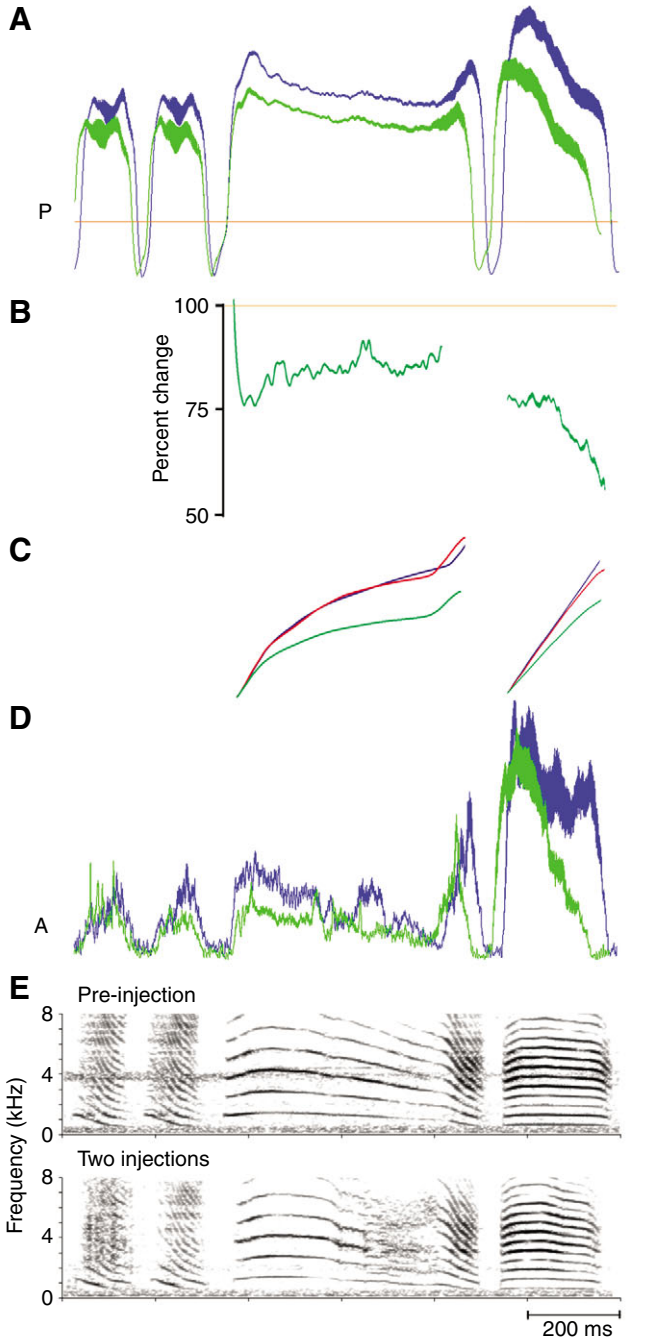

Fig. 8. Zebra finch B4 showed a decrease in duration of two syllables and yet retained air sac pressure and airflow throughout. In A, the change in duration of the long expiratory pulse (612 ms) is evident by the comparison between pre-injection pressure (blue) and pressure after two injections (green). B shows the decreased pressure in each syllable in areas where the pressure pulses remain similar in spite of the duration change. The descent of the difference in the second syllable is due to change in duration. C shows the cumulative flow of each syllable, which shows the overall decrease in airflow from pre-injection (blue) to one injection (red) and two injections (green). Sound is shown as rectified and integrated amplitude (D) and spectrographically (E). The change from smooth frequency modulation (pre-injection) to a complex harmonic structure (two injections) can be seen in the long syllable in the spectrograms.

modified one minibreath of the motif during his pre-injection song, which extended motif duration. The modified minibreath occurred in 13% of pre-injection motifs. After the injections, the modified inspiration appeared more frequently than in pre-injection song (in 26% of motifs). The duration of expiratory pulses did not change. Another bird's motif contained a long syllable, whose duration decreased with each successive injection (Fig. 8). The mean duration prior to injection was 612 ms and decreased after two injections by 9% to 554 ms.

Table 3. Duration of expiratory pressure pulses during song

	Injection	Mean % of pre-injection	% Syllables significantly different*	Range for all syllables
W42	One	100.8	20	98.3–102.6
	Two	103.2	20	98.7–111.9
V74	One	100.2	20	97.8–105.3
	Two	101.0	0	98.7–106.9
B4	One	94.6	50	92.5–99.5
	Two	96.5	50	90.8–102.0
V52	One	101.8	16.6	98.4–104.9
	Two	101.3	16.6	98.4–104.7
Y34	One	98.9	0	96.7–101.2
	Two	97.1	0	96.9–97.3
B10	One	106.2	100	99.3–102.7
	Two	103.9	80	98.1–107.5
R9	One	104.1	75	101.8–109.3
	Two	101.5	100	92.1–104.8
Missed first injection				
V76	One	101.8	0	100.9–105.1
	Two	104.3	80	98.0–113.8
P71	One	104.1	100	101.9–107.0
	Two	103.4	75	101.3–105.8
W19	One	100.8	57.1	99.6–101.7
	Two	104.9	71.4	102.7–107.6
P65	One	102.4	80	101.8–103.4
	Two	103.4	100	101.8–105.2

*Significance was tested with a two-tailed *t*-test ($P<0.05$). Tests (control *vs* one injection; control *vs* two injections) were run for each syllable of the motif separately, using between 10–30 measurements for each.

The temporal structure of song bouts changed in five individuals. Mean motif length decreased in four birds due to variation in how much of the full motif was sung. Pre-injection song in these birds occasionally included incomplete motifs, but their occurrence increased after injections. One bird sang 97% full motifs prior to injection and only 29.2% after two injections. One individual stayed within 5% of pre-injection motif length. The change could have been based on small sample size. Three other birds always sang their full pre-injection motif and continued to do so throughout the experiment.

Mean bout duration decreased significantly after the first injection. Duration was normalized to the maximum recorded bout duration for each individual to allow pooling of data between birds. Mean bout duration prior to injection was 0.45±0.03 of maximal bout duration and dropped to 0.28±0.02 after the first injection and 0.24±0.03 after the second (Student's *t*-test; pre- *vs* first injection, $t=4.45$; $P<0.00001$, d.f.=166; first *vs* second, $t=0.99$, $P=0.32$, d.f.=160). However, birds were still able to sing long bouts, but did so less frequently (Fig. 9). Because bout duration also depends on motivation, it is possible that this decrease reflects reduced motivation and not a direct respiratory effect of the injections.

Acoustic changes

Concurrent with the decrease in air sac pressure amplitude, sound amplitude decreased in all birds with injections into the posterior thoracic air sacs (Table 1). Sound amplitude consistently decreased with the first injection, followed by a further significant decrease with the second injection (Fig. 4A, Fig. 5). After two injections, sound amplitude was, on average, 45.9% of the pre-injection value (a mean decrease of 6.8 dB, with a range of 2.15–13.3 dB in birds with no missed injections) (Table 1). Although all syllables were affected, the amplitude of syllables with high fundamental frequency decreased more than that of low-frequency syllables. High-frequency sounds were either generated during expiration or inspiration. Syllables produced during either respiratory phase were shortened or lost completely after injections (Fig. 10), with the exception of one bird, whose inspiratory notes remained intact.

The potential effects of injections on the acoustic characteristics of song, other than the decreased amplitude, were assessed by visual comparison of spectrograms. Only two notable changes to sound characteristics were noticed after injections. In zebra finch B4, a syllable generated during a long expiratory pressure pulse of 612 ms changed after two injections. The pre-injection syllable was a smooth frequency-modulated harmonic sound, but after two injections it contained an abrupt frequency jump followed by a more complex harmonic structure, suggesting different frequency

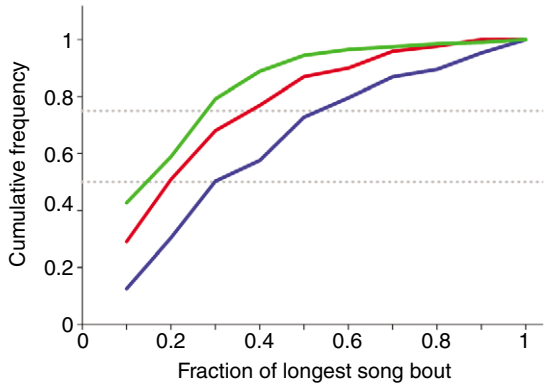

Fig. 9. Birds sang with shorter bout duration after injections. Cumulative frequency plots of relative bout durations expressed as a percentage of the longest pre-injection bout (using 10% bins) are shown for the three treatments (blue, pre-injection, $N=99$; red, one injection, $N=80$; green, two injections, $N=101$) for all birds.

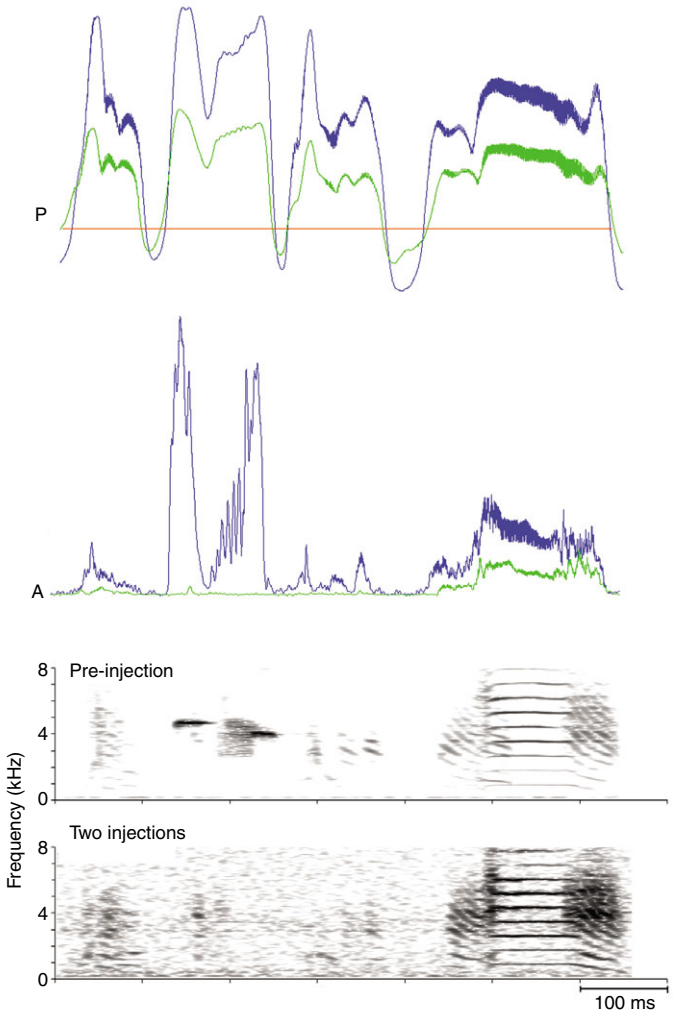

Fig. 10. Air sac pressure (P) and sound amplitude (A) during song were increasingly reduced after two injections (green) into the posterior thoracic air sacs of one bird, V74, as compared with pre-injection (blue). The reduction or total loss of high frequency notes is shown in the sound amplitude trace (A) and in the spectrogram (bottom).

contributions of the two sound generators (Fig. 8). This same syllable was also shortened.

In zebra finch Y34, short segments of song were omitted (arrows in Fig. 5). Sound production during these segments was prevented by closing the syringeal valves to airflow, as indicated by zero tracheal flow. One of these segments was an entire, short syllable. The acoustic structure of these segments does not suggest an obvious reason for why closure of the labial valves occurred.

Injection does not increase EMG activity in expiratory muscles

In six birds, we measured EMG activity of the abdominal expiratory muscles to assess whether injection into posterior thoracic air sacs caused changes in activity. Because the experiments lasted 3–4 days, changes in EMG activity have to be interpreted carefully. Impedance at the electrode tips can change over the course of a few days and might therefore result in differences in the recorded EMG amplitude.

In five birds, the amplitude of EMG bursts associated with specific expiratory pulses of the song did not increase after injections (Fig. 11). Because air sac pressure was lower after injection, the points for each expiratory pulse are shifted to the left, indicating lower EMG activity for the specific syllable but higher EMG activity for the achieved air sac pressure than during pre-injection song (Fig. 11B–D). These results indicate that a similar expiratory effort results in generation of lower expiratory pressure and that no compensation for the reduced air sac pressure is evident from the EMG recordings. One bird showed an increase in EMG activity following the first injection. However, EMGs during quiet respiration also increase substantially after the first injection, suggesting a change in the impedance and not a compensatory adjustment.

Missed injections do not have the same effect on air sac pressure and sound amplitude

In four birds, the first injection of dental impression medium missed the posterior thoracic air sac and occupied space next to the air sac. Missed injections resulted in a small, significant decrease in air sac pressure amplitude and sound amplitude (Table 1, Fig. 4A), but the mean decrease was to 94.4% of pre-injection air sac pressure and only a 0.44 dB decrease in sound amplitude. These values were significantly different from those measured in birds where the first injection filled the air sac (Table 1, Fig. 4A). The second injection in these birds was directly into the opposite posterior thoracic air sac and generated a similar effect on air sac pressure and sound amplitude as the first injection in the other group of birds.

DISCUSSION

In this study, we investigate how reducing the air volume in the air sac system affects the respiratory pattern of song in the zebra finch. After reducing the volume of the posterior thoracic air sacs, respiratory activity increased during quiet respiration. During song, however, air sac pressure amplitude was reduced compared to that before volume reduction, resulting in reduced airflow and decreased sound amplitude. Electromyograms of expiratory muscles indicate that no compensatory activation of the abdominal expiratory muscle sheet occurs during song. Together, these results give insight into the integration between respiratory control and the motor program for song production.

Mechanics of breathing and injections of dental impression medium

Injections of dental impression medium replace part or all of the volume of an air sac and therefore reduce the air reservoir available for singing. The reduction of the total volume of air available for expiration ranged from approximately 8 to 19%, but injections into the posterior thoracic air sacs reduced the air volume for perfusion of the lung during expiration by as much as 25.6%. In chickens, filling of all thoracic air sacs with cotton wool had a minimal effect on breathing during rest and running (Brackenbury et al., 1989). Because the cotton wool became suffused with fluid within the air sacs, the whole air sac volume was unusable to the bird. Singing requires much less increase in oxidative metabolism than running (Oberweger and Goller, 2001; Franz and Goller, 2003), suggesting that in our experiment gas exchange might not have been limited either. A potentially important difference between the experiment on chickens and this study is that we injected the posterior thoracic air sac without similar injections into the anterior thoracic air sac. Such an asymmetric injection might have altered flow patterns differently from those achieved after asymmetrical filling of the anterior thoracic air sacs or the symmetrical filling of all thoracic air sacs in the chicken (Brackenbury et al., 1989).

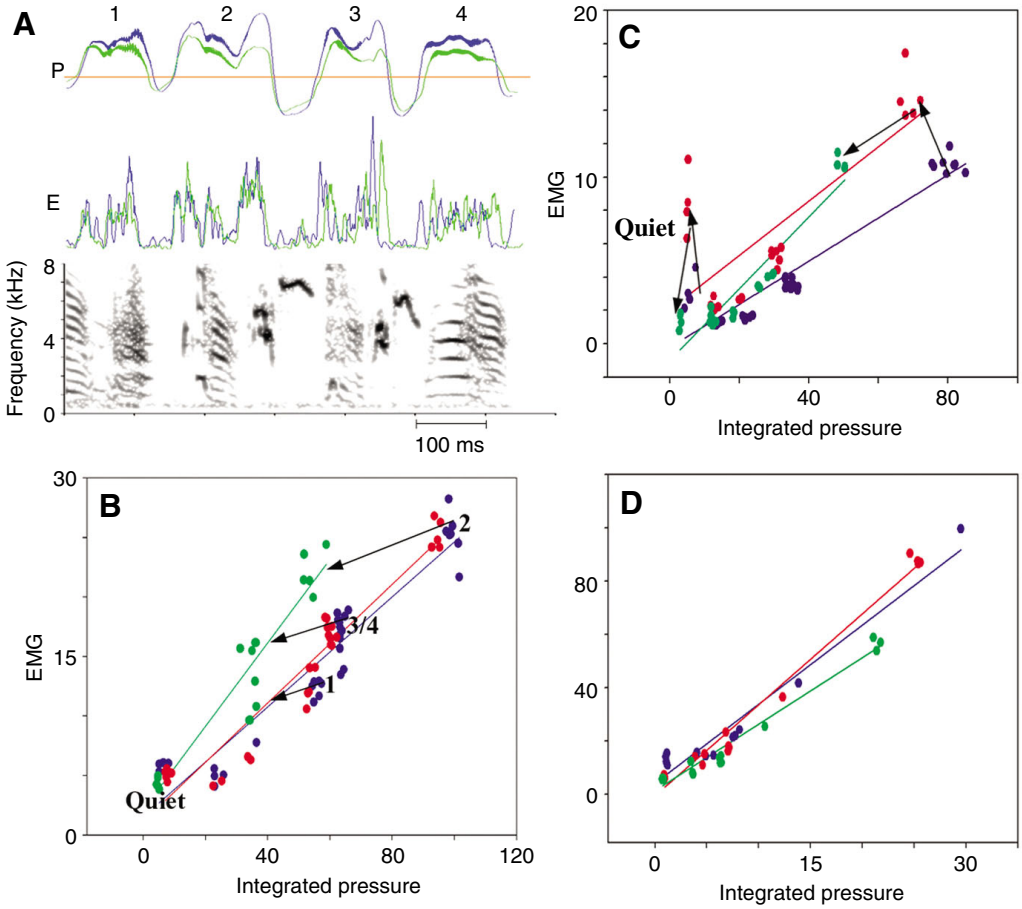

Fig. 11. Electromyogram (EMG) activity does not indicate a compensatory increase in muscle activity after injections. (A) Example of data for one zebra finch illustrates the decrease in air sac pressure (P) after two injections (green) compared with pre-injection (blue) to two injections and the accompanying EMG activity (E; rectified and integrated). The song is illustrated spectrographically (bottom). (B–D) Air sac pressure and corresponding EMG activity (integrated voltage) for the three zebra finches for quiet respiration and song. Colors indicate the three recording conditions (blue, pre-injection; red, one injection; green, two injections). The data in B are from the individual whose song is depicted in A, and numbers correspond to the four expiratory pulses of song. Arrows indicate the shift in air sac pressure after injections for the four expiratory pulses of song. C shows that the first injection was followed by an increase in EMG activity, which is most pronounced in the syllable in the upper right corner. However, a corresponding shift in EMG activity is seen in quiet respiration, at the bottom left of the graph, likely indicating an impedance change.

The cured dental impression medium could also present a physical obstacle to the thoracic movements associated with respiration. Dorso-ventral and lateral motion of the rib cage during breathing (McLelland, 1989; Fedde, 1976) could be impeded by the addition of a solid mass within the air sacs. Although the reduced effect of injections that missed the air sac partially controls for mechanical interference, a physical obstruction of respiratory movements cannot be ruled out completely.

The missed injections provide a good control for the added mass resulting from replacing air with dental impression medium. Because this additional mass must be moved with every breath, it is possible that metabolism might have been increased as a consequence. However, birds with missed injections had to accelerate similar additional mass with every breath as did the birds with air sac injections but did not show increased ventilation, thus making it unlikely that the observed changes are caused by the addition of mass to the thorax.

Respiratory airflow and injections

In order to assess the potential effects of the injections on song, flow patterns of air during both respiratory phases need to be considered. Because no data are available for songbirds, we base this discussion on the avian model obtained from anesthetized non-songbirds (e.g. Bretz and Schmidt-Nielsen, 1972). The posterior set of air sacs (posterior thoracic and abdominal air sacs) serves as an air reservoir (McLelland, 1989), which fills during inspiration and provides air that flows through the lung during expiration (Fig. 2). The routing of air is thought to be controlled by aerodynamic valving (e.g. Jones et al., 1981; Banzett et al., 1987; Brown et al., 1995), which is possibly enhanced by physical modifications (Maina and Africa, 2000). The efficiency of the valving is decreased at low airflow (Banzett et al., 1987).

When air space was removed from the posterior thoracic air sacs, we measured a slight increase or no significant change in air sac pressure during quiet respiration. Air sac pressure amplitude can vary substantially with fluctuating levels of activity, making these comparisons between treatments difficult. However, injections into the posterior thoracic air sacs resulted in a consistent increase in respiratory rate (Table 2). These data therefore suggest that the reduction in available air volume did affect gas exchange during quiet breathing and birds compensated with increased ventilation. Surprisingly, a much larger reduction in volume in chickens did not

cause significant changes in gas exchange during rest and running (Brackenbury and Amaku, 1990; Brackenbury et al., 1989).

Volume of air needed for singing

The effects of the reduced air reservoir on song generation must depend on the volume of air exchanged during different song syllables. The posterior reservoir, composed of posterior thoracic and abdominal air sacs, holds about 0.9 ml of air. Two large injections of 0.22 ml each into the posterior thoracic air sacs cause a reduction of at least 48%, leaving nearly 0.46 ml of air in the posterior reservoir. Similarly, in cases where the ostium was blocked by the injections, a reduction of the same amount was possible. Goller and Daley used calibrated tracheal airflow measurements to estimate the volume of air exhaled during individual expiratory pulses, corresponding to song syllables (Goller and Daley, 2001). The largest expired volume for a syllable in songs of three zebra finches approached 0.3 ml, which would use 50–75% of the available posterior air volume after large bilateral injections. This estimate indicates that long song syllables, which are generated with high airflow, might be limited by the available air volume.

It is striking that air sac pressure and airflow were typically reduced for the entire song motif, independent of the variable volume of air exchanged during different syllables. This global reduction in airflow was unexpected. Instead, we expected to find that long syllables with large air requirements would be reduced in duration when air supply is exhausted, whereas short syllables should not be affected by the reduced air supply at all. Only one bird (B4) appeared to reach the limit of air supply and reduced the duration of an exceptionally long syllable from 612 ms to 554 ms (Fig. 8). However, even in this bird all syllables were produced with reduced air sac pressure amplitude after the injection.

Another possibility for how injections might affect song is a passive decline in air sac pressure as the available reservoir is depleted. In this case, a growing decrease in air sac pressure throughout long syllables would be predicted. This pattern was found only in two zebra finches (W42 and R9), and the progressively growing decline in air sac pressure amplitude during the course of a syllable was seen in short and long syllables alike (Fig. 7). Why these individuals showed such a different response to injection is unknown.

With the above-mentioned exception, the temporal pattern of the song motif did not change despite reduced amplitude of air sac pressure, airflow and sound amplitude. This suggests that the stereotyped respiratory motor program of song is generated unless physical limitations prevent its completion. The consequences of reduced airflow on gas exchange during song are not known, but quiet respiration after song did not indicate a limitation. Reduced bout duration after injection, however, could indicate a potential limitation caused by need for gas exchange, although a motivational explanation for this reduction cannot be ruled out.

Somatosensory feedback

Respiration is regulated by feedback from an array of different sensory systems. Chemoreceptors in the avian lung, especially carbon dioxide receptors, can affect respiratory rate and depth of breath on a breath-by-breath time scale (Gleeson and Molony, 1989). The decreased air volume in the posterior air reservoir reduced flow through the lungs and altered gas exchange during quiet respiration. This change was presumably effected by chemoreceptors. During song, only one bird hyperventilated, as indicated by periods of apnea after song bouts. Apnea was reduced after injections, indicating reduced gas exchange during song. The other individuals were not apneic after song, and injections did not change respiration after song noticeably (data not shown). In general, these observations indicate that gas exchange during song was not sufficiently compromised by the injections to cause altered respiratory patterns after song. This strongly suggests that during song in zebra finches, gas exchange is enhanced relative to quiet breathing. This confirms the indirect evidence from oxygen consumption measurements, which do not show an increase in oxygen consumption after song (oxygen debt) and also suggest hyperventilation during song in some individuals (Oberweger and Goller, 2001; Franz and Goller, 2003).

Mechanoreceptors in the respiratory system are potentially important for controlling the timing and duration of song bouts (Wild, 2004). These mechanoreceptors are most likely located in the air sac system (Kubke et al., 2004) and probably respond to volume changes, particularly during the inspiratory phase (Ballam et al., 1982; Molony, 1974). In cardinals, an increase in air sac volume by injection of small air pulses into the anterior thoracic air sac during song resulted in decreased EMG activity in the abdominal expiratory muscles (Suthers et al., 2002). This compensatory reduction in muscle effort suggested regulation of air sac pressure and airflow. In contrast to these results, an air sac permanently filled by an injection of dental impression medium elicited no compensatory response in the abdominal muscles to maintain air sac pressure and airflow for song. Comparisons of absolute amplitudes of EMG activity across several days of recording can be problematic if the impedance at the electrode tips changes over this time. Nevertheless, by comparing EMG activity during quiet respiration before and after injection, we can rule out large changes in impedance. Compensatory changes in EMG activity during song after injections are not present in any of the birds. In one bird, EMG activity during quiet respiration and song increased similarly after the first injection (Fig. 11B), which is consistent with an impedance change and probably does not indicate compensation. This interpretation is confirmed by the data after the second injection, where the pattern is the same as that found in all the other birds (Fig. 11).

Mechanoreceptors fire preferentially during inspiration, when volume increases and pressure decreases (Gleeson and Molony, 1989). Perhaps the compensatory response in the cardinal occurred because the injection of air simulated an inspiratory event. In our experiment, the injected air sacs were permanently full and therefore volume changes did not occur in these air sacs. Because volume change is the most likely physical variable to alter the firing rate of these receptors (Ballam et al., 1982; Molony, 1974), feedback information may not have been available to correct for our manipulation.

Auditory feedback

During song, birds also receive auditory feedback information. However, altered auditory feedback does not cause rapid changes to the temporal pattern of song in the zebra finch. Changes occurred only after several days of receiving altered acoustic feedback (e.g. Leonardo and Konishi, 1999; Cooper and Goller, 2004). Because our experiments were completed within 3–4 days and acoustic changes were generally small, we did not expect altered auditory feedback to cause changes to the temporal pattern of song.

The lack of response to the reduced sound amplitude, which the bird must have perceived through auditory feedback, is surprising. Zebra finches show an increase in song intensity by 1–3 dB if the receiver distance is increased (Brumm and Slater, 2006). In addition, a significant Lombard effect was present, with increases

in song amplitude by 8–10 dB, when birds sang in elevated background noise (Cynx et al., 1998). Theoretically, neither of these responses requires zebra finches to use auditory feedback from song output to adjust song amplitude. In the case of distance adjustments, visual estimates of distance may be the stimulus for changing song amplitude. In the case of the Lombard effect, the bird may be responding to the level of background noise and not the amplitude of its song. However, budgerigars (*Melopsittacus undulatus*) and nightingales (*Luscinia megarhynchos*) respond with a Lombard effect if noise occupies the same frequency band as their vocalizations (Manabe et al., 1998; Brumm and Todt, 2002), suggesting that birds monitor their vocal output or the signal-to-noise ratio.

Data from this study suggest a possible reliance on background noise. Two large injections reduced the volume of song by as much as 13 dB in some syllables. If birds had used auditory feedback to evaluate their sound output, we would expect an increase in abdominal muscle activity to compensate for this amplitude decrease, but no compensation was found. This suggests that increased background noise is the main stimulus for the Lombard effect in the zebra finch. If this sensitivity to background noise is most effective in the frequency band of the song, our interpretation is also consistent with the results in budgerigars and nightingales (Manabe et al., 1998; Brumm and Todt, 2002). However, it is possible that the lack of amplitude control in our study may be a non-specific effect of the injection procedure.

Conclusions

The reduction of air volume by filling posterior thoracic air sacs with dental impression medium has very little impact on the timing of song. Only very long syllables are shortened, but otherwise the temporal pattern remains intact. Although oxygen exchange must be reduced by the treatment, as indicated by the change in quiet respiration and reduced apnea, this reduction appears to be insufficient for causing temporal changes to the motif structure. Air sac pressure amplitude for all syllables is increasingly reduced by the consecutive injections of medium, resulting in reduced airflow and reduced sound amplitude. EMG recordings do not indicate that birds attempt to compensate for the reduced pressure and sound amplitude, suggesting that this chronic alteration may not generate feedback information that leads to corrective changes in the motor gestures. These two observations indicate that the stereotyped temporal pattern of song arises from a motor program, which remained surprisingly unmodified by the chronic reduction in air supply.

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