The Vocal Sac Increases Call Rate in the Túngara Frog *Physalaemus pustulosus*

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ABSTRACT

In most anurans, the production of advertisement calls is accompanied by the inflation of a vocal sac. Current functions of the vocal sac, however, are not fully understood, although several hypotheses have been proposed. One hypothesis suggests that the vocal sac decreases the intercall interval (i.e., increases call rate) by reinflating the lungs more rapidly than is possible with the buccal pump. We investigate this hypothesis by analyzing audio and video recordings of calling túngara frogs. We compare the first two call bouts emitted by an originally uninflated male. The first call bout requires lung inflation via buccal pumping, but in the second, the male is already inflated because of capture of air and reinflation of the lungs by the vocal sac. Lung inflation to typical field levels requires 26–51 buccal pumps, which takes at least 4.4 s. This estimate is more than 2.5 times the typical intercall interval with lung reinflation via a vocal sac (ca. 1.7 s). Evidence from phonotaxis tests demonstrates that these differences in intercall intervals are salient to females and that female *Physalaemus pustulosus* prefer the shorter intercall interval/higher call rate. Acoustic analyses demonstrate that the first call of bout 1, which requires buccal pumping, is usually shorter, of lower amplitude, and spans a smaller frequency range than the first call of bout 2, which does not require buccal pumping. Because females prefer longer, more intense calls, these results suggest that the vocal sac not only increases call rate but also allows males to produce more calls of increased attractiveness to females.

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Introduction

A fundamental problem for animals that vocalize is coupling sound vibrations produced inside the body to the surrounding medium (reviewed in Bradbury and Vehrencamp 1998). In aquatic animals, sound energy is relatively easily transferred between the organism and the aquatic environment because the density of water is similar to the density of the sound-producing animal. In terrestrial animals, however, the acoustic impedance is much higher because the density of air is much less than the density of the sound producer, so sounds are reflected back into the body. A common solution to this problem is reliance on structures that decrease acoustic impedance, and common among these structures are vocal sacs, which occur in howler monkeys, gibbons, orangutans, hammerhead bats, several species of grouse, and many frogs (often called throat sacs in nonanuran species; Bradbury and Vehrencamp 1998). In addition to acting as acoustic couplers, vocal sacs may have other important functions in vocalization. Here, we investigate one hypothesized function for the anuran vocal sac: that it serves to rapidly reinflate the lungs after calls, thereby negating repeated use of the slower buccal pump and allowing animals to call at a faster rate.

Frogs rely extensively on acoustic communication (Ryan 2001; Gerhardt and Huber 2002). Although females of a few species produce calls (reviewed in Schlaepfer and Figeroa-Sandí 1998), males produce the vast majority of anuran acoustic signals. The most familiar and well-studied call is the advertisement call, which adult males emit to attract mates and to establish territories (Wells 1977). In nearly all male anurans, the production of advertisement calls is accompanied by the inflation of a vocal sac. The result is a striking sexual dimorphism in the occurrence of this secondary sexual trait.

Anuran vocal sacs are distensible, balloonlike structures that inflate with pulmonary air expelled from the lungs when a male produces advertisement calls. These structures vary in size and number but when inflated can be nearly as large as the animal itself (e.g., Dudley and Rand 1991). Some species have a single subgular vocal sac, while others have paired lateral sacs (Liu 1935). Vocal sacs result from a diverticulum of the lining of the buccal cavity that protrudes ventrally through apertures in the floor of the mouth (i.e., vocal slits), resulting in a chamber in the underlying superficial mandibular muscles. The wall of the vocal sac is formed by the musculus interhyoideus and, in some species, the more anterior musculus intermandibularis (terminology following Tyler 1971, 1974). The musculus in-

terhyoideus is lined externally with a network of elastic fibers (Jaramillo et al. 1997; Savitzky et al. 1999). When inflated, the vocal sac contacts the gular skin, which is highly modified in males of some species and is frequently considered a part of the vocal sac.

Most frogs begin the production of advertisement calls by inflating their lungs via buccal pumping (description following de Jongh and Gans 1969; Gans 1973). A buccal pump begins when the muscles in the floor of the buccal cavity are relaxed, which expands the buccal cavity and draws air in through the nares. The nares are then closed, the larynx is opened, and the buccal floor muscles are contracted, forcing air into the lungs. Buccal pumping is repeated until the lungs are adequately inflated. To produce a call, the muscles of the abdominal wall contract, forcing pulmonary air through the larynx, vibrating the vocal cords. Air entering the buccal cavity with the nares closed is forced through one or two vocal sac apertures into the vocal sac. As the vocal sac expands, the network of elastic and muscle fibers in the vocal sac wall stretches. Once the abdominal muscles are relaxed, elastic recoil of the vocal sac forces air to return to the lungs. Contractions of the submandibular muscles may also be involved in vocal sac deflation, but this has never been demonstrated. Moreover, Gridi-Papp (2003) argues that, at least for the hylids he examined, elastic recoil, without assistance from any submandibular muscles, is sufficient to explain the return of air to the lungs. Subsequent calls can then be produced without additional buccal pumping.

Calling is energetically inefficient (Ryan 1985a) and expensive (Bucher et al. 1982; Wells 2001), but the vocal sac may reduce energy expenditure in several ways. First, as suggested by Ryan (1985b), the elasticity of the vocal sac may store elastic strain energy as the vocal sac inflates during a call and may then use this energy to return air to the lungs. Histological results of Jaramillo et al. (1997) are consistent with this possibility. Second, by capturing the expelled pulmonary air and reinflating the lungs, the vocal sac eliminates the need for repeated bouts of buccal pumping before each call. Fully inflating the lungs in preparation to call requires numerous buccal pumps and, therefore, numerous contractions of the laryngeal and submandibular muscles. Reinflation via the vocal sac should require less energy because it relies on the elasticity of the sac and, potentially, brief contractions of the submandibular muscles.

In addition to increasing energetic efficiency, the vocal sac may also improve temporal efficiency by quickly reinflating the lungs (Dudley and Rand 1991). Without the vocal sac, buccal pumping must precede each call so that the lungs can be inflated. Therefore, males that capture air in their vocal sacs and use it to reinflate their lungs should attain lower intercall intervals (i.e., higher call rates) than males using buccal pumping. This is an important feature because many female animals that respond to acoustic signals prefer lower intercall intervals (Bosch et al. 2000; reviewed in Ryan and Keddy-Hector 1992;

Andersson 1994; Gerhardt 1994). We examine this hypothesis by making video and audio recordings of male túngara frogs *Physalaemus pustulosus* as they inflate their lungs and call. Our goal is to estimate the minimum intercall interval attainable with buccal pumping and to compare this value to observed intercall intervals, which are based on lung reinflation using the vocal sac. We then determine whether these differences in intercall intervals are salient to females by comparing our observed values to previously published phonotaxis test results (Bosch et al. 2000). Even if lung reinflation via the vocal sac increases calling rate relative to reinflation via buccal pumping, this difference may be irrelevant if it is not noticeable to potential mates.

Natural History

The túngara frog *P. pustulosus* is a small leptodactylid distributed throughout the Middle American and northern South American lowlands. It breeds nocturnally in temporary pools during the rainy season (May–December in Panama, where this study was conducted). The main component of the advertisement call of túngara frogs is a whinelike frequency sweep. Males can also facultatively add secondary components (chucks) to the whine. In general, isolated calling males produce only whines, while males interacting acoustically with other males add chucks to their calls (Ryan 1985b). Typically, a male calls in bouts, during which multiple calls are given with fairly short intercall intervals; in our study population, the average intercall interval during a call bout was 1.992 ± 0.397 s ($n = 32$; Bosch et al. 2000). *Physalaemus pustulosus* have two vocal slits and a single, median subgular vocal sac. The vocal sac is uninflated between calls but inflates dramatically during an advertisement call. These frogs are ideal for studying vocal sac inflation because of their extremely large and conspicuous vocal sacs that can increase in volume by a factor of 20–40 during a call and can become almost as large as the frog itself (Dudley and Rand 1991).

Material and Methods

Our goal was to assess differences in inflation and calling associated with the presence or absence of the vocal sac. Without a vocal sac, a male must buccal pump before each call. To determine how much time and how many pumps are needed for a male to fully inflate, we made video recordings of 22 male *Physalaemus pustulosus* buccal pumping and calling in the field at Gamboa, Panama (09°07′0″N, 79°41′53″W) between July 28 and August 6, 2004. Males were filmed at 30 frames/s on Sony Hi-8 analog tapes using the infrared lighting from Sony video cameras (model CCD-TRV128 or CCD-TRV66). Video recordings were taken from the side or front of the frogs to get the best view of any gular movements. Audio recordings were simultaneously collected for some individuals using a Sony Pro-

fessional Walkman (model WM-D6C) and a Sennheiser ME 66 microphone. Preliminary recordings showed that previously uninflated males start calling before they are fully inflated. Moreover, males buccal pump before and after the first few calls of a bout, but once fully inflated, they may not pump or may pump only a few times between subsequent calls. We therefore recorded the inflation and first call bout of each male. In contrast with the first call bout, at the beginning of the second call bout the male is already inflated because the vocal sac reinflated the lungs after the last call of the previous bout. A long sequence of buccal pumping, therefore, is not necessary before the second call bout. Preliminary recordings also suggested that call characteristics may vary between call bouts based on the method of inflation. We will refer to the first and second call bouts as bout 1 and bout 2. Because the male is not fully inflated, the initial calls of bout 1 may be of lower amplitude and/or shorter duration than the initial calls of later bouts. We recorded the second call bout and conducted acoustic analyses to examine this possibility.

To record inflation via buccal pumping, we had to start with uninflated males, and operationally, the call bout after deflation is here termed call bout 1. Upon our approach or when gently touched with a grass stem, males that had called recently deflated. We then recorded any subsequent buccal pumping and the first two call bouts. Call bout 1 was defined to include the first five calls regardless of the intercall interval and to end once the male stopped calling for >10 s, as long as he had called at least five times. Call bout 2 was defined as the calls after this >10-s pause and before the next >10-s pause. This "10-s" rule is based on our observations of call rates in male *P. pustulosus*. After video recording, we measured water temperature at the calling site, and frogs were collected and individually marked by toe clipping before release to ensure that individuals were not recorded multiple times.

Because a mild disturbance is necessary to induce deflation, call bout number is confounded with potential changes in motivation following disturbance. Therefore, observed differences between call bouts could be attributed to differences in motivation rather than differences in the method of lung inflation. Although it is not possible to assess changes in motivation, we attempted to minimize any impact by recording the most actively calling males. Moreover, impacts from changes in motivation are likely to be minimal in the active choruses of *P. pustulosus*.

Video Analyses

To determine the number of buccal pumps and the amount of time spent pumping to reach full inflation, we quantified buccal pumps and assessed when the frog was fully inflated. We used frame-by-frame video analysis for both measures. Using Pinnacle Studio DV (ver. 8, Pinnacle Systems), we digitized each inflation and calling sequence. We then projected the digitized

video onto a viewing screen and cataloged the sequence of buccal pumps and calls using the frame-by-frame counter in Pinnacle Studio. Individual buccal pumps were indicated by movement in the gular region and nearly simultaneous increases in abdomen size. Buccal oscillations, which involve cycling air into and out of the buccal cavity through the nares while the larynx is closed (Gans 1973), also result in movement of the gular region, but these movements are smaller and have no concomitant change in abdomen size. Because buccal pumps can be difficult to distinguish from buccal oscillations, we were able to confidently count buccal pumps for only the 10 males with the highest-quality videos. For these 10 males, we also determined the number of pumps before the first call of bout 1 and the number preceding the first five calls of each call bout.

We also measured the duration of buccal pumps. We could confidently measure pump duration on only some video recordings because each pump occurs very quickly (ca. one-sixth of a second). We therefore recorded duration of each of 10 pumps from each of five individuals and obtained an average value for pump duration. Calculations of the time spent pumping (e.g., time to full inflation) were determined by multiplying the number of pumps by the average pump duration; pauses in buccal pumping were not included.

We measured the vocal sacs of all 22 males by hand directly on a 74-cm flat-screen monitor (Sony Trinitron KV-29FS12) to assess vocal sac distension, which we used as a proxy for lung inflation in the first call bout. Measurements were taken from the video frame that showed the greatest vocal sac distension for each call. Frame-by-frame analysis was conducted with the original analog tapes in a Sony video cassette recorder (EVO-0800A). We measured the longest dimension of the vocal sac and then the length of the axis normal to the primary axis to the nearest millimeter. In all cases, the frogs remained in the same position relative to the camera throughout each call bout. We needed only to make comparisons of vocal sac size within each individual to determine when the vocal sac was fully distended. We did not make comparisons between individuals; thus, size measurements were not standardized among video recordings. We considered the vocal sac to be completely distended, and therefore the frog to be fully inflated, at the first of three sequential calls with equivalent values of vocal sac size. We considered sizes to be equivalent if vocal sac dimensions changed by less than a threshold percentage that represents the typical variation in vocal sac size between calls given by a fully inflated male. This threshold was determined by measuring the maximum percentage change in each vocal sac axis between subsequent calls from 10 calls in each of two individuals. The measured calls were from the middles of lengthy call bouts, well beyond the initial period for which vocal sac size is still increasing with each call. By combining the vocal sac measurements with the 10 chronologies of buccal pumping described above, we determined the number of calls with partial

distension of the vocal sac and the number of buccal pumps required for complete inflation.

Acoustic Analyses

We analyzed acoustic differences among the calls of the first two call bouts because these differences may be correlated with the method of lung inflation. In the first bout, the male has to buccal pump, but in the second bout, the male is already inflated. For the acoustic analyses, we digitized calls using Cool Edit 2000 (Syntrillium Software) at a sample rate of 22 kHz and 16 bits/sample. We measured the intercall intervals (s) between the first six calls of each bout (i.e., the first five intercall intervals) in Cool Edit for the 10 individuals for which we also cataloged buccal pumps. The first calls in a call bout rarely had chucks (the secondary call component that can be facultatively added to the call), but as the bout advanced, males were more likely to add them. For the 12 individuals that produced chucks, we determined the call number at which males produced the first call with chucks and compared these values for the first and second call bouts using a paired t-test. In all other acoustic analyses, we did not analyze the chuck because it was present in only a small proportion of the calls recorded. The chuck was digitally excised from those calls in which it was present, and further analyses were done on only the whine. The chuck occurs after the whine, so excising it does not substantially affect measurements of the whine. We used SIGNAL (Engineering Design) to measure the following call characteristics: relative peak amplitude (V), call duration (ms), rise time (ms), fall time (ms), dominant frequency (Hz), initial frequency (Hz), final frequency (Hz), and frequency shape, calculated as the time from the whine's onset to its midfrequency as a proportion of the duration of the call. We included only individuals with a complete data set for the first five calls of each call bout (i.e., only males that produced at least five calls in each call bout and for which all calls could be distinguished from the background chorus and analyzed) for the analysis of call characteristics and a complete set for the first six calls of each bout for the intercall intervals (i.e., the first five intercall intervals). As a result, we analyzed relative amplitude and the remaining call traits in 11 males and intercall interval in seven males. We compared call duration, dominant frequency, initial frequency, final frequency, the length of the intercall intervals, and the number of buccal pumps between bouts 1 and 2 using a repeated-measures ANOVA for each call trait. In each analysis the subject was the male, and we used two within-subject factors: call bout (first or second bout) and call number or intercall interval (from 1 to 5). Because call duration increased over a call bout, we calculated a Pearson correlation to determine if this change was due to an increase in fall time, initial frequency, and/or final frequency. We also performed paired t-tests to compare duration, relative amplitude, and initial frequency between call bouts for the first call. We consider the effect of

temperature on the call traits to be trivial because water temperatures at calling sites varied only by a maximum of 2°C. All statistical analyses were performed using SYSTAT, version 10. Two-tailed probabilities are given, and the fiducial level of significance, α, was set at 0.05.

We also examined the change in relative peak amplitude between call bouts of the same individual. The first call of bout 2 was usually of greater amplitude than the first few calls of bout 1. Therefore, we determined which call in bout 1 was the first with a relative amplitude equivalent to or greater than the amplitude of the first call of bout 2. Here, we define equivalent amplitudes as differing by an amount equal to or less than the maximum percentage change in peak amplitude between subsequent calls from the middle of an extended call bout. These measurements were taken from two males well after the initial calls of a bout and from regions for which vocal sac dimensions and peak amplitude were stable. As a result, a call of equivalent amplitude to the first call of bout 2 can be of slightly less amplitude if it differs by less than the maximum measured level of variation. For any individuals for which we also obtained chronological sequences of pumping and calling, we determined the number of buccal pumps and the amount of time spent pumping in all of bout 1 before a call was produced with a relative amplitude equivalent to or greater than that of the first call of bout 2.

Results

A typical inflation and calling sequence begins when an uninflated male starts buccal pumping. Before full inflation of the lungs, the male emits his first call that partially distends the vocal sac and that is followed by additional buccal pumping. Subsequent calls distend the vocal sac by greater amounts (Figs. 1a, 2A) and are also followed by buccal pumps but with fewer pumps following each sequential call until the lungs are fully inflated. Once fully inflated, a male's vocal sac distends to a nearly identical size with each call (Fig. 2A), and males usually either do not pump between calls or pump only once. In preparation for the second bout, males may not pump at all or may pump only a few times before calling. The first few calls of the second bout also do not result in full inflation of the vocal sac, even though there is enough pulmonary air for it (Figs. 1b, 2A). As with the later calls in the first bout, no pumps to only a few pumps occur between all calls in the second bout (Table 1). Several call characteristics also increase through the initial calls of each bout, with maximum values reached more quickly in the second bout.

We defined call bout 1 to begin with the first five calls produced, regardless of the length of their intercall intervals, and to end when the male stopped calling for >10 s, as long as he had called at least five times. Three of the 22 males had an intercall interval >10 s in bout 1 before they had called at least five times. All three of the intervals >10 s were between calls
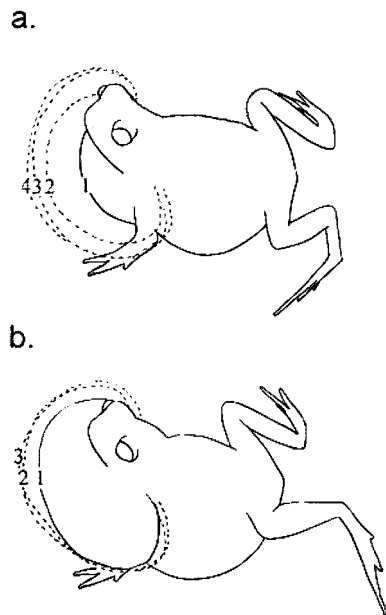
a.

b.

Figure 1. Illustration of the maximum vocal sac inflation reached when producing the first calls during the first (*a*) and second (*b*) call bouts. The extent of vocal sac distension for the first call of each bout is indicated by a solid line, and vocal sac distensions for subsequent calls are indicated by dashed lines.

1 and 2. Two individuals had intercall intervals of approximately 11 s, and the third individual paused for 21 s. These individuals showed typical patterns of buccal pumping, vocal sac distension, and changes in call duration and were included in analyses as long as they met analysis-specific criteria for inclusion. Therefore, we included all three in the vocal sac measurements, two in the relative amplitude analysis, and one in the call trait and call complexity analyses.

Video Analyses

We combined results from the chronologies of buccal pumping and calling, the vocal sac measurements, and the estimate of the duration of a buccal pump to determine the time necessary for a male to fully inflate its lungs via buccal pumping. For establishing our equivalence rule for vocal sac sizes, we determined that the maximum variation in vocal sac axes between subsequent calls never exceeded 3% for the long axis and 5.7% for the short axis. Using this measure, male *Physalaemus pustulosus* required 26–51 buccal pumps (mean = 34.7) to fully inflate their lungs (Table 1). Because one buccal pump lasted 0.168 ± 0.024 s (mean ± standard deviation), full inflation of the lungs required 4.4–8.6 s of continuous buccal pumping (Table 1).

Additional results from the chronologies and vocal sac measurements are summarized in Table 1. Males emitted the first
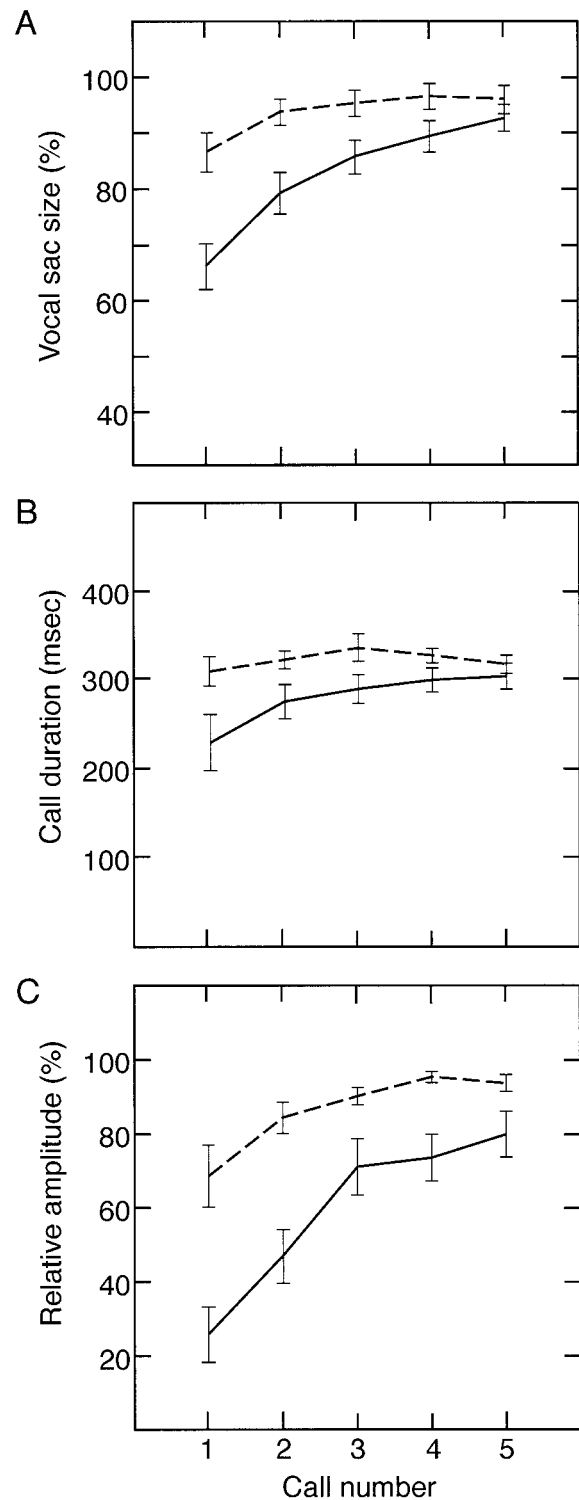

Figure 2. Changes in vocal sac size (*A*) and call properties (*B*, *C*) during the initial calls of a call bout. The percentage of maximum vocal sac size is relative to the highest value recorded for the primary/longer axis. Solid lines indicate call bout 1; dashed lines indicate call bout 2. The mean and standard error are shown.

Table 1: Results of video analyses of calling by *Physalaemus pustulosus*

Individual	Number of Pumps before First Call	Number of Pumps to Full Inflation	Time to Full Inflation (s)	Number of Pumps in First Five Calls of Bout 1	Number of Pumps in First Five Calls of Bout 2[a]	Maximum Number of Pumps in a Row	Number of Calls Lacking Full Vocal Sac Inflation in Bout 1	Number of Calls Lacking Full Vocal Sac Inflation in Bout 2
1	17	35	5.88	36	1	12	3	1
2	29	40	6.72	41	2	3	3	0
3	43	51	8.57	56	0[b]	7	2	≥2
4	22	36	6.05	36	5	8	4	2
5	8	28	4.70	24	1	8	≥6	3
6	20	39	6.55	35	1	3	≥6	2
7	13	27	4.54	27	2	5	4	1
8	3	26	4.37	24	2	7	5	3
9	19	27	4.54	27	4	6	4	1
10	24	38	6.38	41	0	3	3	4
Mean	19.8	34.7	5.83	34.7	1.8	6.2	4	1.9
SD	11.2	7.9	1.33	9.91	1.62	2.86	1.3	1.2

Note. The vocal sac measurements of individuals 5 and 6 had not stabilized by call 6, the last call of their first call bouts, suggesting that full inflation either was not yet reached or was reached in call 6. The mean and standard deviation for the number of calls lacking full vocal sac inflation in bout 1 were calculated assuming that full inflation was reached in call 6 for these two individuals.

[a] No buccal pumps occurred immediately before the first call of bout 2 for these 10 individuals.

[b] Only two calls were produced in this individual's second bout.

call after 3–43 buccal pumps (mean = 19.8), although the first calls of the two individuals with the fewest number of initial pumps, individuals 5 and 8, were of low amplitude and were difficult to detect. For the initial calls of each bout, the vocal sac was larger in bout 2 than in bout 1 (Figs. 1, 2A). Similarly, full distension of the vocal sac was generally not achieved until the fifth call of bout 1 or the third call of bout 2 (Table 1; Fig. 2A). Once inflated, few buccal pumps occurred between calls. For example, males pumped 24–56 times during the first five calls of bout 1, which includes the inflation sequence, but only 0–6 times during the first five calls of bout 2 (Table 1). Interestingly, during the initial calls of bout 2, the vocal sac was only partially distended, even though there was additional pulmonary air available for calling (Fig. 1b). This observation suggests that other factors in addition to lung inflation might limit vocal sac inflation at the beginning of a call bout. One possibility is that the initial calls are a warming-up phase for the muscles and other structures involved in call production; this phase might be particularly important for *P. pustulosus* and other species whose calls result from relatively large transfers of air.

Acoustic Analyses

We conducted acoustic analyses to determine whether call characteristics differed between call bouts. Any differences correlate with the difference in inflation method because bout 1 requires buccal pumping before calling but bout 2 does not. We examined 204 calls from 17 individuals, although not all individuals were appropriate for use in each of the three categories of analysis—call complexity (occurrence of chucks; $n = 12$), call traits (e.g. duration, dominant frequency, etc.; $n = 11$), and relative peak amplitude ($n = 11$). The first and second call bouts differed in several aspects. Males produced chucks earlier during the second call bout than during the first (first bout, 2.83 ± 0.52 calls; second bout, 1.92 ± 0.31 calls; $t = 2.722$, df = 11, $P = 0.02$). Calls in bout 1 were significantly shorter ($P < 0.001$) and of lower initial frequency ($P = 0.006$) than the calls in bout 2 (i.e., an effect of call bout in the repeated-measures ANOVA; Table 2). In addition, the first call of bout 1 was shorter and had a lower amplitude and initial frequency than the first call of bout 2 (paired *t*-test: duration, $t = -3.55$, df = 10, $P = 0.005$; amplitude, $t = -6.749$, df = 10, $P < 0.001$; initial frequency, $t = -2.682$, df = 10, $P = 0.023$; Fig. 2B, 2C; Fig. 3A). As the males produced more calls in bout 1, the calls increased in duration and peak amplitude, and the sweep covered a broader frequency range as a result of an increase in the whine's initial frequency and a decrease in its final frequency (Table 2; Fig. 3). The increase in duration was a result of an increase in fall time (Pearson correlation: $r = 0.841$, $P < 0.001$, $n = 154$), while the rise time remained roughly constant. Call duration increased with both bout number

and call number (Table 2), so that the duration of the fifth call of bout 1 was similar to that of the first call of bout 2 ($t = -1.135$, df = 10, $P = 0.283$; Fig. 2B). Increases in call duration over the call bout, which were more pronounced in bout 1 (Fig. 2B), were related to the increase in the frequencies covered by the whine (Table 2). As duration of the whine increased, its initial frequency increased (Pearson correlation: $r = 0.208$, $P = 0.005$, $n = 179$), while its final frequency decreased (Pearson correlation: $r = -0.504$, $P < 0.001$, $n = 179$). Although the range of frequencies increased in longer calls, there was no effect of call bout number on the dominant frequency of the calls (Table 2). The whine is a descending frequency sweep, and the shape of this sweep (i.e., the curve in a graph of frequency vs. time) differed in the first call of each of the first and second call bouts (Fig. 3C). The shape of the whine relative to call duration, however, did not change after the first call of each call bout.

We measured relative peak amplitude for 11 individuals, including six individuals for whom we also measured vocal sac dimensions, and documented chronologies of buccal pumping and calling. Relative peak amplitude of each call increased during the first few calls of a call bout (Fig. 2C). The first call of bout 1 was of low amplitude. Its relative amplitude averaged 30.8% of the maximum peak amplitude reached by a male and 39.5% of the peak amplitude of the first call of bout 2. (This variable was measured in only eight individuals because the amplitude of the first call of each of three individuals was too low to detect over the background.) Additionally, more calls were nearly always required in bout 1 to reach an equivalent or larger peak amplitude than that of the first call of bout 2 (Fig. 2C). We found that peak amplitudes never varied by more than 9% between calls once acoustic characteristics stabilized after the initial calls. Using this value as our amplitude equivalence measure, we determined that an average of 3.67 calls were required before the relative peak amplitude of a call in bout 1 was equivalent to or greater than that of the first call of bout 2. In order to generate this call, a male had to buccal pump 24–38 times, which required 4.0–6.4 s of pumping.

Because buccal pumping is time consuming, we expect a correlation between the number of buccal pumps and the length of the intercall interval. Although we did find such a correlation (Pearson correlation: $r = 0.323$, $P < 0.001$, $n = 119$), the number of buccal pumps explains only a small amount of the variation in intercall intervals. Most buccal pumps, however, occurred before the first call of bout 1 (Table 1), biasing the analysis toward portions of the call sequence where fewer buccal pumps were produced. The correlation result (i.e., the low r value) and the observation that intercall intervals were longer than is required for zero to a few buccal pumps (as in the second bout; Table 2) indicate that multiple factors, including buccal pumping, probably influence intercall interval. Similarly, we did not find significant differences in intercall intervals between calls or bouts, even though the number of

Table 2: Call characters measured in the first and second call bouts

	Duration (ms)	Dominant Frequency (Hz)	Initial Frequency (Hz)	Final Frequency (Hz)	Intercall Interval (s)	Number of Pumps
Call bout 1:[a]						
Call 1	209.42 ± 24.59	681.48 ± 28.11	907.90 ± 3.98	528.43 ± 38.51	3.04 ± .64	6.71 ± 1.55
Call 2	276.38 ± 14.35	618.71 ± 18.69	931.74 ± 8.92	481.40 ± 15.05	2.04 ± .34	3.00 ± .38
Call 3	285.04 ± 14.64	605.22 ± 13.88	939.43 ± 11.40	454.02 ± 15.77	2.42 ± .53	3.28 ± .36
Call 4	298.02 ± 14.85	595.10 ± 11.94	935.64 ± 10.23	446.35 ± 15.66	2.82 ± .79	1.86 ± .34
Call 5	300.93 ± 16.78	601.85 ± 13.52	919.96 ± 14.56	465.86 ± 7.82	2.00 ± .39	1.57 ± .30
Call bout 2:[a]						
Call 1	305.55 ± 17.25	658.11 ± 18.99	947.47 ± 12.98	496.75 ± 18.77	2.12 ± .46	.43 ± .30
Call 2	317.49 ± 10.80	624.22 ± 10.76	959.28 ± 13.09	454.12 ± 6.80	2.73 ± .52	.86 ± .34
Call 3	335.37 ± 19.76	607.82 ± 12.80	970.96 ± 17.78	461.94 ± 8.32	1.58 ± .22	.57 ± .30
Call 4	321.62 ± 8.89	604.26 ± 13.83	978.69 ± 20.23	461.96 ± 38.51	2.60 ± .37	.57 ± .20
Call 5	313.08 ± 10.57	603.30 ± 11.96	986.76 ± 18.74	461.85 ± 6.02	1.66 ± .22	.71 ± .18
ANOVA results:						
Call bout:						
SS	54,856.44	59.071	47,815.768	1,711.680	1.856	123.56
F	25.586	.015	11.947	.473	.900	7.34
df	1, 10	1, 9	1, 10	1, 10	1, 6	1, 6
P	**<.001**	.904	**.006**	.507	.379	**.001**
CD	.719	.002	.544	.045	.130	.877
Call number:						
SS	43,955.01	74,505.541	12,832.754	49,370.601	7.837	53.51
F	7.243	10.034	2.723	4.408	1.269	6.285
df	4, 40	4, 36	4, 40	4, 40	4, 24	4, 24
P	**<.001**	**<.001**	**.043**	**.005**	.310	**.001**
CD	.420	.527	.214	.306	.174	.511
Number × bout:						
SS	23,071.041	4,168.216	5,171.185	9,674.917	5.838	64.94
F	3.715	1.147	1.016	1.403	1.095	61.66
df	4, 40	4, 36	4, 40	4, 40	4, 24	4, 24
P	**.012**	.350	.411	.251	.381	**.001**
CD	.271	.113	.092	.123	.154	.513

Note. Values for each character are presented as mean ± SE for the first five calls of each bout. Calls from 11 individuals were analyzed for duration and dominant, initial, and final frequency. Intercall interval and the number of buccal pumps were analyzed for seven individuals for which we also cataloged buccal pumps; three of the 10 individuals for which we cataloged buccal pumps, including the one individual with an intercall interval of >10 s, were excluded because they did not have five intercall intervals in bouts 1 and 2. Results from the repeated-measures ANOVA are shown for each call trait, with significant P values in boldface. The factors call bout, call number, and number × bout address the following questions, respectively: (1) Are there significant differences between the two call bouts, regardless of call number? (2) Are there significant differences between different-numbered calls (e.g., first, second, etc.), regardless of call bout? (3) Are there significant differences between call bouts for calls of the same number? CD = Coefficient of determination.

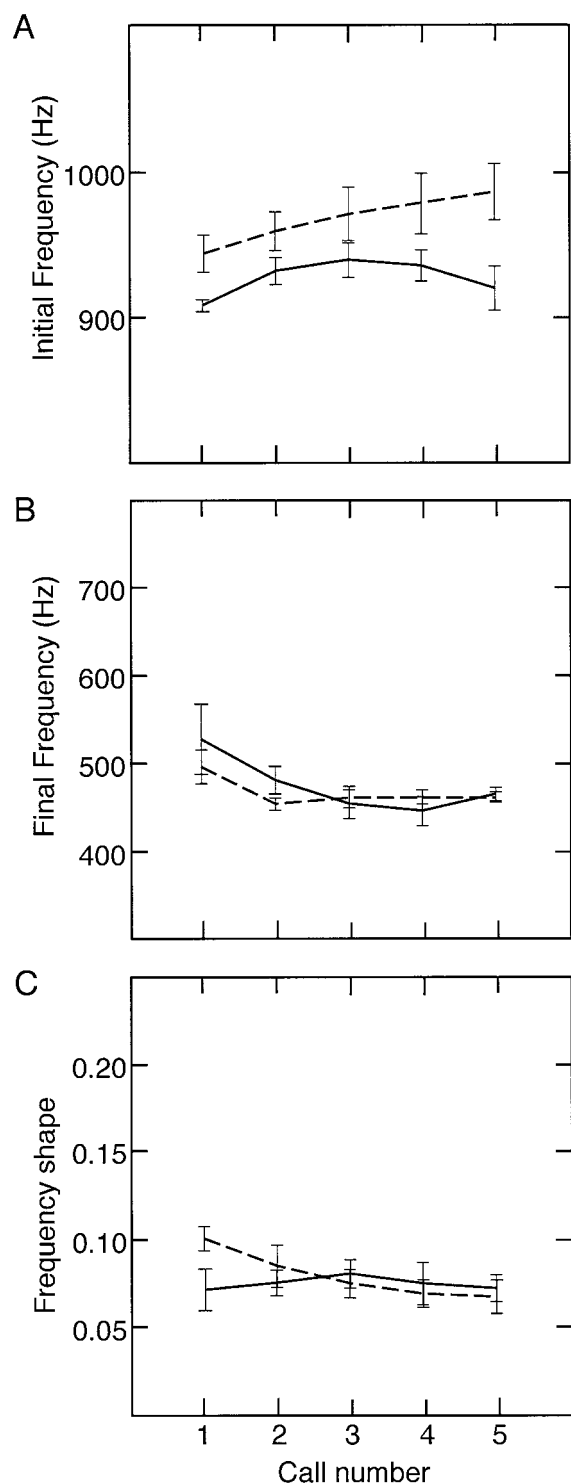[a] The length of the intercall interval and the number of buccal pumps were assessed for each intercall interval.

Figure 3. Changes in initial frequency (*A*), final frequency (*B*), and shape (*C*) of the whine during the initial calls of a call bout. Time to the midfrequency of the whine as a proportion of call duration was measured as an indicator of shape. Solid lines indicate call bout 1; dashed lines indicate call bout 2. The mean and standard error are shown.

buccal pumps decreased throughout the initial calls of bout 1 and between bouts 1 and 2 (Table 2).

Discussion

Most adult male frogs have vocal sacs, and as the name implies, these have something to do with vocalization. A number of functions of vocal sacs have been suggested, and some of these have been documented. Our results show that at least in *Physalaemus pustulosus*, the vocal sac enables increased calling rates, a trait that is favored by sexual selection in a number of taxa (reviewed in Ryan and Keddy-Hector 1992; Andersson 1994; Gerhardt 1994), including túngara frogs (see below).

Without a vocal sac, a male has to inflate his lungs using the buccal pump before every call, but with a vocal sac, the air expelled during a call is captured and rapidly returned to the lungs. The question, then, is how long it would take for a male to fully inflate via buccal pumping. In this study, the male requiring the fewest buccal pumps to reach full inflation still pumped for 4.4 s, and on average, males required 5.8 s of pumping to fully inflate (Table 1). Our estimated minimum intercall interval using buccal pumping, however, is an extremely conservative estimate. This interval requires that a male pumps continuously at least 26 times (i.e., for 4.4 s). However, we never observed a male to pump more than 12 times in rapid succession before pausing (i.e., 2.01 s of continuous pumping; Table 1). Buccal pumping requires the contraction of multiple muscles (e.g., buccal floor, laryngeal, and submandibular muscles; de Jongh and Gans 1969; Gans 1973), and resting these muscles may be necessary after repeated buccal pumps. As a result, a true minimum intercall interval would include several pauses, thereby increasing the time to reach full inflation without a vocal sac. Moreover, our estimate is concerned with only a single inflation series and not the average intercall interval that could be sustained across multiple calls. Nevertheless, our estimated intercall interval using buccal pumping is still much greater than intercall intervals observed in males using the vocal sac. Bosch et al. (2000) reported that the average intercall interval in this population is 1.992 ± 0.397 s. Sustained call rates in the most actively calling males yield slightly lower intercall intervals, at 1.7 s, or approximately one call every 2 s because calls average about 300 ms (Ryan 1985*b*; Ryan and Rand 2003). As a result, the minimum intercall interval using buccal pumping (4.4 s) is at least 2.5 times as long as the average interval observed in sustained calling by active males (1.7 s). Our results confirm the suggestion by Gridi-Papp (2003) that "inflation of the lungs by buccal pumping is probably too slow to support the repetition rate of natural advertisement calls" (p. 145).

Can females differentiate between males with intercall intervals based on inflation via buccal pumping or via the vocal sac? Sexual selection can favor increased temporal efficiency of calling only if the increase is salient to females. Bosch et al. (2000) conducted phonotaxis tests of female preferences for

males calling at different intercall intervals. The test stimuli differed by 1–4 standard deviations (1 SD = 0.4 s). In all tests, female *P. pustulosus* chose the lower intercall intervals more times and significantly preferred ($P < 0.05$) the lower interval when the test stimuli differed by more than 3 SD (Table 3). Inflation with the vocal sac requires at least 2.7 s less than inflation with the buccal pump. This is equivalent to 6.75 SD. These results demonstrate that the difference in intercall intervals between the two methods of lung inflation is salient to females and that females prefer the lower intercall interval that can be produced only by males with a vocal sac. Admittedly, our approach assumes that species lacking vocal sacs would not evolve other means to increase call rate, such as faster buccal pumping or increased volume of the buccal cavity.

The hypothesis that the vocal sac reduces intercall intervals predicts that species that produce advertisement calls but lack vocal sacs should have long intercall intervals. Unfortunately, to our knowledge, intercall intervals have been reported for only one species that produces advertisement calls without a vocal sac. Hedges (1987) documented intercall intervals of 47 and 52 s in *Hyla marianae.* This result is consistent with the prediction of longer intercall intervals in species that must buccal pump before each call. Some species with vocal sacs, however, also have long intercall intervals. Additional call data, especially from clades with some members who have and some who lack vocal sacs, would be useful for further interpreting this and other functions of the vocal sac.

Several acoustic and nonacoustic hypotheses have been proposed as functions of the anuran vocal sac. These hypotheses are not mutually exclusive and may vary among taxa. Although the vocal sac may serve multiple current functions, it is not clear whether all, some, or none of the functions described here are reasons that vocal sacs evolved in ancestral anurans. Vocal sacs may also have several costs, including attracting predators.

Acoustic hypotheses include that the vocal sac is a sound resonator and/or radiator. Capranica and Moffat (1983) and Rand and Dudley (1993) rejected the hypothesis that the vocal sac is a cavity resonator. Resonance in the tissues of the vocal sac wall, however, occurs in several species (Purgue 1995). Sound radiators influence the efficiency with which different frequencies are transmitted to the external environment. Several authors have considered the vocal sac to be a radiator (Watkins et al. 1970; Martin and Gans 1972), but to our knowledge, this has not been demonstrated empirically. Another possibility, as discussed above, is that the thin walls of the vocal sac decrease the acoustic impedance between the inside and outside of the frog relative to the thicker body wall.

The vocal sac may also have nonacoustic functions. For example, movement of the vocal sac and the overlying gular skin can be a visual signal. In some species, including *P. pustulosus*, vocal sac movements during sound production are a visual component of multimodal signals important in mate selection and intrasexual agonistic behaviors (Lüddecke 1999; Narins et al. 2003; Rosenthal et al. 2004). The vocal sac can also function as a visual signal in the absence of sound production (reviewed in Hödl and Amézquita 2001). Additionally, the vocal sac may have important physiological roles, including improving the energetic and temporal efficiency of calling, as described previously.

Our results demonstrate that when a fully inflated male calls, the vocal sac can reinflate the lungs with no or very few additional buccal pumps. As noted by several authors (Ryan 1985*b*; Jaramillo et al. 1997; Gridi-Papp 2003), the elastic properties of the vocal sac aid in lung reinflation without spending mechanical energy. Lung reinflation may require some mechanical energy for muscle contractions (the interhyoideus muscle is the most likely candidate), but this has not been demonstrated. Inflation via buccal pumping, however, requires the repeated contraction of laryngeal muscles and multiple submandibular muscles, including the interhyoideus (de Jongh and Gans 1969). Given the large number of repeated muscle contractions needed to fully inflate the lungs via pumping, the vocal sac should be more energy efficient than buccal pumping for reinflating the lungs after each call. Future studies examining energy consumption during buccal pumping would be useful to quantify the vocal sac's impact on energy conservation.

Table 3: Results of female choice tests for intercall intervals conducted by Bosch et al. (2000)

Experiment	Difference between Stimuli (Number of SD)	Test Stimuli (Intercall Intervals; s)	Number of Female Choices	P for Number of Female Choices (Binomial, One-Tailed)
Mean − 1.5 SD vs. mean − .5 SD	1	1.4 vs. 1.8	12 : 8	.252
Mean − 1.5 SD vs. mean + .5 SD	2	1.4 vs. 2.2	14 : 6	.058
Mean − 1.5 SD vs. mean + 1.5 SD	3	1.4 vs. 2.6	16 : 4	.006
Mean − 1.5 SD vs. mean + 2.5 SD	4	1.4 vs. 3.0	17 : 3	.001
Hypothetical test; our results	*6.75*	*1.7 vs. 4.4*		

Note. The last row, in italics, presents the hypothetical stimuli suggested by our analysis. Note that as the difference between the test stimuli increases, more females choose the call with the lower interval. Population values measured by Bosch et al. (2000) are 1.992 ± 0.397 s (mean ± SD); sample size = 32 individuals.

Within *P. pustulosus*, our research, in combination with previous studies, suggests that the vocal sac has at least three functions. It serves as a visual signal (Rosenthal et al. 2004) and serves to increase the speed of lung reinflation (this study). A comparison of the necessary muscle contractions also suggests that lung reinflation via the vocal sac is more energy efficient than buccal pumping. The vocal sac, however, is not a resonating cavity in *P. pustulosus* (Rand and Dudley 1993), although tissues in the vocal sac may resonate, as they do in several other species (Purgue 1995). The vocal sac also probably functions as a sound radiator and to decrease the acoustic impedance as sound leaves the body. This summary provides a picture of vocal sac function in *P. pustulosus*, but in other species, specific functions will probably vary. For example, vocal sac movements may not be intraspecific visual signals in some species. Other functions may vary with the pattern of vocal sac inflation during call production. Although the vocal sac of *P. pustulosus* completely deflates between calls, other inflation patterns exist. For example, many toads (*Bufo*) partially inflate their vocal sacs before calling, and the sacs may remain this way between calls (G. B. Pauly, personal observation). This observation is likely to be related to the importance of the vocal sac in shuttling air in and out of the lungs while calling, which is necessary for producing the longer pulsed calls of most *Bufo* (Martin 1972). Other frogs, such as *Pseudacris regilla*, also partially inflate their vocal sacs before calling, although they do not appear to shuttle air in and out of the lungs during their relatively short calls (G. B. Pauly, personal observation).

Our results also suggest that the method of lung inflation can influence the volume of air in the lungs, which, in turn, influences acoustic properties of the call. If we assume that *P. pustulosus* without a vocal sac would emit calls similar to the first call of the first bout, then the resulting calls would be of shorter duration and lower amplitude and frequency range than calls produced with lung reinflation via a vocal sac. Females generally prefer calls of greater total energy, and this can manifest itself as preferences for higher call rates, longer call durations, and/or greater call amplitudes (Ryan and Keddy-Hector 1992; Andersson 1994). With a vocal sac, more calls can be emitted in a given period of time, and these calls will be longer and more intense. Because the vocal sac stretches the gular skin, sound is also radiated more effectively (i.e., at a greater amplitude) than if the sound were transmitted across a thicker, uninflated region of skin (Martin 1971). Therefore, in species that lack vocal sacs but produce advertisement calls, the calls are likely to be shorter and of lower amplitude than the calls of related taxa that have vocal sacs.

The differences in calls produced with lung inflation via buccal pumping and via the vocal sac may also be behaviorally mediated. Longer and louder calls, like those produced after lung reinflation with the vocal sac, could be produced with buccal pumping if a male increases the number of pumps, and therefore the volume of pulmonary air, used for each call. This results in a trade-off between fewer higher-quality calls and a larger number of poorer-quality calls. Therefore, we propose that the vocal sac may also function to produce more calls that are of increased attractiveness to females and/or are transmitted through the environment more effectively. This suggestion combines aspects of the sound radiation, energy efficiency, and temporal efficiency hypotheses for vocal sac function.

Acknowledgments

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