Vocal and anatomical evidence for two-voiced sound production in the greater sage-grouse *Centrocercus urophasianus*

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SUMMARY

Greater sage-grouse, *Centrocercus urophasianus*, have been a model system in studies of sexual selection and lek evolution. Mate choice in this species depends on acoustic displays during courtship, yet we know little about how males produce these sounds. Here we present evidence for previously undescribed two-voiced sound production in the sage-grouse. We detected this 'double whistle' (DW) using multi-channel audio recordings combined with video recordings of male behavior. Of 28 males examined, all males produced at least one DW during observation; variation in DW production did not correlate with observed male mating success. We examined recordings from six additional populations throughout the species' range and found evidence of DW in all six populations, suggesting that the DW is widespread. To examine the possible mechanism of DW production, we dissected two male and female sage-grouse; the syrinx in both sexes differed noticeably from that of the domestic fowl, and notably had two sound sources where the bronchi join the syrinx. Additionally, we found males possess a region of pliable rings at the base of the trachea, as well as a prominent syringeal muscle that is much reduced or absent in females. Experiments with a live phonating bird will be necessary to determine how the syrinx functions to produce the whistle, and whether the DW might be the result of biphonation of a single sound source. We conclude that undiscovered morphological and behavioral complexity may exist even within well-studied species, and that integrative research approaches may aid in the understanding of this type of complexity.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/22/3719/DC1

Key words: acoustic location system, Galliformes, lek, microphone array, syrinx, syringeal muscle.

INTRODUCTION

Birds use complex sounds to communicate in a variety of intrasexual, intersexual and inter-species contexts. Interest in the morphological basis of this vocal complexity has focused on the syrinx, the avian sound source (Gaunt, 1987; Greenewalt, 1968; King, 1989; King and McLelland, 1984; Stein, 1968). The syrinx lies at the junction of the trachea and the primary bronchi; sounds may be produced by either paired or unpaired syringeal elements, depending on the species (King, 1989). In songbirds, sounds are produced by a two-voiced system in which two sets of labia may vibrate independently to produce different tones. For example, songbirds can produce songs with two simultaneous, independent sounds or use the two sound generators sequentially (Suthers, 1999; Suthers and Zollinger, 2008). The left side of the syrinx typically generates lower frequencies than the right side, thus increasing the possible range of frequencies and enhancing the ability to generate rapid frequency sweeps and/or permit more rapid frequency sweeps (Suthers, 1997; Suthers, 2001; Suthers and Goller, 1997). Two-voiced sound production is not unique to songbirds (Greenewalt, 1968); for example, signatures of individual identity are encoded in the partially independent tones of penguin vocalizations (Aubin et al., 2000; Lengagne et al., 2001); differences in vocal tract asymmetries also explain individual vocal differences in the oilbird (Suthers, 1994). Additionally, biphonation from a single sound source can occur as a result of nonlinear phenomena; in mockingbirds, simultaneous non-harmonically related tones can be the result of

biphonation or the action of a dual sound-source syrinx (Zollinger et al., 2008).

Game birds (order Galliformes) have been vital model systems for studies of avian anatomy and physiology. Although the vocal tract morphologies of several galliform birds have been described, functional investigations of sound production are limited primarily to the best-studied exemplar, the domestic fowl (*Gallus gallus*). In this species, detailed physiological studies seem to indicate that males lack both the morphological basis as well as the neuromuscular ability to produce two independent sounds (Gaunt and Gaunt, 1977; Gaunt et al., 1976). In domestic fowl, sounds are generated at the base of the trachea (Fig. 1). Sound production in other galliform species is not well understood; obvious differences in vocal tract morphology (Bayram and Liman, 2000; Bottino et al., 2006; Burke et al., 2007) hint that the domestic fowl may not be an appropriate model for all game birds.

Greater sage-grouse (*Centrocercus urophasianus*, Bonaparte) are large-bodied lek-breeding galliform birds with a complex courtship display (i.e. the 'strut') involving mechanically produced sounds as well as vocal elements (Fig. 2a; Audio File 1 in supplementary material). Male vocalizations are important for long-distance attraction of females as well as in short-range courtship (Gibson, 1989; Gibson, 1996; Gibson et al., 1991). A conspicuous component of the strut display is the rapid distension and inversion of an esophageal air sac behind a pair of pliable apterygia on the breast (i.e. the vocal sacs). Unlike respiratory air sacs common to all birds,
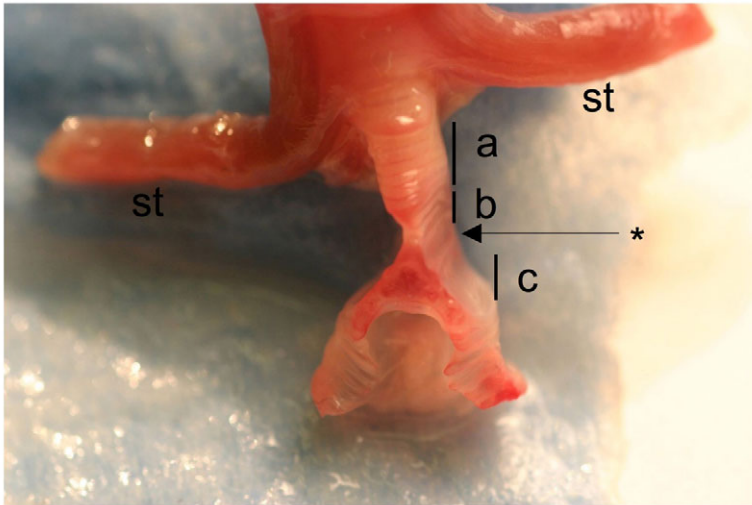
Fig. 1. Photograph of the ventral surface of the syrinx of a male wild-type domestic fowl. Region a is composed of hardened tracheal rings. Region b is the region of tracheal narrowing and more flexible tracheal rings. Vibrations occur below this region, as indicated by the asterisk. Although there are large membranous areas on either side of the syrinx at c, we could not induce these to produce vibrations in either of two rooster specimens. Lateral projections (st) are m. sternotrachealis.

the vocal sacs result from extreme inflation and manipulation of a distensible segment of the esophagus (Clarke et al., 1942; Honess and Allred, 1942). These vocal sacs are probably responsible for an unusual multi-lobate pattern of sound radiation of the 'whistle' note, which is up to 24 dB quieter directly in front of a displaying male than it is lateral to or behind the male (Dantzker et al., 1999).

We first observed two apparently simultaneously produced, non-harmonically related tones in the spectrograms of recordings obtained during the course of acoustic monitoring of a sage-grouse lek in Wyoming, USA (Fig. 3; Audio File 1 in supplementary material). Our study addresses four aspects of the apparent 'double whistle' (DW). First, we analyzed multi-channel audio recordings in conjunction with video recordings of sage-grouse courtship to rule out the possibility that the apparent DW is an artifact of multiple males calling simultaneously. Secondly, we surveyed recordings from other populations of greater sage-grouse to determine whether this two voiced system might be unique to our population or common to other populations. Thirdly, we examined the relationship between male mating success and DW expression to address possible fitness consequences for variation in DW production. In the work reported here we test two equally plausible hypotheses: that males with more complex whistles might achieve higher mating success, or alternatively, that DWs may be the result of poor coordination during the display and males with fewer or less obvious DWs might mate more frequently. Finally, we conducted anatomical dissections to investigate the potential sources of two non-harmonically related tones. Specifically, we describe the structure of the syrinx in light of the potential capability for containing multiple sites of sound generation.

MATERIALS AND METHODS
Study site and recording

We conducted this research at Monument Lek, on US Bureau of Land Management land approximately 30 km from Lander, Wyoming, USA. This region is characterized by relatively undisturbed sage-brush habitat, and contains a number of greater sage-grouse leks. The main display area on Monument Lek is a clearing approximately 100 m×80 m surrounded by short (<0.5 m height) sage brush, and it is >1 km from human habitation and power lines. We focused on a subset of approximately 40 males that held territories in and around this main clearing; maximum attendance at the lek was 99 males. Territorial males were identified based upon individual differences in the pattern of the

white-tipped under-tail coverts presented during display (Wiley, 1973a).

To aid in collecting positional data on individual male sage-grouse, we installed short survey stakes in a regular 10 m×10 m grid. On a subset of 32 of these stakes, we attached a short length of PVC conduit to serve as a microphone holder. We then buried microphone cables in shallow trenches, with the ends of each cable terminating in a single observation blind (hide) at the edge of the lek. Cables ranged from 13 m to 115 m. Although we established locations for 32 microphones in 2007, we only deployed 24 microphones (Sennheiser ME62 with K-6 power capsules; Sennheiser, Wedemark, Germany) at one time. The locations of each stake (including those supporting microphones) were georeferenced using a ProMark 2 global positioning system providing x–y axis
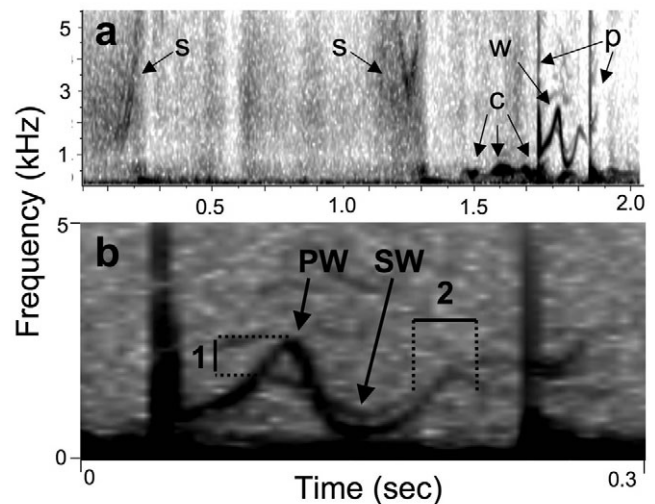

Fig. 2. Spectrogram of the courtship display of the male greater sage-grouse. (a) The entire display consisting of mechanically generated 'swishes' (s) followed by low frequency 'coos' (c), broadband 'pops' (p) and the frequency-modulated whistle (w). Note the evident double whistle (DW). (b) A spectrogram of a DW, which is composed of the more frequency-modulated primary whistle (PW) and less modulated secondary whistle (SW). Along with the proportion of calls showing a DW, we measured three aspects of the DW: (1) the difference in maximum frequency of the PW and SW, (2) the relative time of termination of the PW and SW, and the relative darkness (amplitude) in the spectrogram (see Fig. 4).
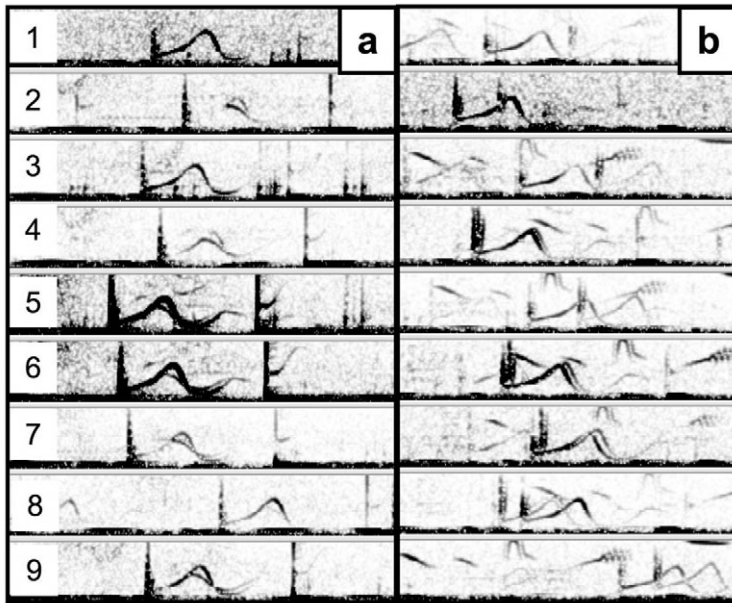
Fig. 3. Multi-channel spectrograms of sage-grouse vocalizations. Each row depicts the recording from one microphone of the 24-channel recording array; only nine channels are depicted here. (a) A display from a single male. In channels with both frequency-modulated tones in the whistle (channels 2, 4–7 and 9), these tones always appear in the same relative position, and there is never more than one pop note prior to the whistle. Only the primary whistle is visible in channels 1, 3 and 8. (b) The overlap of the vocalizations of two males. In channel 4 the two whistles appear almost coincident, but show increasing temporal offset in channels 6,7 and 8. See Fig. 2 for description of the acoustic display.

accuracy of better than 0.1 m (Ashtek Solutions, Magellan, Santa Clara, CA, USA).

All audio recordings were made between 06:00 h and 08:30 h from 05 April to 28 April 2007. For recording, microphone cables were connected to one of three eight-channel digitizing preamplifiers (one Mark of the Unicorn [MOTU] 896 and two MOTU 896HD preamplifiers; Mark of the Unicorn, Cambridge, MA, USA) that provided phantom power for the microphones. These preamplifiers were connected in series using Firewire 400 cables, with the final unit connected to a Macintosh Powerbook G4 laptop. We recorded 16 bit, 44.1 kHz sampling rate audio in each of the 24 channels using the acquisition and editing software MOTU Digital Performer 4.5. Digital sound files were recorded onto an external 7200 r.p.m. hard drive that was connected to the laptop with a Firewire 800 cable. We ensured synchronous recording by generating Word Clock time code with a MOTU Timepiece AV generator and MOTU Clockworks software. One preamplifier received Word Clock from the laptop with a USB cable and the time code was passed to the additional preamplifiers using coaxial cable. All equipment was powered using a power inverter and an 80 Ah deep cycle battery.

Acoustic analysis

In order to assign the audio record of a display to a male of known identity, we used an acoustic location system (Mennill et al., 2006; Spiesberger and Fristrup, 1990). Specifically, we used the freeware program Syrinx (www.syrinxpc.edu; John Burt, University of Washington) to select and annotate calls on the multi-channel spectrograms that appeared to show no overlap with other grouse displays. Calls with evident overlap from another male's vocalization were avoided because we noticed during preliminary analyses that they often failed to localize or, if they did localize, unpredictably returned a source location for either the intended call or the overlapping call. We passed these annotations to a series of Matlab algorithms (John Burt, University of Washington) that used the microphone array geometry and estimated of the relative time of arrival of a sound in each channel to determine a putative point of origin for the sound. Speaker playback tests suggested that this process yields accuracy of approximately 0.5 m for birds within the array (A.H.K., J. Burt and G.L.P., unpublished data). Finally, we

compared the time and location of acoustic localizations to visual estimates of male positions during display in order to assign calls to individual males; as part of another study (Patricelli and Krakauer, in press), we noted the position and time of male displays. To avoid misinterpreting overlapping calls from multiple males as DW calls, we only analyzed calls assigned to males that were approximately 6 m or more from their nearest neighbor. This eliminated the possibility that calls from multiple males would overlap in more than a few channels, since this inter-male distance would result in readily observable time-of-arrival differences of up to 0.018 s for their respective calls, or almost 10% of the time between the first and second 'pop' note of a display.

To further address the possibility that the DW might be the result of overlapping calls from multiple males, we examined the distribution of maximum whistle frequencies in calls containing and lacking a DW. In describing the two-voiced system of the sage-grouse, we designate the primary whistle (PW) as the more frequency-modulated tone and the secondary whistle (SW) is designated as the less frequency-modulated tone (Fig. 2b). Not all recorded vocalizations include a double-whistle note ('No DW'); these calls may either consist of a single whistle with additional whistles undetectable or absent, or alternatively, two perfectly coincident tones that appear as a single primary whistle. We generated spectrograms using the software package Raven 1.3 (Cornell Laboratory of Ornithology, Ithaca, NY, USA), and measured the frequency at the first frequency peak of calls lacking DW, as well as the PW and SW of calls containing the DW. If the range of maximum frequencies of calls containing no DW encompassed the range of maximum frequencies of both the PW and SW, it would suggest DWs could be the result of overlapping calls. However, if the maximum frequencies of SW calls fall outside of this range, then it would suggest the SW is not the result of overlapping calls from different males. Using SPSS v.16, we performed a one-way ANOVA with *a priori* contrasts to compare non-DW calls with both PW and SW calls.

Survey of non-focal populations

In order to search for the presence of DWs in other populations, we obtained a set of four field recordings from the Macaulay Library

Table 1. Presence of double-whistle calls in various populations of greater sage-grouse

Location	Recording	Calls with DW
Monument Lek (Lander, WY, USA)	Krakauer and Patricelli, unarchived recording	16
Alberta, Canada	CLO acc. no. 59284	15
Mono Basin, CA, USA	Marc Dantzker, unarchived recording	8
Colorado, USA	Gordon Hempton, unarchived recording	12
Oregon, USA	CLO acc. no. 50119	35
Oregon, USA	CLO acc. no. 111166	9
Saskatchewan, Canada	CLO acc. no. 2385	4

Number of calling males is unknown. Each sample consists of the first 50 calls in which the whistle was clearly visible on the spectrogram and was not obviously overlapped by other vocalizations. The value for Monument Lek represents a count of 50 calls from a single channel recording taken from the multi-channel array. Since recording conditions varied across sites, these rates of double whistle (DW) detection are not accurate estimates of the rate of its occurrence.

CLO=Macaulay Library at the Cornell Laboratory of Ornithology, Ithaca, NY, USA.

of the Cornell Laboratory of Ornithology, as well as additional recordings from private recordists (Table 1). We visualized these recordings as spectrograms in Raven, and a single observer (A.H.K.) examined the first 50 sage-grouse displays in which the whistle could be seen clearly and in which the whistle was not obviously overlapped by the pops or whistle of another bird; we scored these calls for the apparent presence or absence of a DW. Since these were typically single-channel or single-location stereo recordings, we could not rule out the presence of completely synchronous displays from two or more males based upon acoustic localization of calling males. Similar to the analysis of the vocalizations of males within our microphone array, we compared the maximum frequency of primary whistles (PW), secondary whistles (SW) and non DW calls to determine whether the distribution of maximum frequencies of non-DW calls overlapped the distribution of maximum frequencies of the SW.

Relationship to male mating success

We used data on the mating success of individually identified males to examine whether characteristics of the DW may be related to male fitness. Mating success (MS) was determined by summing the number of copulations observed in the field and those recorded on video during each morning of observation (Patricelli and Krakauer, in press). A previous study of paternity of a small sample of sage-grouse nests showed that mating success is a good, but not perfect, predictor of male reproductive success (Semple et al., 2001).

We examined four acoustic features. First we considered the proportion of calls attributed to a male in which we detected a DW. Second, in calls containing a DW we measured the frequency difference between the first peak in the PW and the first peak in the SW (Fig. 2b). Third, we measured the relative time of termination of the PW and SW as a proportion of the inter-pop interval (IPI; the total duration from Pop 1 to Pop 2) divided by the duration of the PW relative to the IPI (Fig. 2b). Finally, we used a qualitative measure of relative amplitude of the PW and SW, based upon relative darkness in the spectrogram, with 0 indicating that the PW was of much higher amplitude than the SW, and 3 indicating that the SW appeared approximately equal in amplitude to the PW (Fig. 4). Mean values of each variable were considered in univariate analyses, and were also combined in a principal components analysis (PCA) with Kaiser normalization and varimax rotation. The first two components yielded eigenvalues greater than one, and came before a natural break point in the eigenvalue scree plot before the third component; therefore PC1 and PC2 were used in subsequent correlations with male mating success (Quinn and Keough, 2002). Mating success was highly skewed and could not be

transformed to achieve normality, therefore non-parametric Spearman rank correlations were employed. All analyses were conducted using SPSS v.16.

Anatomical dissections

Greater sage-grouse are a species of conservation concern throughout their range, making it difficult to obtain breeding-condition specimens for dissection and experimentation. We obtained two male and two female sage-grouse from Wyoming Game and Fish officials – these were road-killed birds collected in or near the breeding season. Specimens were stored frozen and thawed prior to dissection. We excised the vocal tract by removing the tongue and trachea, severing the sternotrachealis muscles and cutting through the bronchi several rings below the syrinx. After photographing the vocal tract, we removed the tongue and anterior
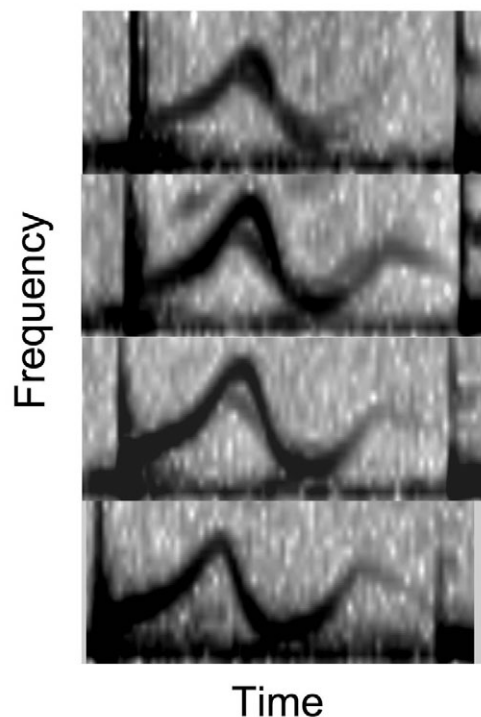

Fig. 4. Spectrograms of whistles illustrating variation in the qualitative measure of relative amplitude (sound intensity) between the more frequency-modulated primary whistle (PW) and less modulated secondary whistle (SW). Scores range from 3 (top) to 0 (bottom). Spectrograms are 0.25 s in length and extend from 0 kHz to 3 kHz; all are from different male sage-grouse.
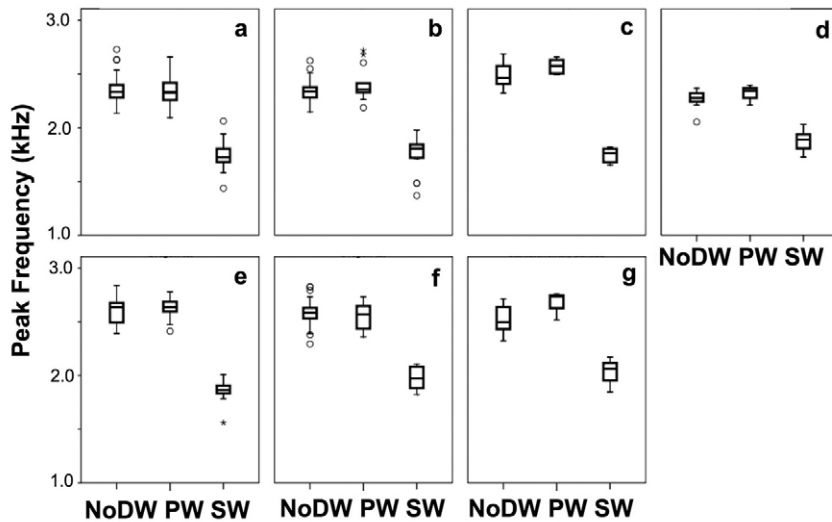
Fig. 5. Distribution of maximum whistle frequencies of calls lacking a double whistle (NoDW), and the primary whistle (PW) and secondary whistle (SW) of DW calls. Populations are (a) our focal lek Monument Lek, near Lander, WY, USA; (b) Alberta, Canada; (c) Mono Basin, CA, USA; (d) Colorado, USA; (e) Oregon, USA; (f) Oregon, USA; (g) Saskatchewan, Canada.

portion of the trachea. We then inserted surgical tubing into the anterior end of the remaining vocal tract, and sealed this with suture and surgical glue to create an air-tight seal. This surgical tubing was then connected to a standard laboratory vacuum suction pump. To identify potential sources of vibration, we initiated suction and modulated tension on the vocal tract by gently pulling on the bronchi with forceps (Movie 1 in supplementary material). Airflow was adjusted until it was strong enough to induce vibrations. Once the sound source was identified, airflow was increased in steps until further increase resulted in collapse of the trachea. This method of inducing vibrations in excised syringes allows identification of the principal sound sources, but probably does not duplicate details of the vibratory behavior of the sound generating structures in the intact animal (Dürrwang, 1974; Fee et al., 1998; Rüppell, 1933). When syringeal vibrations were induced, we used a blunt probe to gently push on various membranes to modify tension and change the pitch of the induced tone. To determine whether our putative sound sources had the potential to vibrate in a bilaterally independent manner, we inserted small segments of surgical tubing into one bronchus, thus allowing air to pass through but preventing vibration on that side of the syrinx.

RESULTS
Acoustic evidence for two-voiced sound production
We found the range of maximum frequencies of secondary whistle (SW) did not overlap the range of maximum frequencies of non-DW calls (Fig. 5a), demonstrating that DW calls are not simply overlapping whistle vocalizations from a number of different birds. In a sample of 67 calls lacking a DW from 23 males, the mean maximum frequency was 2341±112 Hz. For 198 calls from 28 males

in which a DW was identified, the mean maximum frequency of the primary whistle (PW) was 2340±129 Hz, whereas the mean maximum of the SW was 1739±91 Hz. The mean maximum frequencies of these three call components (non-DW calls, PW and SW) are significantly different (one-way ANOVA, F=1644.2, $P<0.0001$, d.f.=2); *a priori* contrasts demonstrate that the non-DW calls are different from SW (t=38.2, $P<0.0001$, d.f.=460) calls but not the PWs (t=0.09, P=0.993, d.f.=460). Thus the two tones present in the double whistle (DW) could not be attributed to the overlap of calls from more than one male.

Geographic extent of the double whistle
To examine whether DWs are widespread in sage-grouse or restricted to our study population in Wyoming, we examined audio recordings from six other populations throughout the species' range. Apparent DWs were observed at all six recording sites (Table 1). The occurrence of DW scored from a single channel from the Monument Lek microphone array was within the range of DW occurrence found in other populations. Identical to the microphone array results, the PW of DW calls had a similar maximum frequency to that of non-DW calls in each population, but there was no overlap between the maximum frequencies of SW and non-DW calls, suggesting that the apparent DWs we observed were not the result of overlapping calls from more than one male (Fig. 5).

Variation among males
All 28 males on Monument Lek produced a DW in at least one call. Males varied in the expression of DWs, with some males rarely producing a DW and other males typically producing prominent DWs (Table 2, Fig. 6). In spite of this variation we found no

Table 2. Summary of the principal components analysis examining variation in the double-whistle and its relation to male mating success

|	Raw distribution (mean±s.d.)	PC1	PC2
Frequency difference	578.1±106.6 Hz	0.795	−0.70
Relative time of termination	0.70±0.11 s	0.181	0.873
Relative amplitude (scale 0–3)	1.91±0.67	0.807	−0.006
Proportion with DW	0.68±0.26	0.357	−0.769
Eigenvalue	n.a.	1.583	1.219
Percentage of variance explained	n.a.	39.6	30.4
Spearman correlation with mating success	n.a.	−0.182	0.039
P-value for significance of Spearman correlation	n.a.	0.35	0.845

DW, double whistle; n.a., not applicable.
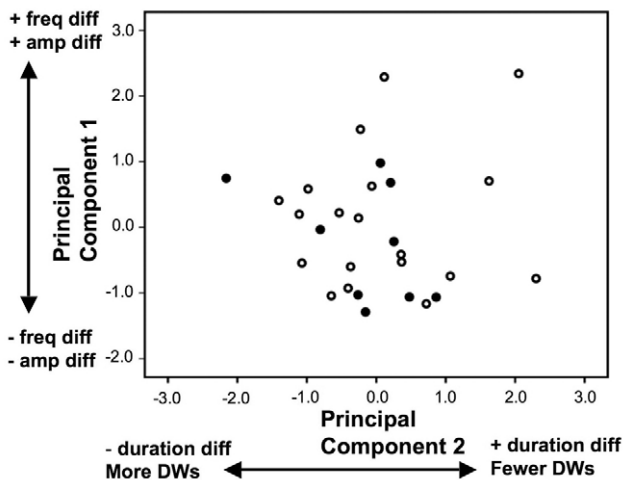
Fig. 6. Plot of PC1 and PC2 from the principal components analysis showing variation in the expression of the double whistle (DW). There was no significant difference in the scores of copulating males (filled circles) or non-copulating males (open circles). Results of the principal components analysis are presented in Table 2.

correlation between the DW and male mating success, when considering either PC1 and PC2 from the principal component analysis (Table 2), or the four DW variables independently (proportion of calls with DW: R_S=–0.18, N=28, P=0.38; maximum frequency of the SW – maximum frequency of the PW: R_S=–0.15, N=28, P=0.46; relative duration of SW relative to the duration of the PW: R_S=–0.19, N=28, P=0.35; estimated relative amplitude of PW and SW: R_S=–0.03, N=28, P=0.88).

Functional anatomy of the syrinx

We examined the vocal tracts of two female and two male sage-grouse (Fig. 7). The basal region of the trachea of female sage-grouse exhibited a consistent diameter and moderate ossification of the cartilaginous rings. In this same region, male sage-grouse showed similarly consistent size and shape of tracheal elements, although the first six tracheal rings anterior to the pessulus were pliable, and could collapse easily if modest pressure was applied. Both males and females therefore lacked the 'tympanic drum' and extreme tracheal narrowing present in domestic fowl (Table 3, Fig. 1) (Gaunt et al., 1976; King and McLelland, 1975; Myers, 1917). Both males and females showed relatively large external tympaniform membranes that are located posterior to the pessulus.

We also found a muscle present on the syrinx and basal portion of the trachea that covers almost the entire ventral surface of the syrinx and basal portion of the trachea. In males, this muscle was robust and somewhat thicker laterally (Fig. 7b); its anterior insertion appeared to be on the fourth to sixth tracheal rings anterior to the pessulus, with a posterior insertion on the syrinx on the second ring anterior to the anterior-most bronchial ring. Although the actual muscle fibers were somewhat difficult to make out because the specimens had been frozen and thawed, this muscle did not appear to be a part of either the tracheolateralis or sternotrachealis muscles. In the female sage-grouse, this muscle is either absent or drastically reduced, such that it was not possible to identify it under a dissecting microscope.

We were able to induce phonation in the syrinx of both the female and male sage-grouse. When air was pulled through the vocal tract, vibrations were evident externally in the tissue between the basal syringeal elements. These external vibrations appeared to be a consequence of vibrations of internal membrane folds at the lateral occlusion of the medial tympaniform membrane (medially on each bronchus) and the first or second bronchial ring (laterally) on each side of the syrinx (Fig. 7a,c). Pressing on the vibrating syringeal membranes increased the frequency of the induced sound (rather than eliminating the sound). The larger vocal tract of males allowed us to observe directly the opposition of these lateral and medial elements while looking at the syrinx through the cut end of the bronchi. We found that vibrations could be induced independently in the left and right bronchi – when elements on one side were prevented from vibrating by the insertion of a tube in that bronchus, the other side continued to vibrate. We did so in both female specimens, and one male specimen (the second specimen of a male sage-grouse had a torn left bronchus, therefore we were only able to induce phonation in the right bronchus).

We also suggest the possibility of a third sound source in the vocal tract of the male sage-grouse. This suggestion is based upon a single bout of induced phonation in the first male specimen. During this event, we observed the region of pliable tracheal rings at the base of the trachea collapsing and re-opening at a relatively low frequency (approximately 100 Hz) and creating a guttural pulse-tone that was unlike the higher frequency tones produced in the bronchi. We were unable to replicate this vibration in the (partially damaged) second specimen. When examining the interior of the trachea in this region we did not notice any modifications of the rings or tissue between the rings that might indicate specific locations where airflow could be occluded. However, the dimorphic intrinsic muscle appears to be positioned to act by folding the pliable region of the trachea into the air stream as this region is suspended between the two sites of attachment of this muscle.

DISCUSSION
Acoustic evidence for a two-voiced system

The courtship display of greater sage-grouse has been a subject of research for decades, and audio recordings of the displays from

Table 3. Anatomical and functional differences in the vocal tract of domestic fowl (*Gallus gallus*) and the greater sage-grouse (*Centrocercus urophasianus*)

	Domestic fowl	Sage-grouse (male)	Sage-grouse (female)
Tracheal tympanum (fused rings near base of trachea)	Present	Absent	Absent
Caudal tracheal cartilages	Laterally compressed region of 4–5 cartilages	Six pliable rings, similar in size and shape to rest of basal trachea	Cartilages similar in size, shape and ossification to rest of basal trachea
Site of sound production	Base of trachea (unpaired)	Bronchial–syringeal junction (paired)	Bronchial–syringeal junction (paired)
Intrinsic syringeal musculature	Absent	Very robust	Absent or virtually absent

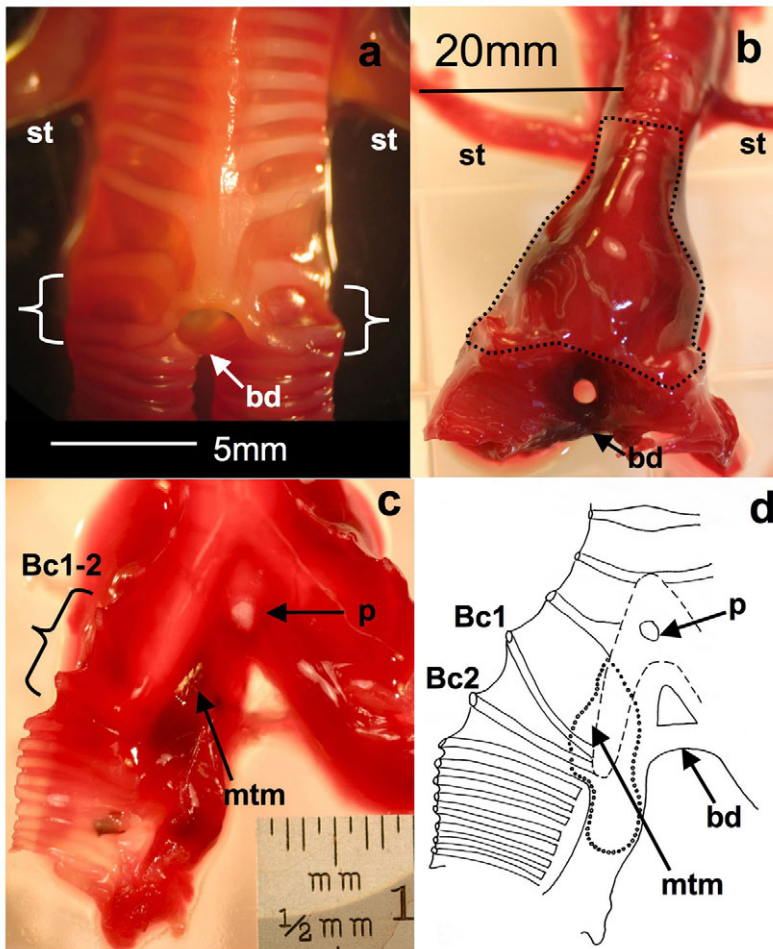See Figs 1 and 7 for photographs.

Fig. 7. The ventral views of the syrinx of a female (a) and male (b) greater sage-grouse. The sites of syringeal vibration in the female are marked with brackets, st, m. sternotrachealis; bd, bronchidesmus. Panel b shows the large intrinsic syringeal muscle found in the male sage-grouse (the red tissue covering the syrinx: the rough extent of this muscle is indicated by the dotted line). (c,d) Ventral view of the male sage-grouse syrinx with the ventral half cut away, so the inner surface of the dorsal half is exposed. p denotes the pessulus, the extent of which is marked in d by the dashed line; the circle indicates the connection to the ventral portion of the syrinx. One of the two sets of vibrating elements is identified with a bracket in c: the medial tympaniform membrane (mtm) and first two bronchial cartilages Bc1-2. This particular specimen was damaged and the vibrating elements on the right side (bird's left) were no longer intact. In all panels the trachea extends up towards the mouth, and the two bronchi towards the bottom of the photograph would extend to the lungs.

numerous populations have been examined (Gibson, 1996; Gibson et al., 1991; Hartzler, 1972; Taylor and Young, 2006; Wiley, 1973b; Young et al., 1994). Despite this prolonged interest in male sage-grouse vocalizations, we are the first to describe two-voiced sound production in this species. In our focal population on Monument Lek, we found that all males produced DW vocalizations. We used multi-channel audio recordings combined with video-taped observations of male display locations to reject the possibility that the apparent two-voiced system is the result of the overlap of calls from more than one male. Two lines of evidence allowed us to do this. First, our observations of the time and location of male displays makes call-overlapping an unlikely explanation for the DW we observed. Our video data confirm that the calls were not produced by two synchronously calling males in close proximity. Although two more distantly separated males could produce calls that arrive synchronously at a given microphone, these calls would arrive asynchronously at other microphones in the array. Secondly, we found no overlap in the maximum frequencies of calls lacking a DW and the secondary whistle (SW) of DW calls, suggesting that the secondary whistle is not merely the whistle note of a second nearby male. Instead, strut vocalizations with no visible DW appear to be just the primary whistle (PW; i.e. no vocalizations were found with only an SW and no PW).

In addition to the recordings from our multi-channel microphone array, we also analyzed recordings from six other populations, and found evidence of DW in all of these populations. As with the calls we recorded in Wyoming, in all six populations the maximum

frequencies of the SW were without exception lower than the maximum frequencies of non-DW calls, whereas non-DW calls and PW calls did not significantly differ. We therefore believe that the apparent DW calls we detected in one- or two-channel recordings are not due to overlap of different male calls, and consequently, that DW production may be typical of males in most if not all populations of greater sage-grouse.

Given the previous attention to sage-grouse vocalizations, why might the DW have remained undiscovered until now? It is likely that previous recordists who may have observed DW in their recordings assumed the independent tones represented overlapping calls from simultaneously displaying males (R. Gibson, J. Bradbury and M. Dantzker, personal communication). Additionally, detecting the DW requires a relatively high quality recording since the (SW) component is typically much lower in amplitude than the primary whistle (PW). DWs were frequently identified in no more than a few of the 24-channels in our microphone array (Fig. 3a), thus DW detectability may vary with distance from the microphone and possibly orientation of the male (A.H.K. and G.L.P., unpublished data). Because all of these factors can influence the likelihood of detecting a DW, and since these factors varied among recordings analyzed here, the rates of detection listed in Table 1 are not estimates of the true rate of occurrence of DW vocalizations.

Relationship to male mating success

Examining the calls of individual males, we found differences among males in both the frequency of DW production and the relative pitch

and amplitude of the SW relative to the primary whistle (PW). Some males rarely produce DWs or produce a DW in which the SW is visible for only a small portion of the whistle, whereas other males consistently produced DWs in which the secondary whistle was much more prominent in our spectrograms. We did not find support for either of the alternative hypotheses regarding the functional significance of the DW for males: (1) males with more DW would achieve higher mating success, perhaps because of female preference for complexity, or (2) males with less DWs would have higher success, since this may indicate better bilateral coordination during vocal production and/or a louder PW. Our results could indicate that two-voiced sound production by males is neither favored nor disfavored under sexual selection. We are currently examining how variation in DWs relates to other aspects of display, including signal directionality, acoustic amplitude and display effort, which may be under selection by female choice (J. Bradbury, unpublished data) (Dantzker et al., 1999; Gibson, 1996) (A.H.K. and G.L.P., unpublished data). Additional data such as these may reveal the functional significance, if any, of variation in this aspect of the display.

Anatomical basis of sound production

Our anatomical dissections have revealed previously undescribed diversity in vocal tract morphology of galliform birds. While the vocal tracts of several species have been described (Bayram and Liman, 2000; Bottino et al., 2006; Burke et al., 2007; King and McLelland, 1975), to our knowledge these reviews of syringeal anatomy have not considered the possibility of lateral biases in sound production. We found important structural differences between the syrinx of greater sage-grouse and that of domestic fowl. Both males and females lacked the heavily ossified tympanum and characteristic narrowing at the base of the trachea of domestic fowl. The syrinx of the female sage-grouse looks more similar to published illustrations of other galliform birds such as Japanese quail (Bayram and Liman, 2000), with no narrowing and uniformly rigid rings at the basal end of the trachea. Male sage-grouse had pliable rings in the same region where the domestic fowl trachea is narrowed [i.e. 'first caudal cartilages' (King and McLelland, 1984)], although in the sage-grouse this pliable region contains six rings rather than four in the fowl.

The presence of a well-developed intrinsic syringeal muscle in the male sage-grouse deviates from other galliform syringes. The muscle appears analogous to the intrinsic syringeal muscle, m. broncholateralis (also named m. syringealis) in the oilbird (King, 1989; Suthers and Hector, 1985). Intriguingly, in the oilbird the broncholateralis muscle is involved in gating of airflow and thus sound production, as for example in the 'double-click' (Suthers and Hector, 1985), a vocalization that superficially resembles the two 'pops' of the male sage-grouse display. The sexual dimorphism in this muscle in the greater sage-grouse is also noteworthy. Although syringeal muscles in both passerines and non-passerines are dimorphic in mass (e.g. Miller et al., 2007; Wade and Buhlman, 2000), this is the first case, to our knowledge, in which males possess such a well-developed syringeal muscle that is entirely or nearly absent in females.

The structural features of the vocal tract of sage-grouse may have implications for production of the whistle note. Most attention thus far has focused on how the large esophageal air sac may be involved in generating sounds during display (Clarke et al., 1942; Dantzker and Bradbury, 2006; Dantzker et al., 1999; Honess and Allred, 1942). Expansion of these sacs probably influences resonance filtering, impedance matching with the environment, and acoustic directionality (Dantzker et al., 1999; Fletcher et al., 2004). Our results indicate that the syrinx itself could be more important than initially believed for contributing to the acoustic complexity of the male's courtship display. Specifically, our finding that males have two sound sources, as well as possessing intrinsic syringeal musculature that could act to modulate tension on the syrinx during phonation, leads us to the conclusion that the syrinx could be the origin of the DW. It is unclear to what degree the two sound sources of the sage-grouse syrinx can act independently. The close temporal synchrony between the PW and SW, as well as similar frequency modulation patterns, suggest that some coupling between the two sources may exist during the production of the whistle. Similarly, since we could not reproduce actual whistle notes during our airflow experiments or measure pressure and airflow in a live, phonating bird, biphonation of a single sound source remains a potential explanation for the DW (Zollinger et al., 2008). If this were true, it suggests there could be differences in airflow in the two bronchi during whistle production, or, alternatively, that a separate unpaired sound source such as the possible tracheal source we identified could be the site of nonlinear phenomena, which give rise to the two non-harmonically related tones observed in the DW.

We are currently examining the role of the vocal sacs in sound production by using high-speed video and synchronized audio recordings of grouse displays to correlate the dynamics of the vocal sacs with the coincident sounds (M. S. Dantzker, A.H.K. and G.L.P., unpublished data). In the future we plan to examine directly the role of the syrinx in sound production using either field-implantable devices or captive animals. This will help to determine whether calls without DWs represent perfect coordination of both sides of the syrinx, the use of only one side of the syrinx, or calls in which the SW is too quiet to detect. Regardless of the outcome of future investigations, our study has uncovered unexpected vocal and morphological complexity in an otherwise well-known species. Moreover, our findings highlight the usefulness of microphone arrays, and suggest that future studies employing multi-channel recording techniques may uncover additional instances of previously undetected signal complexity in vocalizing animals.

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