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A laryngeal fibrous mass impacts the acoustics and attractiveness of a multicomponent call in túngara frogs (*Physalaemus pustulosus*)

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ABSTRACT

The evolution of elaborate signals can emerge from changes in anatomical signaling structures. In the male túngara frog, a simple advertisement call ('whine') can be ornamented facultatively with a suffix ('chuck') to produce a more attractive complex call, or 'whine-chuck'. A fibrous mass (FM1) supported by the vocal cords plays a role in chuck production. Here, we examine the effects of FM1 ablation on a large set of spectral and temporal features of both the whine and chuck and we test the hypothesis that FM1 ablation reduces call attractiveness to females. Both call components were impacted by FM1 ablation, but especially the suffix. The proportion of energy in the suffix's odd compared to even harmonics diminished markedly as did the relative amplitude, effectively eliminating the chuck percept. FM1 ablation also reduced the whine's frequency and its rate of frequency modulation. Moreover, post-surgery chucks no longer enhanced the attractiveness of the simple call and whines also appeared to diminish in attractiveness. Together, our results demonstrate that FM1 plays a role in the production of both call components in a way that stimulates the female auditory system and that the growth of FM1 had potentially positive sexual selection implications for the pre-existing simple call.

Introduction

Female preferences can drive the evolution of elaborate male secondary sexual characteristics (Darwin 1871; Andersson 1994). In many animals, these exaggerated traits are behavioural, can be facultatively expressed and are underpinned by a complex suite of anatomical adaptations. For example, the elaborate and multimodal courtship displays of male neotropical manakins (*Pipridae*) are made possible by specialized feather and bone morphologies, hypertrophied wing muscle fibres and a suite of neuromuscular adaptations (reviewed in Fusani et al. 2014). At the other end of the continuum, a discrete anatomical adaptation can underlie exaggerated sexual traits. For example, the acoustic complexity

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of advertisement songs shared among hundreds of species of oscine passerines illustrates how elaborate sexual signals ('two-voiced' song) can be underpinned in part by a discrete anatomical trait – a bifurcated syrinx (reviewed in Zeigler & Marler 2008).

In contrast to oscines, the vocalizations of anurans (frogs and toads) are typically less elaborate, due in part to a simpler laryngeal morphology (Gerhardt & Huber 2002; but see Feng et al. 2002). However, a few members of the *Physalaemus pustulosus* species group – a clade of neotropical leptodactylids – represent a rare exception. Males can facultatively ornament the terminal portion of the simple advertisement call with harmonically complex appendages (Rand & Ryan 1981; Ryan 1985). Because of this unique feature, a volume of research has explored the proximate and ultimate causes for its evolution (Ryan 2011). Among other findings, this research has demonstrated that strong inter-sexual selection has favoured complex vocalizations, and more recently, shown that a discrete anatomical mechanism – a fibrous mass supported by the vocal cords – is responsible for producing it (Gridi-Papp et al. 2006).

Túngara frogs – the most studied species in this group – are small anurans (ca. 30-mm snout-vent length) distributed throughout much of Mesoamerica (Weigt et al. 2005). During the breeding season (May–December), males vocally advertise to females using a species-typical call, known as the 'whine' or simple call (Ryan 1985). This call consists of a harmonic stack of frequency sweeps with the dominant frequency (which is also the fundamental for most of the call) beginning at approximately 1000 Hz and terminating at about 400 Hz and is about 300 ms in duration. Males can produce a 'complex call' by ornamenting the whine with one to seven suffixes known as 'chucks', producing what are known as 'whine-chuck' calls (Ryan 1985; Bernal et al. 2007). The chuck has a fundamental frequency that is half that of the whine and is also harmonically structured, but unlike the whine the higher frequency harmonics contain more energy than the lower frequencies with most of the energy above 1500 Hz. A single chuck is much shorter than the whine, typically 30 ms in duration. Bilateral fibrous masses (FM1) attached to the vocal cords can be facultatively engaged during the terminal phase of expiration of the whine and are key to the production of the chuck. Evidence for this laryngeal mechanism for chuck production comes from three sources: (1) an ontogenetic study in túngara frogs found a male-biased sexual dimorphism in the size of FM1 (Guerra et al. 2014); (2) a comparative study of species in this clade demonstrated that the presence and size of FM1 correlates with chuck production (Boul & Ryan 2004); and (3) surgical ablation of FM1 results in a significant reduction of the odd harmonics in the chuck (Gridi-Papp et al. 2006).

In nature, females use calls to localize an individual male amongst a chorus and then select a mate by making physical contact, after which the male mounts and clasps the female in a posture known as amplexus. Although the whine is necessary and sufficient to attract adult females to mate, females strongly prefer the complex call to the whine only (probability of 0.86; 4327 vs. 707 choices; exact binomial, $p < 10^{-6}$).

Here, we extend earlier studies in this species by examining the spectral and temporal effects of FM1 ablation on both the whine and the chuck. We then test the hypothesis that call changes resulting from FM1 ablation decrease call attractiveness to females and we explore which call characters are linked with changes in attractiveness.

Methods

Animals

Protocols for collecting and testing frogs were approved by the Institutional Animal Care and Use Committees of the University of Texas at Austin (06041701) and Autoridad Nacional del Ambiente approved scientific permits in the Republic of Panamá. We conducted all experiments during the breeding season in July 2006 and July 2007 at facilities for the Smithsonian Tropical Research Institute in Gamboa, Panamá (9° 07.0′ N, 79° 41.9′ W). Protocols for collecting and testing females followed those we have described previously (Ryan et al. 2003). Females were returned to their site of collection within 12 h. To prevent resampling, females were marked after testing with a unique toe-clip combination following the *Guidelines for the Use of Live Amphibians and Reptiles in Field Research* (Beaupre et al. 2004).

We used the pre- and post-manipulation recordings conducted as part of a previous study (for details see Gridi-Papp et al. 2006). Briefly, the complex calls of males were recorded at their breeding aggregations in the field. These field recordings served as the 'pre-manipulation' recordings. Males were then returned to the lab and randomly assigned to one of three treatment groups: (1) *experimental surgery*: surgical ablation of FM1 under anaesthesia; (2) *sham surgery* control: incision and anaesthesia but no FM1 ablation; and (3) *no surgery* control. After treatment, males were allowed to recover and then were recorded in the lab ('post-manipulation'). All recordings were filtered with a high-pass filter (cut-off frequency = 80 Hz; Butterworth, 4th order) prior to measurement.

Acoustic stimuli: measurement of calls

For the pre- and post-manipulation whine–chuck calls of each male (Figure 1), we measured the following eight temporal characters for both call components separately: (1) *Chuck lag* (s): the interval in seconds separating the peak amplitudes of the whine and the chuck; because some post-manipulation whines exhibited a large decay prior to the chuck, it was occasionally difficult to estimate whine duration and therefore this measurement facilitates interpreting whether a short duration whine merely has low energy during the final decay phase or if it actually terminates early and is thus followed by an early chuck; (2) *Duration Call* (ms): because the exact demarcation between whine and chuck can be arbitrary, we operationally defined the end of the whine as the minimum amplitude between the maximum amplitude of the whine and the maximum amplitude of the chuck; (3) *Duration rise* (ms): duration from call onset to maximum amplitude; (4) *Duration decay* (ms): duration from max amplitude to call offset; (5) *Shape rise*: duration of time from 10 to 50% of the peak amplitude during the rise divided by the duration of time from 10 to 90% of the call component's peak amplitude; (6) *Shape decay*: duration of time from 50 to 10% of the peak amplitude during the decay divided by the duration of time from 90 to 10% of the call component's peak amplitude; Shape rise and Shape decay yield the same value if the call is symmetrical; they are equal to 0.5 if the amplitude change is linear between 10 and 90%; they are < 0.5 if most of the amplitude change occurs near the end and they are > 0.5 if most of the amplitude change is concentrated near the peak; (7) *Energy rise/decay*: proportion of energy in the rise relative to the decay of the amplitude envelope; (8) *Relative amplitude Whine/Chuck*: for the complete complex call, we measured the relative peak amplitude of the
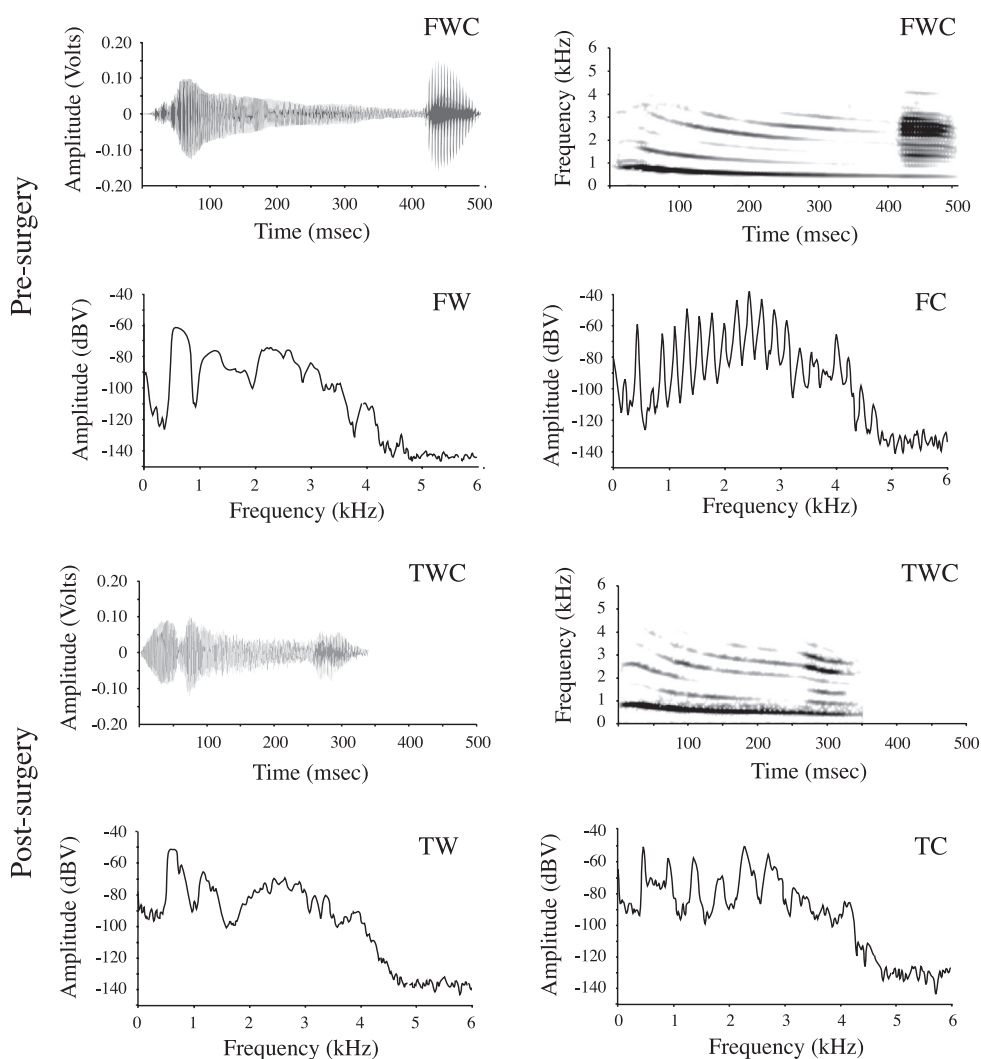
Figure 1. Representative oscillograms, spectrograms and power spectra (re 1 V) of advertisement calls from one experimental (FM1 ablation) male (#1701). FWC: pre-surgery whine–chuck; TWC: post-surgery whine–chuck; TW: post-surgery whine; TC: post-surgery chuck.

whine compared to the chuck, which is known to be important in the relative attractiveness of natural male calls (Baugh & Ryan 2011).

Likewise, we measured the following seven spectral characters: (1) *Dominant frequency* (Hz); (2) *Fundamental frequency* (Hz); (3) *Minimum frequency* (Hz): the lowest value that the dominant frequency assumes during the duration of the call component; (4) *Maximum frequency* (Hz): the highest value that the dominant frequency assumes during the duration of the call component; (5) *Energy harmonics even/odd*: proportion of energy in the even relative to the odd harmonics. Note that in order to facilitate comparison between the whine and the chuck for this measurement, we defined the actual fundamental frequency (ca. 800 Hz) of the whine as the second harmonic – i.e. so that the first harmonic would be aligned with the actual first harmonic of the chuck (ca. 400 Hz). (6) *Half FM*: the proportion

of the duration of the call character at which half of the frequency modulation is reached; in the whine this measure approximates the slope of the frequency sweep with positive change values indicating that the slope of the whine becomes more shallow; (7) *Dominant harmonic*: the harmonic of highest amplitude (chuck fundamental = 1; whine fundamental = 2; see note for (5) above).

Female mate choice tests

Mate choice tests were conducted inside a rectangular, sound-attenuating chamber (2.7 × 1.8 × 1.78 m, L × W × H; ETS Lindgren, Cedar Park, TX) equipped with acoustic foam to reduce reverberation and located inside a temperature-controlled (~27 °C) laboratory. Acoustic stimuli were output through the soundcard of a desktop (Dell Dimension 4600) using SIGNAL 4.0 (Engineering Design, Berkeley, CA; 20 kHz sampling rate, 8 bit depth), amplified with a Crown XLS 402 amplifier (Crown Audio USA, Elkhart, IN), and broadcast through one or two A/D/S L210 speakers (Directed Electronics, Vista, CA) located 2.6 m apart at opposite ends of the sound chamber at equal distances from the centre of the chamber. Stimulus SPLs were calibrated to 82 dB SPL (fast root-mean-square [RMS], C-weighted; re 20 µPa) using a GenRad, 1982 sound level meter (SLM; IET Labs Inc., Roslyn Heights, NY), with the microphone directed at a 90° angle from both speakers at a central release point at the centre of the chamber, equidistant from the two playback speakers. The subjects' behavioural responses were monitored using a wide-angle infrared camera (Fuhrman Diversified Inc., Seabrook, TX) mounted from the centre of the sound chamber ceiling and connected to a TV monitor located outside the chamber.

We haphazardly selected a pre- and post-surgery whine–chuck call from each of the eight experimental treatment males. All stimuli were matched for peak amplitude of the whine (1 V). The chuck from each call was excised digitally (SIGNAL 4.0) with care taken to avoid generating artificial transients. This allowed us to construct four categories of stimuli: (1) FW_{1-8}: field-recorded (i.e. pre-surgery) whine-only calls for males 1–8; (2) FWC_{1-8}: field-recorded whine–chuck calls; (3) TWC_{1-8}: treatment (i.e. post-surgery) whine–chuck calls; (4) $FWTC_{1-8}$: field-recorded whine with the post-surgery chuck from the same male (Figure 2).

We tested female preferences in response to four different two-choice discrimination tests: (a) FWC vs. TWC; (b) FWC vs. FWTC; (c) FW vs. FWTC; and (d) FW vs. TWC. Test (a) evaluated the full cost of FM1 ablation on complex call attractiveness. Test (b) assessed the extent to which the attractiveness of the chuck is affected, i.e. is a post-surgery chuck less attractive then an intact chuck. And test (c) further determined if a post-surgery chuck is able to merely enhance an intact whine. Finally, test (d) determined whether the full post-surgery call is perceived as similarly attractive as an intact whine. In each trial, the call of a given male was paired with his own alternative (e.g. Male #1 FW vs. Male #1 FWTC), thus controlling for all acoustic parameters except the intended one.

Each trial began by placing a single female frog under an acoustically transparent cone at the centre of the chamber (origin). The two stimuli were then broadcast antiphonally (180° out of phase) for 2 min at a rate of one stimulus per 2 s – simulating two actively calling males (Ryan 1985). Following this 2-min period, the cone was lifted remotely and subjects could move freely within the sound chamber. We scored a 'choice' if the subject entered a choice zone that extended 10 cm from the speaker in all directions without simply following the chamber wall in less than 15 min. A 'no choice' was recorded if the frog (a) remained
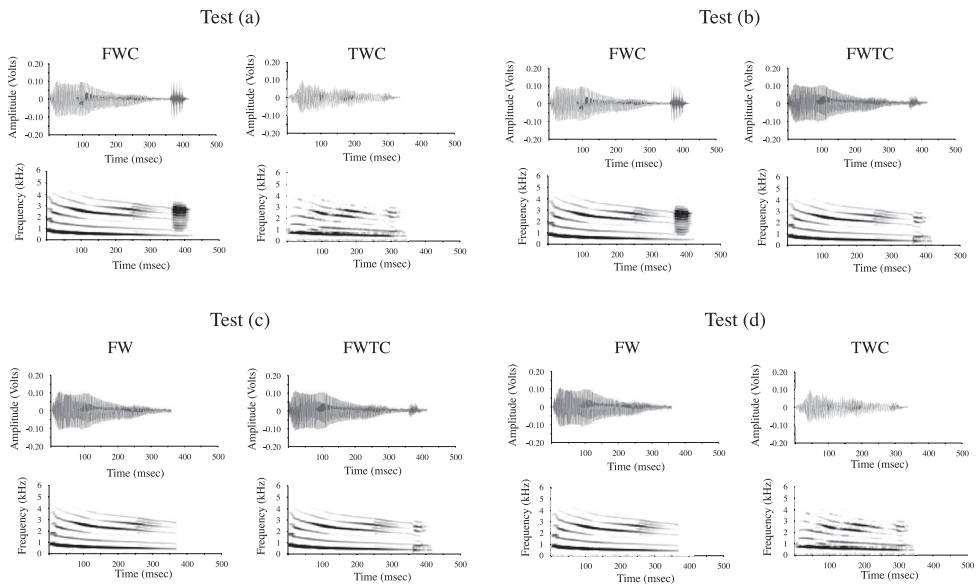
Figure 2. Oscillograms and spectrograms from one experimental (FM1 ablation) male (#1706) illustrating the two alternative stimulus pairs used in the four female preference test conditions. FWC: pre-surgery whine–chuck; TWC: post-surgery whine–chuck; TW: post-surgery whine; TC: post-surgery chuck.

motionless at the origin for 5 min after the cone was raised; (b) remained motionless for 2 min after exiting the origin; or (c) failed to make a choice within 15 min (Ryan & Rand 1993). We alternated the side of the chamber broadcasting each alternative stimulus to eliminate any potential side bias and alternated which stimulus in each pair was broadcast first to control for a potential leading caller preference (Greenfield & Rand 2000; Bosch & Márquez 2002). Additionally, we measured latency to choice (after raising the cone).

In order to achieve choices from 24 females in each test condition (a–d), we tested 27–31 females (three females tested for each of the 8 males' call sets), for a total of 96 choices from 50 frogs. Individual females were never tested more than once in the same test condition, but females did serve as subjects across multiple test conditions. In this species, there is no evidence of carry-over effects for repeated testing (Kime et al. 1998), and memory of stimuli decays to null expectations in less than 120 s (Akre & Ryan 2010), which is faster than the interval separating repeated trials.

Statistics

We calculated the mean and 95% confidence intervals for the acoustic measurements for each call component in both the pre- and post-manipulation recordings. Because we predicted that FM1 ablation would principally impact the chuck – by reducing or eliminating much of its energy, particularly in the odd harmonics – we used repeated measures ANOVAs to test the a priori hypothesis that FM1 ablation would increase the relative amplitude of the whine compared to the chuck and increase the proportion of energy in even compared to odd harmonics of the chuck (between subjects factor: treatment group; within subjects factors: pre- vs. post-manipulation; Bonferonni corrections were applied to

post hoc comparisons). For the female preference data, we performed binomial exact tests (two-tailed) for each of the four acoustic conditions.

Results

Effect of surgery on call structure

The post-surgery calls that we describe as 'complex' involved males attempting to produce the chuck. One of the diagnostic characters of the chuck – that it increases the amplitude at the end of the call – was present in post-surgery recordings (Table 1, Figure 1, S1). The absence of the chuck's odd harmonics, however, violated a second diagnostic character. To the human ear, suffixes recorded after FM1 ablation did not sound distinct from the whine (Table 1; Figure 1; see S1 for complete data-set).

Some call measurements appear to be influenced by the sham and no surgery-control treatments. For example, the amplitude of the chuck and duration of the whine both decreased after treatment in all three groups. This is likely due to the fact that all post-manipulation recordings were performed in the lab, while the pre-manipulation recordings were conducted in the field. Lab-housed animals are likely less motivated due to captivity stress and a reduction in auditory stimulation from an active chorus. With these control effects accounted for, there are a several clear effects of FM1 ablation per se on the whine and chuck. For the whine, the rate of frequency modulation decreased after FM1 ablation (i.e. *Half FM* increases considerably) and there were decreases in the whine's dominant, fundamental and maximum frequencies. There was also a large increase in the whine's minimum frequency, which is probably due to the shortening of the whine's duration and its more shallow frequency sweep.

For the chuck, FM1 ablation caused a decrease in the duration of the chuck and changes in its amplitude envelope during the rise phase. Moreover, we statistically tested the a priori predictions that surgery would reduce the chuck's relative amplitude and energy in its odd harmonics. We found support for both of these predictions: there was a significant main effect of treatment ($F_{2,17} = 10.17$, $p = 0.001$) and an interaction between treatment and pre-versus post-manipulation ($F_{2,17} = 4.92$, $p = 0.02$). Post hoc tests demonstrated that FM1 ablation resulted in a decrease in the relative amplitude of chuck compared to the whine ($p = 0.001$) and a decrease in the proportion of energy in the odd compared to the even harmonics of the chuck ($p = 0.001$). Neither of these measurements were impacted in the two control treatments (all $p > 0.17$).

Effect of surgery on female call preferences

FM1 ablation made the calls less attractive to females. The attractiveness of the chuck was rendered neutral and the whine's attractiveness appeared to diminish as well (Table 2). Neither of these call components became aversive after surgery – they elicited positive phonotaxis in females, but with clear deficits in attractiveness. In the FW versus FWTC test (c) there were approximately equal choices for the two alternatives, and therefore we were able to evaluate whether latencies to choice differed for these two stimuli; as with choices, latencies did not differ (mean ± SD (s): FW: 177.8 ± 190.5; FWTC: 126.3 ± 79.4; $t_{22} = 0.83$, $p = 0.38$, two-tailed; equal variances not assumed), suggesting that post-surgery chuck does

Table 1. Mean values (± 95% CI) for the 15 acoustic measurements of the whine (W) and chuck (C) from males that were assigned to one of three treatment groups: (1) Experimental surgery (FM1 ablation; $N = 8$); (2) Sham surgery ($N = 6$); (3) No surgery ($N = 6$). Each measurement was made from the field recording (i.e. pre manipulation) and then later in the lab (i.e. post-manipulation). The Δ values (italics) are post- minus pre-manipulation, hence negative Δ values indicate surgery decreased the call measurement and vice versa. Bolded Δ values indicate that the manipulation (experimental, sham or no surgery) caused large changes in the mean call component – specifically, that the 95% CI did not overlap zero for the Δ values.

Treatment	Call component	Chuck lag (s)	Duration call (ms)	Duration rise (ms)	Duration decay (ms)	Shape rise	Shape decay	Energy rise/decay	Relative amp W/C	Dominant freq (Hz)	Fundamental freq (Hz)*	Minimum freq (Hz)	Maximum freq (Hz)	Harmonics even/odd	Half FM	Dominant harmonic
Experimental surgery	W-pre	0.305 (0.042)	319.51 (45.60)	44.40 (24.30)	242.75 (32.56)	0.53 (0.14)	0.71 (0.12)	1.00 (0.06)		834.96 (78.39)	417.48 (37.20)	498.05 (19.14)	986.33 (65.91)	74.51 (57.20)	0.26 (0.04)	2.00 (0)
	W-post	0.227	165.97	55.03	98.38	0.63	0.75	1.00 (0.05)	Pre: 0.67 (0.13)	771.48 (57.42)	385.74 (28.71)	634.77 (82.80)	898.44 (47.99)	321.85 (352.20)	0.42 (0.12)	2.00 (0)
	ΔW	*−0.077* (0.038)	*−153.54* (76.71)	*10.63* (35.51)	*−144.37* (64.97)	*0.11* (0.24)	*0.04* (0.14)	*0.00* (0.07)	Post: 1.66 (0.96)	*−63.48* (−57.76)	*−31.74* (−28.88)	*136.72* (85.60)	*−87.89* (78.92)	*247.34* (345.37)	***0.16*** (0.13)	*0.00* (0)
	C-pre	NA	55.05 (12.00)	14.37 (3.90)	35.20 (9.29)	0.65 (0.15)	0.49 (0.04)	0.94 (0.18)	Δ: 0.99 (1.03)	2780.76 (128.38)	231.84 (9.68)	2690.43 (126.91)	2817.38 (140.98)	1.61 (0.91)	0.30 (0.18)	12.00 (0.37)
	C-post	NA	45.96 (9.53)	16.45 (6.51)	29.51 (5.95)	−0.18 (0.36)	0.42 (0.12)	1.34 (0.32)		937.50 (609.92)	232.42 (6.14)	883.79 (547.47)	971.68 (637.11)	6.06 (3.00)	0.36 (0.22)	4.00 (2.57)
	ΔC	NA	*−9.09* (6.90)	*2.08* (6.21)	*−5.69* (7.49)	*−0.82* (0.41)	*−0.07* (0.13)	*0.40* (0.40)		***−1843.26*** (714.55)	*0.58* (14.97)	***−1806.64*** (669.75)	***−1845.70*** (745.77)	***4.44*** (3.28)	*0.06* (0.30)	***−8.00*** (2.80)
Sham surgery	W-pre	0.303	340.90 (28.30)	54.94 (27.31)	282.47 (12.21)	0.42 (0.18)	0.76 (0.12)	1.01 (0.13)		803.91 (79.83)	401.95 (39.92)	574.22 (85.58)	983.35 (35.59)	23.64 (14.47)	0.15 (0.10)	2.00 (0)
	W-post	0.260 (0.035)	207.72 (75.68)	35.07 (13.19)	158.18 (72.49)	0.42 (0.18)	0.80 (0.16)	0.98 (0.06)	Pre: 0.60 (0.12)	839.80 (87.18)	419.89 (43.59)	574.22 (88.31)	954.63 (80.57)	46.64 (27.91)	0.25 (0.16)	2.00 (0)
	ΔW	*−0.044* (0.037)	*−133.19* (85.76)	*−19.87* (26.64)	*−124.29* (78.48)	*0.01* (0.32)	*0.04* (0.21)	*−0.03* (0.13)	Post: 1.11 (0.33)	*35.89* (159.29)	*17.94* (79.64)	*0.00* (149.41)	*−28.71* (96.65)	***22.99*** (17.86)	*0.10* (0.12)	*0.00* (0)
	C-pre	NA	58.83 (15.36)	13.42 (6.05)	41.08 (6.43)	0.48 (0.28)	0.36 (0.12)	0.96 (0.20)	***Δ: 0.51*** (0.23)	2835.21 (166.93)	236.27 (13.91)	2655.76 (143.61)	2878.27 (161.14)	1.12 (0.31)	0.31 (0.29)	12.00 (0.53)
	C-post	NA	72.80 (24.47)	20.32 (11.37)	52.48 (13.70)	0.28 (0.24)	0.53 (0.16)	1.13 (0.21)		2103.08 (886.80)	235.37 (15.15)	1981.06 (843.73)	2131.78 (902.82)	1.85 (0.64)	0.44 (0.35)	9.00 (3.75)
	ΔC	NA	*13.97* (29.99)	*6.90* (13.65)	*11.41* (15.65)	*−0.19* (0.41)	*0.17* (0.24)	*0.18* (0.38)		*−732.13* (735.53)	*−0.89* (20.86)	*−674.71* (791.12)	*−746.48* (763.94)	***0.73*** (0.66)	*0.13* (0.33)	*−3.00* (3.75)

No surgery	W-pre	0.281	305.60	52.98	252.62	0.34	0.73	0.97		803.91	401.95	559.86	997.71	14.46	0.20	2.00
		(0.044)	(47.05)	(29.10)	(47.02)	(0.21)	(0.08)	(0.05)		(50.72)	(25.36)	(56.62)	(41.73)	(4.16)	(0.08)	(0)
	W-post	0.272	227.87	60.25	155.99	0.66	0.94	0.96	Pre: 0.58	868.51	434.25	602.93	1004.88	46.64	0.23	2.00
		(0.037)	(70.34)	(26.27)	(55.65)	(0.19)	(0.04)	(0.03)	(0.21)	(71.18)	(35.59)	(89.20)	(40.29)	(23.61)	(0.18)	(0)
	ΔW	−0.010	−77.73	7.27	−96.63	0.33	0.21	−0.02	Post: 1.14	64.60	32.30	43.07	7.18	32.19	0.03	0.00
		(0.043)	(56.73)	(36.95)	(59.81)	(0.26)	(0.07)	(0.06)	(0.21)	(89.20)	(44.60)	(108.97)	(25.94)	(24.10)	(0.19)	(0)
									Δ: 0.57							
									(0.26)							
	C-pre	NA	44.14	12.85	31.28	0.36	0.43	0.97		2777.78	237.89	2691.65	2820.85	1.03	0.29	11.67
			(9.82)	(3.32)	(7.28)	(0.27)	(0.13)	(0.23)		(259.71)	(6.03)	(294.02)	(267.81)	(0.08)	(0.27)	(0.97)
	C-post	NA	43.83	13.70	30.12	0.06	0.22	1.21		1299.17	253.20	1284.81	1313.53	1.94	0.15	5.00
			(6.28)	(3.78)	(5.96)	(0.11)	(0.11)	(0.22)		(867.86)	(13.13)	(865.66)	(885.29)	(0.28)	(0.14)	(3.16)
	ΔC	NA	−0.31	0.85	−1.16	−0.30	−0.22	0.24		−1478.61	15.30	−1406.84	−1507.32	0.91	−0.13	−6.67
			(12.02)	(4.99)	(10.15)	(0.28)	(0.21)	(0.24)		(1048.54)	(17.18)	(1029.34)	(1090.58)	(0.26)	(0.36)	(3.88)

*To facilitate comparison of the even/odd harmonics between the whine and the chuck, we defined the actual fundamental frequency of the whine (ca. 800 Hz) as the second harmonic – i.e. so that the first harmonic would be aligned with the actual first harmonic of the chuck (ca. 400 Hz).

Table 2. Female choice results for each of the four test conditions and *p*-values from exact binomial tests (two-tailed).

Test condition	Alternative #1	Alternative #2	No. females choosing #1	No. females choosing #2	No. females no choice	Exact bino- mial test
(a)	FWC	TWC	21	3	3	*p* = 0.0002
(b)	FWC	FWTC	19	5	7	*p* = 0.0066
(c)	FW	FWTC	13	11	5	*p* = 0.84
(d)	FW	TWC	19	5	5	*p* = 0.0066

not influence female behaviour, positively or negatively. There were 3–5 trials in each of the tests b–d in which females selected the less preferred stimulus (post-surgery alternative). The majority of these 'exception to the rule' trials occurred during playback of two particular males' calls (ID: 2710, 2712). These calls stand out as having among the smallest effects of surgery on the proportion of energy in the even compared to the odd harmonics as well as the relative peak amplitude of the whine to the chuck (see Supplemental Data).

Discussion

Removal of FM1 resulted in temporal and spectral changes to both the whine and the chuck. Previously, Gridi-Papp et al. (2006) examined the impact of FM1 ablation on the energy in even/odd harmonics and showed attenuation of the odd harmonics in the chuck (but no effect on the whine). In the present study, we examined 14 additional acoustic characters. In general, FM1 ablation resulted in larger and more consistent changes to the chuck than to the whine. The principal effects of FM1 ablation on the chuck were large decreases in the relative energy in its odd harmonics and amplitude, and a reduction in the chuck's duration – owing largely to a shortening of the decay phase. To the human ear, these changes effectively eliminated the normal percept of the chuck. The whine's spectrum was also impacted by FM1 ablation. Compared to the two control treatments, the post-surgery whines of experimental males were shifted down in frequency by 30–90 Hz on average. We speculate that the removal of the connection with the fibrous masses may have reduced the dynamic tension on the vocal cords, allowing them to vibrate at a lower frequency. The rate of frequency modulation in the whine was also reduced, yielding a more shallow frequency down sweep. Lastly, after surgery the relative amplitude of whine compared to the chuck increased.

The perceptual implications of these acoustic effects were demonstrated in the female choice tests. Females treated the post-surgery chuck as though it were non-salient – the FW versus FWTC test showed that the post-surgery chuck was neither attractive nor repulsive, as observed in both the choice results and latencies to choice. It is unclear whether females even perceive the post-surgery chuck. Because FM1 ablation had multiple impacts on the chuck, including reductions in duration, relative energy in the odd harmonics, relative amplitude of the chuck compared to the whine, and the dominant and related frequencies, it is difficult to ascertain which trait changes (or their combinations) were responsible for the reduction in attractiveness. Although the present study was not designed to evaluate the independent or multivariate effects of call characters on female preferences (Ryan & Rand 2003; Gerhardt & Brooks 2009), we know from previous studies that certain features of the whine and chuck are particularly salient to female receivers, and overlap with three

acoustic effects of FM1 removal. First, the reduction in the relative amplitude of the chuck is known to strongly degrade complex call attractiveness, explaining a considerable amount of variance (57%) in female preferences for natural call variation (Baugh & Ryan 2011). Second, the increase in the minimum frequency of the whine coupled with the decrease in the chuck's dominant harmonic is relevant because of how spectral cues are processed in the túngara frog auditory system. Specifically, reception of the complex call involves the simultaneous processing the call's lowest frequency (the terminal portion of the whine's frequency sweep, processed by the amphibian papillae) and its highest frequency (the initial portion of the chuck, process by the basilar papillae). This simultaneous stimulation of the largest dynamic spectral range in the call across both auditory end organs is essential to enhancing call attractiveness (Wilczynski et al. 1995). FM1 ablation reduces this dynamic range at both ends, which likely causes a decrement in attractiveness. Lastly, the relative reduction of energy in the odd harmonics might contribute to the deficit in chuck attractiveness, but it is probably not be the only important acoustic feature. Previous studies have reported that the details of the chuck's harmonic structure are not essential to enhancing the whine's attractiveness to females (Wilczynski et al. 1995; Akre et al. 2014). For example, merely amplifying the whine's third harmonic towards the end of the call significantly increases call attractiveness (Ryan unpub. data).

Although not specifically tested, females appeared to strongly prefer the call alternatives containing the pre-surgery whine, suggesting that FM1 ablation degraded the attractiveness of the whine. Given the acoustic effects of FM1 ablation on the whine, this reduced attractiveness was predicted. The reduction in whine duration, especially the decay phase, and the changes in the amplitude envelope indicate that post-surgery whines contain less energy, which is a major determinant of signal attractiveness in male túngara frogs and most acoustically communicating species (Ryan & Keddy-Hector 1992; Ryan & Cummings 2013). This reduction in call energy may not be sufficient, however, to explain the large reduction in whine attractiveness, because appending the post-surgery chucks – which increases the total energy in the call – does not elevate the attractiveness of the post-surgery whine (test c). FM1 ablation, however, not only reduced the duration of the whine, but also decreased the slope of the whine's frequency sweep (approximated by *Half FM*). Male túngara frogs are capable of differentiating FM sweeps rates and discriminate against rates that depart from the species-typical range (Zelick et al. 1991). Likewise, FM1 ablation caused an increase in the whine's minimum frequency and female túngara frogs tend to prefer lower frequency whines, which are correlated with larger male body size (Bosch et al. 2000). Finally, the post-surgery whines appear to also have a degraded structure in the upper harmonics – though this is unlikely to influence female preferences (Ryan & Rand 1990; Rand et al. 1992). Collectively, a reduction in whine energy and FM rate and an increase in whine frequency would be expected to degrade whine attractiveness. A complete test of the hypothesis that FM1 is necessary and sufficient to induce call attractiveness that is equivalent to a natural whine or whine–chuck will require further study, including an evaluation of female preferences for sham and no-surgery control calls.

In summary, we have demonstrated that a discrete laryngeal mass supported by the vocal cords in male túngara frogs plays an essential role in the normal production of both components of the sexual advertisement call. This finding both confirms previous research indicating that chuck production is made possible by FM1 (Boul & Ryan 2004; Gridi-Papp et al. 2006; Guerra et al. 2014), and furthers our understanding of the broader impacts of

FM1 on chuck and whine production. This finding suggests that the evolution of FM1 made possible not only the sexually stimulating chuck, but also had positive repercussions on the taxonomically conserved (pre-existing) whine. This directional effect on whine and chuck may have accelerated fixation of the complex call in this clade.

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