

Female and male behavioral response to advertisement calls of graded complexity in túngara frogs, *Physalaemus pustulosus*

Ximena E. Bernal · Karin L. Akre ·
Alexander T. Baugh · A. Stanley Rand ·
Michael J. Ryan

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Abstract We investigated the natural dynamics in a sexual signal that combines different call components and explored the role of call complexity in sexual selection using a neotropical frog. Male túngara frogs, *Physalaemus pustulosus*, facultatively add up to seven short, multi-harmonic components (chucks) to the simple form of their calls (whines). Female túngara frogs are preferentially attracted to whines with chucks over whines without chucks, and males also call more in response to calls containing chucks. Because acoustic predators prefer complex calls, in the context of simple (no chucks) versus complex (any number of chucks) calls, the variably complex call appears to have evolved in response to the opposing selective forces of natural and sexual selection. There is no evidence, however, for the function of increasing the number of chucks within complex calls. We tested two aspects of increasing call complexity: natural patterns of use of call types in males and how both sexes respond to variation in multi-chuck calls. Males incrementally change call complexity by the addition or subtraction

of a single chuck and usually do not produce more than two chucks. Variation in call complexity, for calls with at least one chuck, does not influence response calling in males or phonotaxis in females. Our results suggest that one reason for not increasing call complexity beyond a single chuck is the diminishing effectiveness on the responses of both sexes.

Keywords Call repertoire · *Physalaemus pustulosus* · Evoked vocal response · Female choice

Introduction

In many species, males produce a single type of sexual display to attract females and compete with rival males (Berglund et al. 1996). Variation in such sexual displays may therefore influence males, females, or both. Within-individual graded variation of sexual signals, in particular, can provide receivers with information about the signaler's intent or degree of motivation. In aggressive contexts, for instance, the intensity of the display increases as the contest progresses, and thus it is thought to convey information about aggressive intention (Morton 1982). In such cases, females attending to the same signals could also extract information relevant for mate decisions. Interactions with females, however, could be driving graded variation of male sexual signals. The role that female choice plays in influencing within-individual variation of male sexual signals has been recognized recently (Balsby and Dabelsteen 2002; Kime et al. 2004; Patricelli et al. 2004). Female satin bowerbirds (*Ptilonorhynchus violaceus*), for instance, modulate the intensity of male courtship behavior through a gradual increase in crouching behavior (Patricelli et al.

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X. E. Bernal · K. L. Akre · A. T. Baugh · M. J. Ryan
Section of Integrative Biology C0930, The University of Texas,
Austin, TX 78712, USA

A. S. Rand · M. J. Ryan
Smithsonian Tropical Research Institution,
P.O. Box 0843-03092, Balboa Ancón, Republic of Panamá

Present Address:

X. E. Bernal (✉)
Department of Biological Science, Texas Tech University,
Box 43131, Lubbock, TX 79409, USA
e-mail: ximena.bernal@ttu.edu

2004). A complete understanding of how sexual selection shapes dual-function graded signals requires integrating the selective pressures imposed by both of the intended receivers of these signals, males and females. Unintended receivers such as predators and parasites can also impose costs that influence the evolution of sexual signals (Endler 1978, 1983; Tuttle and Ryan 1981; Zuk et al. 2006). Using a neotropical frog, we investigate the natural dynamics of calling behavior in a call that combines different components, and we explore the role of such graded variation in male–female and male–male interactions.

In most anurans, males produce a stereotyped mating call that is repeated with little variation for several hours at their breeding areas (Gerhardt and Huber 2002). These redundant calls potentially reduce recognition errors and increase localizability. Relative to many anurans in which males only produce a single call element, males of some species produce mating calls with multiple elements (Schwartz and Wells 1984a, b, 1985; Ryan 1985; Narins et al. 2000). The túngara frog, *Physalaemus pustulosus*, has a call that varies in complexity in a way unusual for frogs and toads (Rand and Ryan 1981). Males produce a frequency modulated whine to which they can facultatively add secondary multi-harmonic ornaments called chucks (Fig. 1; Rand and Ryan 1981; Ryan 1985). Chucks are produced by fibrous masses attached to the vocal folds (Gridi-Papp et al. 2006). These fibrous masses are likely to have evolved by sexual selection. While whines can be produced alone, chucks always follow whines. Males produce whines without chucks most often when they are calling by themselves, while calls with chucks are more common in choruses with several males (Ryan 1985). Males can add from zero to seven chucks to a whine (Bernal et al. 2007a, b).

Female túngara frogs are preferentially attracted to calls with chucks over simple whines, and males also call more in response to calls with chucks (Rand and Ryan 1981; Ryan and Rand 1998). Frog-eating bats (Ryan et al. 1982) and blood-sucking flies (Bernal et al. 2006) also are attracted preferentially to calls with chucks over simple whines. Thus, the variably complex call appears to have evolved in response to natural and sexual selection, at least in the context of simple (no chucks) versus complex (any number of chucks) calls. There is presently no demonstration, however, of the function of increasing the number of chucks beyond one.

In the wild, about half of the calls produced by male túngara frogs have chucks. The majority of those complex calls have one chuck. Calls with two chucks are less common, while calls with three or more chucks are unusual (Bernal et al. 2007a, b). Despite these recent findings about the natural signal variation at choruses in the wild, we do not know what sequence males follow to increase call complexity and whether males enjoy any advantage when producing more than one chuck. In this study, we quantify how males use calls with multiple chucks in their call bouts and test the hypothesis that greater call complexity increases call response (number and complexity of vocalizations) in rival males, call attractiveness to females, or both.

Materials and methods

Male and female frogs were collected during the rainy season, between May and August 2000–2006, at breeding ponds around the facilities of the Smithsonian Tropical Research

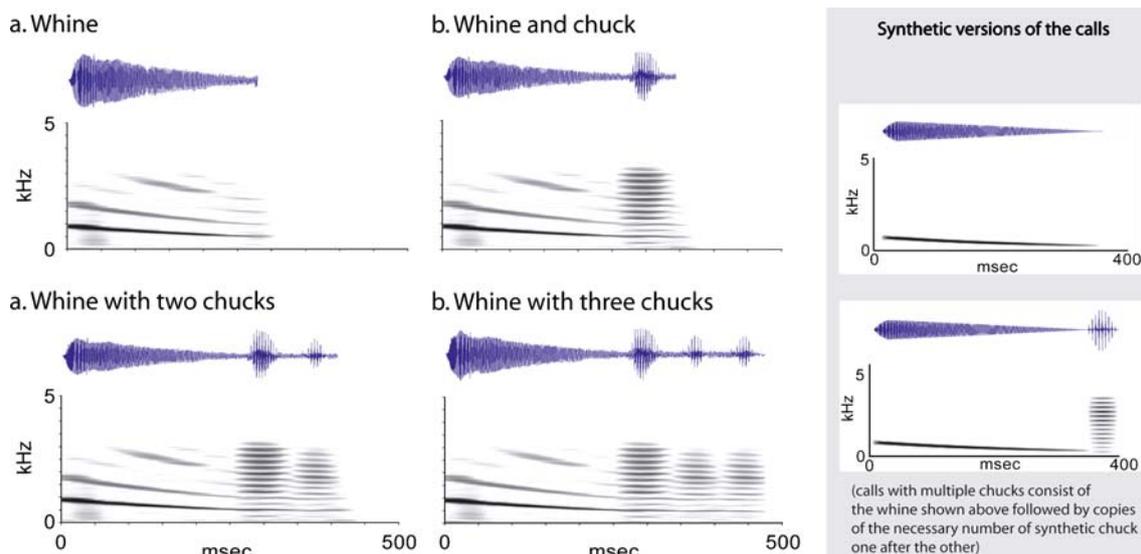


Fig. 1 Complexity series of the advertisement call of *P. pustulosus*, the túngara frog (a–d). Natural versions of the calls are shown with zero to three chucks. Synthetic versions of the calls with zero and one chuck are presented in the *box on the side* for comparison

Institute in Gamboa, Panama (9°07.0' N, 79°41.9' W). All frogs were brought to the laboratory and subsequently returned to their capture sites. Prior to returning the frogs, we gave them a unique toe-clip number to prevent them from being retested or their calls recorded again.

Sequence analysis of call complexity

Túngara frogs call in bouts in which a few males initiate calling. Other males then join them until the chorus reaches a peak of calling activity, after which individual frogs cease calling until all are silent. The entire process starts anew when a few leading males begin to call again (Pauly et al. 2006). We recorded 90 call bouts of individual focal males using a WM-D6C Sony Tape recorder and Sennheiser ME-66 shotgun microphone. We recorded complete bouts, including always the first call of the bout, placing the microphone 1 m from the calling frog. After recordings were complete, males were captured, measured for snout-vent length, and marked.

For each male, we categorized their calls according to the number of chucks added to each whine and the sequence in which these variably complex calls were produced. We then analyzed this vocal sequence to understand how males transition between calls of varying complexity. We organized preceding and following call types into a transition probability matrix in which each cell represents the total instances of call type j following call type i (Table 1). We calculated the expected value for each transition by multiplying the column frequency by its corresponding row total. To establish if there is an association between preceding and following call types,

we used a chi-square goodness of fit using the Yate's correction for continuity (Zar 1996). The total chi-square value for the entire matrix is equal to the sum of the total row chi-square values. From the transition matrix, it was necessary to establish which of the dyads in a row were significant. To do so, we used a modified chi-square value with one degree of freedom per cell (Clark 1994). To calculate the transition probabilities, we divided the total instances of a given call type by the corresponding row total. To evaluate decision making, we first calculated the probabilities for all possible transitions. Because we were interested in the transitions that generate changes in call complexity, we calculated the transition probabilities between call types without including cells representing call transitions that did not incur changes of chuck number.

Behavioral response of males and females to increased complexity

Acoustic stimuli

We used whines with a variable number of chucks, ranging from zero to six, as experimental stimuli. We performed for both sexes one set of experiments with synthetic calls, and a second one with natural calls. Evaluating the response of túngara frogs to natural calls allowed us to explore any effects related to inter-individual variation in acoustic properties undetected in the experiments using synthetic calls. Synthetic versions of the average whine for the population successfully elicit phonotaxis in females (Ryan and Rand 1990) and calling behavior in males (Bernal et al. 2007a, b). Synthetic calls offer high internal validity but do not completely replicate natural

Table 1 Túngara frog call complexity transition matrix for whines with up to three chucks ($n=90$ males)

Preceding call type (number of chucks)	Following call type (number of chucks)				Row total	Row chi square	
	0	1	2	3			
0	obs	919**	144**	9**	0	1,072	1,118.66
	exp	393.33	440.95	231.72	6.00		
1	obs	121**	956**	83**	0	1,160	813.94
	exp	452.62	477.15	250.75	6.49		
2	obs	9**	76**	519**	7	611	1,460.59
	exp	224.18	251.32	132.07	3.42		
3	obs	0	0	7	9**	16	803.18
	exp	5.87	6.58	3.46	0.09		
Total		1,049	1,176	618	16	2,859	4,196.39**
Frequency		0.367	0.411	0.216	0.006		

Top number of the row is the observed value (*obs*), and the bottom number is the expected value (*exp*). Row chi-square values using the Yates' correction are given in the far right column, and the chi-square value for the entire matrix is shown at the bottom of such column. Statistically significant dyads are shown in bold

** $P < 0.001$

calls. Synthetic and natural túngara frog calls differ in several traits (Fig. 1). Natural whines, for instance, have a series of harmonic frequencies instead of having only the fundamental frequency (also the dominant frequency). Previous studies have shown that females do not discriminate on average between natural and synthetic whines (Rand et al. 1992).

We synthesized stimuli based on the mean values of the parameters of the calls in the population by shaping sine waves using custom software (J. Schwartz, Pace University at Pleasantville, NY; sample rate 20 kHz and 8 bit). Mean values for the population were calculated based on the calls from 50 males recorded in July 1996 with a Marantz PMD 420 recorder and a Sennheiser ME 80 microphone with K3U power module on magnetic cassette tape. Additional information on the call parameters used and the synthesis procedure can be found in Ryan and Rand (2003a).

The natural calls are recordings of males from the field or the laboratory. The calls used in the experiments with the males were recorded in the laboratory in individual acoustically isolated chambers with a Sony WM-D6 cassette recorder and a Radio Shack miniature condenser microphone. The chambers were lined with sound absorbent material to minimize echo and attenuate sounds arriving from outside the chambers. We chose recordings with minimal background noise from ten males that produced calls with different numbers of chucks. In most cases, males added from zero up to two chucks to a whine, but a few males produced three chucks. To obtain whines with three and six chucks for each male, we digitally duplicated the terminal chuck of the call and appended it at the end with a 4 ms inter-chuck interval. We repeated this procedure as many times as necessary to achieve the desired number of chucks for the experiment.

The natural calls used in the experiments with the females were recorded in the field with a Marantz PMD 420 recorder and a Sennheiser ME 80 microphone with K3U power module on magnetic cassette tape. We selected the calls of 20 males based on previous studies of female preference using the same calls (details in Ryan and Rand 2003a). The call of each male was modified to obtain the desired number of chucks for each test duplicating the initial chuck of the call and appending it to the end of the whine as many times as required. We used each pair of calls of different call complexity from each male only once.

Male evoked vocal response experiments

Males may increase call complexity in response to conspecific calls, and there are two different pressures that may influence this calling behavior: (1) if females find calls of increased complexity more attractive or (2) through vocal competition among males. In the first case, if females find calls with a greater number of chucks more attractive, males

enjoy an advantage by increasing their vocal response to those call types as this will increase their chances of attracting a mate. On the other hand, interactions among males may influence call complexity independently from the selective pressures imposed by female choice, although the advantage of doing so is not known. Even though calling male túngara frogs do not defend specific sites or resources, they defend a space around them maintaining an inter-neighbor distance (Ryan 1985). Vocalizations play a central role mediating such social interactions, and increased vocal complexity might be relevant in that context. Here, we evaluated the role of call complexity in vocal competition among males.

Calling males were captured at their breeding sites and brought to the lab where each male was placed inside individual acoustically isolated chambers (30.5×46×30.5 cm) following Bosch et al. (2000b, 2002) and Bernal et al. (2007a, b). To record their calling behavior, we placed males with sufficient water to call inside plastic bags previously shown to be acoustically transparent by Ryan and Rand (1998). We broadcast the experimental stimuli using a small, wide-frequency range speaker (Cambridge SoundWorks, Ensemble IV) and recorded the response of the males with a Radio Shack miniature condenser microphone into a Sony WM-D6 cassette recorder. We presented the stimuli using a JVC XL-PG7 CD-player through a Realistic SA-10 amplifier at 90 dB SPL (re. 20 μPa) at 0.5 m measured by a GenRad sound pressure level meter (model 1982). We digitized the tapes using CoolEdit 2000 (Syntrillium Software Corporation) at a sampling rate of 44.1 kHz and 16 bits/sample.

We tested males in evoked vocal response experiments using both synthetic and natural calls. In 2003, we used synthetic versions of the average whine and chuck in the population (see section on “Acoustic stimuli”), recording the calling response of 12 males in each test (i.e., whine only, whine with one chuck, etc). In 2004, we performed a second series of tests using natural calls to explore further the responses of males to calls with multiple chucks. In this second experiment, in addition to using natural calls, we used an experimental design that would allow us to account for variability between males to explore further any potential effects of call complexity on behavior. We recorded whines with variable numbers of chucks from ten males and presented each male's complexity series in random order to a single male (see section on “Acoustic stimuli”). We tested ten males, each one with the calls of a randomly selected but different male (i.e., a repeated measures design).

In both kinds of evoked vocal response experiments, we used the same methodology to present the stimuli. We stimulated the males with a túngara frog chorus recorded on 2 October 1990 from the same population. Once a male was calling, we tested him singly. Each test consisted of a set of five 60 s intervals in the following order: (1) control

stimulus: white noise shaped with the amplitude envelope of the whine (0–10 kHz), (2) silence, (3) experimental stimulus: whine with zero, one, two, three, or six chucks, (4) silence, and (5) control stimulus (see also Ryan and Rand 1998; Bosch et al. 2000a; Bernal et al. 2007a, b). After completing a test, a male was required to call again before starting the next test. Males were tested until they participated in all the experimental stimuli or ceased calling. We broadcast the calls at a rate of one call every 2 s, a typical calling rate for túngara frogs (Ryan 1985). Additional details are presented in Bernal et al. (2007a, b).

To evaluate male calling behavior, we counted the number of whines, number of chucks, and maximum number of chucks in a single call produced by each male in response to the experimental and control stimuli. To account for any changes in male motivation during the experiments, we examined the strength of male calling in response to each stimulus by averaging the calling response of the two controls to calculate the ratio of responses: experimental/(average control+1) following Bosch et al. (2000a, 2002). We then characterized the overall response of males using the first component of a principal components analysis (PCA) after combining the ratios of responses in the number of whines, number of chucks, and maximum number of chucks in a single call. We performed separate PCA for the experiment using synthetic calls and the one using natural calls; thus, the independent measurements of overall calling response were obtained for each set of tests. In the experiment using synthetic calls (between-groups design), we evaluated the effect of adding chucks to a whine on male response with a Kruskal–Wallis nonparametric analysis of variance (ANOVA) followed by post hoc comparisons with Dunn's test using SigmaStat (Systat Software, California, USA). For the data obtained using natural calls (repeated measures design), the Friedman test was used, and Dunn's test was used for post hoc comparisons.

Female phonotaxis experiments

Female túngara frogs use the calls produced by males to locate and choose among them, thus exerting their preference. As a result, call variation influences the decisions of females, and increased complexity of the calls may determine call attractiveness. Here we investigate how call complexity influences female choice.

We performed standard two-speaker phonotaxis tests offering females a choice between calls that differed in the number of chucks appended to a whine. We placed each female under a funnel in the center of a sound-attenuating chamber (Acoustic Systems, Austin, TX, USA; 1.8×2.7 m), while the stimuli were broadcast from speakers placed in the center of walls opposite one another. After 3 min, we remotely removed the funnel allowing the female to move

freely in the arena. We broadcast the test stimuli antipodally such that the peak amplitude of the whine of each test call at the center of the arena was 82 dB SPL (re. 20 µPa). Speakers were balanced for sound pressure level using a 500 Hz continuous tone. We scored a choice when the female approached within 10 cm of either speaker without simply following the wall. If a female was motionless at the release point for 5 min, did not move for any 2 min span of time after exiting the release point, or spent more than 15 min roaming the arena without approaching a speaker, no choice was recorded. We observed the behavior of the females on a monitor using a wide-angle lens video system with an infrared light source (Fuhrman Diversified).

We tested females in phonotaxis using both synthetic and natural calls. In 2000, we used synthetic calls created based on the mean values of the call parameters for the population. We evaluated the responses of females to all possible combinations of calls with zero to three chucks, for a total of six paired tests (0 vs. 1, 0 vs. 2, 0 vs. 3, 1 vs. 2, 1 vs. 3, 2 vs. 3). In 2006, we further explored the potential attractiveness of multiple chucks by examining female choice in response to natural calls. Details about the natural and synthetic calls can be found in the “Acoustic stimuli” section.

We used an exact binomial test for each pair of stimuli to determine the effect of call complexity on female mate choice. Because we predicted that calls with more chucks are preferred compared to those containing fewer chucks, we used a one-tailed probability. We experimentally determined the expected proportion (EP) for the exact binomial test presenting female frogs with the same call (synthetic whine with one chuck) at both speakers. In 2000, when the tests using synthetic calls were performed, the frogs had about the same probability to approach each side of the arena. Thus, we used an EP of 1:1. In 2006, however, we found a bias favoring one side of the arena. Since the side from where each call was broadcast was determined randomly, in each experiment, the number of times each stimulus was presented from each side was not necessarily equal. Taking into account the side bias and the number of times each stimulus was presented on each side of the arena, we determined the EP for each test (EPs ranged from 0.75:1 to 0.87:1; details in Table 2). Finally, we combined the outcome of the experiments using synthetic and natural calls to obtain an overall significance test for the effect of call complexity using the Fisher's method for combining probabilities (Sokal and Rohlf 1995).

Results

Sequence analysis

We recorded a total of 90 call bouts of individual focal males. Males produced call bouts with an average of 32.63

Table 2 Responses of túngara frog females in phonotaxis tests to calls that vary in the number of chucks appended

Test	Choices	<i>P</i>	Power	<i>n</i> (Power=0.80)
Synthetic calls ^a				
WH 0CH vs. WH 1CH	4/16	0.006	0.817	15
WH 0CH vs. WH 2CH	2/18	<0.001	0.996	7
WH 0CH vs. WH 3CH	2/18	<0.001	0.996	7
WH 1CH vs. WH 2CH	9/11	0.412	0.072	616
WH 1CH vs. WH 3CH	9/11	0.412	0.072	616
WH 2CH vs. WH 3CH	7/13	0.132	0.259	67
Natural calls ^b				
WH 0CH vs. WH 1CH a	1/19	<0.001	>0.99	5
WH 0CH vs. WH 2CH b	4/16	0.028	0.817	15
WH 1CH vs. WH 2CH c	8/12	0.162	0.083	107
WH 1CH vs. WH 3CH d	7/13	0.039	0.639	31
WH 2CH vs. WH 3CH e	11/9	0.195	0.288	85

The choices represent the number of females attracted to less/more chucks (*CH*) appended to a whine (*WH*). *P* is the probability of rejecting the null hypothesis using a one-tailed exact binomial test. The power of the test and the sample size required given the observed effect to achieve statistical power of 0.80 are also shown. Expected proportions (*EP*) were experimentally determined

^a EP=1:1

^b EP were calculated for each test: *a* 1:0.75, *b* 0.75:1, *c* 1:0.87, *d* 1:0.75, *e* 0.75:1. Complete description in “Materials and methods”

calls (SEM=3.58) with a broad range of variation (minimum value=4 calls, maximum value=255 calls). The maximum number of chucks appended to a whine was three. Approximately one quarter of males did not produce complex calls (25.6%), and these males had shorter bouts than males that increased call complexity (*t*=3.349, *df*=88, *P*=0.001).

The degree of a given call's complexity depended on the complexity of the preceding call ($\chi^2=4196.39$, *df*=9, *P*<0.001, *n*=90; Table 1). This was true even when cells including transitions to the same state were excluded from the analysis ($\chi^2=381.30$, *df*=9, *P*<0.001, *n*=90). By estimating the chi-square value for each cell of the matrix, we extracted significant dyads, shown in bold in Table 1. For all call types, whines with the same number of chucks (i.e., no change in complexity) were produced in greater frequency than expected, and transitions that produced increases and decreases of chuck number between zero and two chucks were produced in lower frequencies than expected.

Transition probabilities to the same state were high; calling males were likely to maintain the same number of chucks (from/to: 0/0=0.86, 1/1=0.83, 2/2=0.85, 3/3=0.56). Since we were interested in the transitions that generate changes in call complexity in particular, we performed the analysis excluding transitions to the same state. The transition probabilities between calls with different numbers of chucks are shown in Fig. 2. Transitions involving the addition or subtraction of a single chuck occurred with high probabilities. Males producing single whines usually increase call complexity by adding

only one chuck. Calls with one chuck had a slightly higher chance of going back to zero chucks than appending an additional chuck. Males producing whines with two chucks were highly likely to reduce call complexity to one chuck in the next call. Whines with three chucks are reached only from calls with two chucks, and males reduce call complexity producing whines with two chucks. Males never transitioned from three to one or zero chucks in either direction.

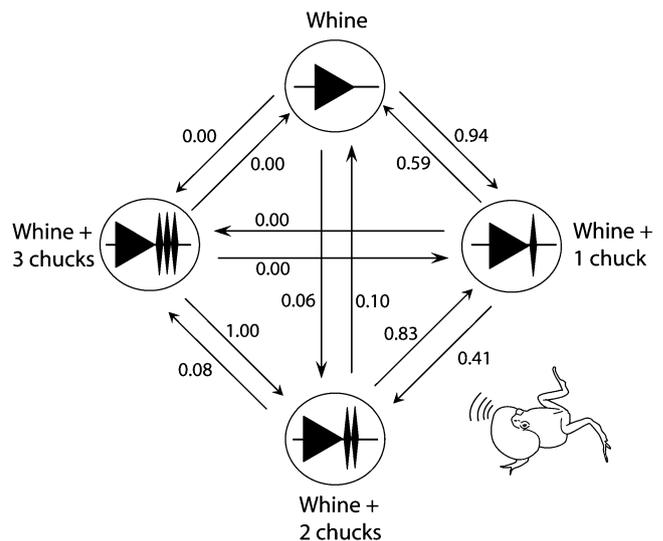


Fig. 2 Sequence diagram of male túngara frog calling behavior. Numbers indicate the transition probabilities from one call type to the subsequent call type

Male-evoked vocal response

Males produced simple and complex calls in response to the stimuli. To describe calling response, we combined the number of whines, number of chucks, and maximum number of chucks in a single call into a single measurement using a PCA. We used the first component of the PCA, here PC1, as a proxy for male calling response. We used separate PCAs for each set of stimuli, and the variation explained by the analysis was similar in both cases (synthetic calls, 71.89%; natural calls, 75.36%). Call complexity has the highest loadings on PC1, followed closely by call rate (synthetic calls: number of calls=0.851, number of chucks=0.912, max number of chucks per call=0.760; natural calls: number of calls=0.769, number of chucks=0.945, max number of chucks per call=0.881).

Males change their calling behavior in response to playbacks of variable call complexity (Fig. 3; synthetic calls: Kruskal–Wallis $H=20.59$, $df=4$, $P<0.001$; natural calls: Friedman $Q=23.12$, $df=4$, $P<0.001$). Overall, the calling responses of males fit a logistic regression, with a sharp increase in calling due to whines with one chuck but a lower increase in response to additional chucks (synthetic calls: $P<0.001$, natural calls: $P<0.001$). The total variation explained by such curve, however, is low and about equal to the variation explained by a linear regression (synthetic calls: $R^2=0.34$, natural calls: $R^2=0.39$).

Male calling responses are similar in the experiments using synthetic and natural calls. In both cases, males call significantly more in response to a whine with any number of chucks than to a single whine ($P<0.05$ in all cases). Calling behavior, however, is not significantly different in response to calls with one, two, or three chucks ($P>0.05$ in

all cases). Given that males called only during silent intervals, it is possible they reached their maximum calling response given the experimental design (e.g., 2 s of silence between playback calls). Calls with six chucks, however, do significantly increase calling response over calls with three chucks using synthetic calls ($P<0.05$) and up to two chucks using natural calls ($P<0.05$). This suggests that the lack of increased calling response to two and three chucks is not due to a constraint in our design but to lower motivation.

Female phonotaxis response

Females strongly preferred a whine followed by one or more chucks to a whine without any chucks (Fig. 4a, c). When using both natural and synthetic calls, there was a trend for females to prefer calls with more chucks over those with fewer chucks (Fig. 4b, d). The preference for calls with more than one chuck was statistically significant only for the test comparing whines with one and three chucks using natural calls (Table 2, Fig. 4). Based on the responses of females in these experiments, power analyses showed that we would need a sample size between 67 and 616 females to adequately address this preference statistically (statistical power of 0.80).

On the basis of all the phonotaxis tests together, we conclude that increasing the complexity increases attractiveness to females ($\chi^2=79.82$, $df=22$, $P<0.001$). This general trend, however, is generated by the strong preference of females for calls with any chucks over simple whines. In five of the six tests in which calls with more than one chuck were paired (1 vs. 2, 2 vs. 3, etc), more females were attracted to the whine with more chucks. Combining across these six tests together, there was no significant

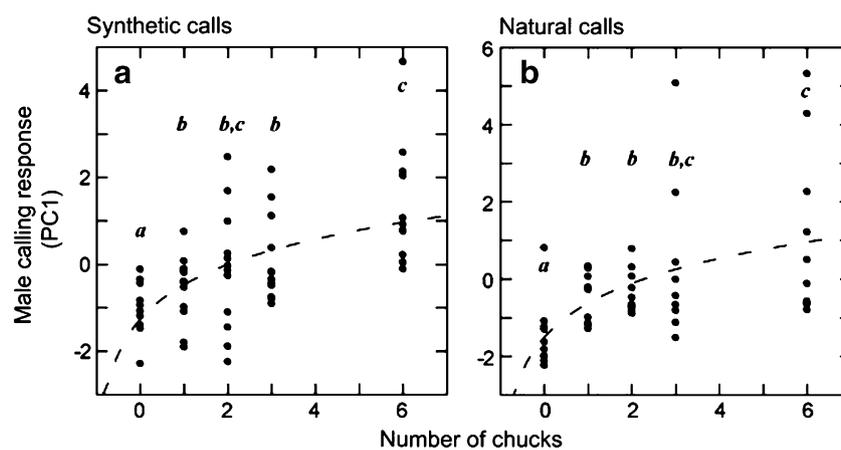
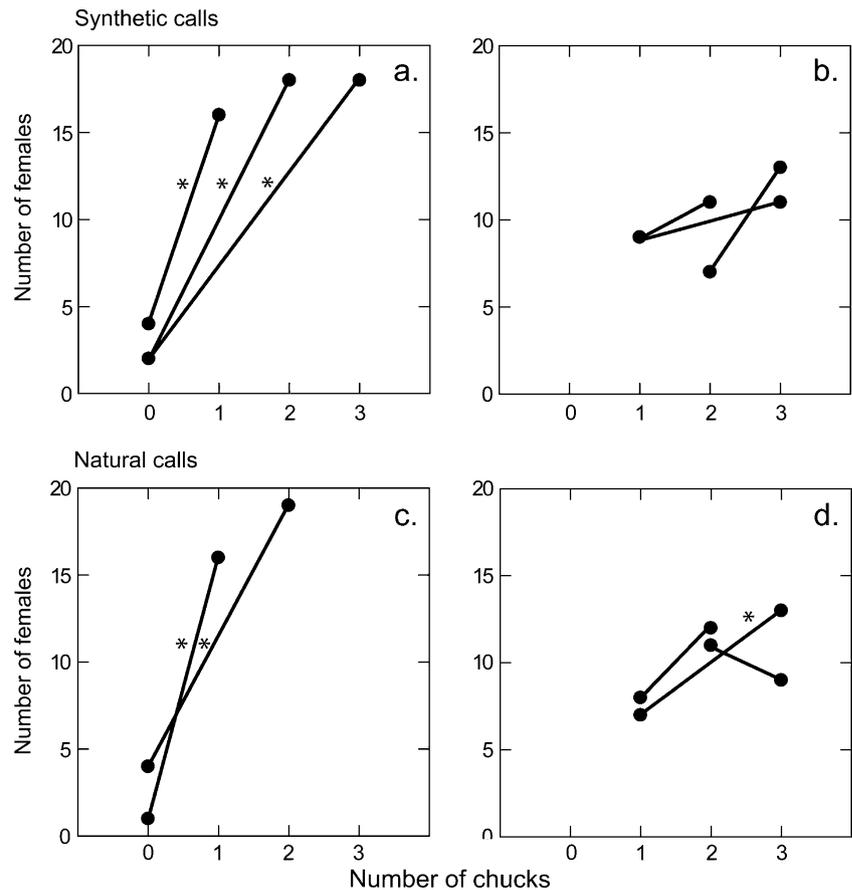


Fig. 3 Male-evoked vocal response to synthetic (a) and natural (b) calls that vary in the number of chucks appended to the whine. Male calling response is expressed as the first component of a PCA (PC1) combining the ratios of responses in the number of whines, number of chucks, and maximum number of chucks in a single call. Separate

PCA were performed for the responses to synthetic and natural stimuli (more details in “Materials and methods”: Male-evoked vocal response experiments). The letters indicate significantly different groups based on a Kruskal–Wallis nonparametric ANOVA and post hoc Dunn’s test

Fig. 4 Number of positive responses by females performing phonotaxis to synthetic (a, b) and natural (c, d) calls that vary in number of chucks appended to the call. Calls paired in each phonotaxis experiment are *linked by a line*. * $P < 0.05$ using a one-tailed exact binomial probability test that each pair of stimuli



preference for greater number of chucks beyond one ($\chi^2 = 20.99$, $df = 12$, $P = 0.055$), but females showed a general tendency to prefer whines with higher number of chucks. Finally, adding one chuck to whines that already have chucks does not significantly increase their attractiveness to females ($\chi^2 = 12.73$, $df = 8$, $P = 0.12$).

Discussion

Male túngara frogs produce calls of varying complexity in response to the counter-selection forces generated by sexual and natural selection. Calls with chucks are more attractive to females but increase predation risk from frog-eating bats (Ryan et al. 1982) and parasitism risk from blood-sucking flies (Bernal et al. 2006). The reason males produce calls with more than one chuck, however, is not understood. Here, we show that males gradually change call complexity by the addition or subtraction of a single chuck. Bernal et al. (2007a, b) showed that ca. 78% of calls had zero or one chuck, while more than 99% of the calls had two or fewer chucks. Thus, although males can add up to seven chucks, it is unusual to produce more than two. The lack of calls with many chucks might be due to constraints in signal production. Our results, however, suggest that one reason

for not increasing the number of chucks more drastically in the wild might be the diminishing effectiveness on the response of both males and females. Such low benefits are probably outbalanced by the negative consequences of increased attraction of frog-eating bats to calls with greater number of chucks (R. A. Page, unpublished data).

Dynamics of call complexity

Analysis of sequential calls reveals a gradual increase in call complexity in túngara frogs. Consecutive calls are highly likely to remain at the same degree of complexity, and if not, it most often will be through the addition or subtraction of single chucks. Why is there a gradual change in call complexity? Chucks are produced by a fibrous mass that extends into the bronchus and is supported by the vocal folds (Gridi-Papp et al. 2006). There are no reasons to assume that mechanical constraints would force a gradual increase in call complexity, and our data show that males are physically able to add or remove two chucks in a single transition, though they do so rarely. The pattern of increase in call complexity that we find is probably shaped by the social environment. The call bouts recorded in the wild reflect male calling strategies when interacting in breeding aggregations. At choruses, males organize their calls into

bouts in which one or two males initiate calling, and other males join them until the chorus reaches a peak of calling activity. This gradual addition of males to the chorus generates a slow increase in acoustic interactions probably leading to a smooth increase in call complexity in single males.

Males in natural choruses rarely produce more than one chuck (Bernal et al., 2007a, b), and the call transition probabilities we found are consistent with this fact. Males producing simple whines have a high probability of adding one chuck but are much less likely to add more than one chuck. Males giving calls with two or three chucks are more likely to subsequently give calls with fewer chucks. The stimuli that cause calling males to add or subtract chucks are not known, and these results highlight our questions about the payoffs of calls with more than one chuck.

Behavioral responses to call complexity

Our study confirmed that both males and females are more responsive to complex calls with one chuck than to a whine without chucks. Moreover, we found that any number of chucks increases call attractiveness to females and calling response of males compared to a whine alone. Nonetheless, approximately half of the calls produced by túngara frog males in nature do not have chucks (Bernal et al., 2007a, b). A relatively high production of simple calls could be explained by the costs imposed by adding chucks to a whine (Ryan 1985). Specifically, despite the lack of an additional energetic cost of chuck production (Bucher et al. 1982), complex calls increase the likelihood of attacks by frog-eating bats (Ryan et al. 1982) and blood-sucking flies (Bernal et al. 2006).

When both sexes were exposed to calls with multiple chucks, neither females nor males were more responsive to calls with greater numbers of chucks. One exception was the preference of females for calls with three chucks over calls with one chuck using natural calls. Thus, it seems that the addition of more than one chuck to an already complex call is necessary to increase the attractiveness of the call to females. Calls with differing numbers of chucks vary in the amount of sensory stimulation to the receiver. Females of several species of anurans and insects prefer signals with traits of greater quantity such as calls that are longer, louder, and delivered at a faster rate (Ryan and Keddy-Hector 1992; Andersson 1994; Gerhardt and Huber 2002). Female túngara frogs follow this pattern to some extent; they are preferentially attracted to whines with any number of chucks over whines only and to whines with three chucks over whines with one chuck. This behavior is paralleled in some ways by males. Although after the first chuck is added to a whine, one or two additional chucks do

not considerably increase male calling response, calls with six chucks do significantly increase this behavior. Calls of such high complexity, however, are rare in nature. Less than 0.1% of calls produced by male túngara frogs in nature have three or more chucks (Bernal et al. 2007a, b).

The general lack of elevated responses from both sexes in response to calls with a greater number of chucks was unexpected. An insufficient sample size is a common source of criticism of studies, like this one, reporting negative results. It is unlikely, however, that there is a preference or increased vocal response that we did not detect as a result of our sample size. The probability of rejecting the null hypothesis, the statistical power, depends on the sample size as well as effect size at a given alpha level (Cohen 1969). While a probability level associated with a given statistical test informs about the strength of evidence that a null hypothesis is incorrect, the effect size measures the degree to which such a null hypothesis is wrong (Grissom and Kim 2005). The effect size is a standardized measurement of the degree of difference between groups or treatments. Small effect sizes most likely generated the low power in the tests from this study and do not result from inadequate sample size. We used methodologies and sample sizes shown to be appropriate to evaluate female and male behavior in túngara frogs and other anurans (reviewed in Gerhardt and Huber 2002; Ryan and Rand 2003b). Moreover, even though synthetic calls are successful at eliciting female phonotaxis (Ryan and Rand 1990, 1995; Ryan et al. 2003) and male-evoked vocal response (Bosch et al. 2002, Bernal et al. 2007a, b), we considered the possibility that the lack of enhanced response was due to the use of synthetic versions of the calls that lack potentially relevant properties for our particular question. In this regard, the experiments with natural calls did reveal a previously unknown female preference for whines with three chucks over one chuck, but overall, the results confirmed the majority of our previous findings with synthetic calls. Although drastic and unpractical increases in our sample size could eventually result in significant results (see Table 2), we would likely err on the side of statistical significance over practical importance (Rosenthal et al. 2000). In such cases, the biological relevance of the results would be highly questionable.

Our study explores the role of increased complexity using simple behavioral paradigms in the laboratory; thus, readers should bear in mind that increased complexity beyond a single chuck may be relevant in a specific context we did not evaluate. Túngara frogs, for instance, make their mating decisions amid noisy environments with conspecific and heterospecific males calling. It is possible that multiple chucks are preferred when perceived against this naturally complex acoustic background. An effect of background noise on call discrimination has been detected in other

species of frogs (Schwartz and Gerhardt 1998; Wollerman and Wiley 2002). Improved discrimination in the presence of noise, however, is unexpected in any auditory system (but see Schwartz and Gerhardt 1998). The potential role of background noise in discrimination of calls with greater call complexity is currently being explored in female and male túngara frogs.

Ryan (1990, 1998) and Ryan and Keddy-Hector (1992) suggest that female preferences can result from greater sensory stimulation, and those preferences ultimately contribute to the evolution of complex signals. Our results, however, suggest that, although túngara frog males can produce signals of greater complexity, females do not appear to have driven the evolution of call complexity. The slight preference in females for greater chuck number, combined with the lack of an increased male vocal response to such calls, probably underlie the low frequency of calls with more than one chuck in nature. Whines with two or more chucks are about one tenth of the calls produced by calling males (Bernal et al. 2007a, b). Without an apparent benefit from female choice or male–male competition, the current function (if any) of increasing call complexity remains intriguing.

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