Female Preferences for Call Properties of Giant Spiny Frog (*Quasipaa spinosa*)

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A B S T R A C T

The advertisement call is an important recognition feature in Amphibian. For example, previous call of two-choice discrimination studies on the genus *Quasipaa spinosa* detected the phonotaxis of the female frog for the calls from different lineages. In this work, we used a single-stimulus design to analyse phonotactic response to stimulus and subsequently estimated the values of female preference functions for call duration and call rate, and recorded and analysed the properties of 756 advertisement calls from 40 males at five reproductive stages and conducted single-stimulus phonotaxis experiments to estimate female preference for different acoustic parameters. Our studies have shown *Q. spinosa* may have female mating preferences and possible variations in calls at five reproductive stages (from April to September). Correlations were established between certain call properties, i.e., pulse rate was positively correlated with fundamental frequency (r=0.151, P<0.01) and negatively correlated with dominant frequency (r=-0.141, P<0.01). The females frog had the shortest response time to calls of 4-note duration and rate of 15 n/min. Our results may provide acoustic information of the spiny frog to further develop effective conservation recommendations for this economically important but threatened animal.

INTRODUCTION

Vocalizations are some of the most important means of animal communication, and acoustic signals may convey information about the species, such as physical condition and heritable fitness of a mate (Henderson and Gerhardt, 2013; Fang *et al*., 2014, 2015). Individuals of one sex (usually male) emit acoustic signals, and individuals of the other sex recognize the signals and use these to choose an appropriate sexual partner (Andersson, 1994; Rosso *et al.,* 2006; Fang *et al*., 2012). In anurans, the male advertisement calls are the primary mating displays. Male advertisement calls are usually recognized to have follow functions: to facilitate species recognition and to provide females with criteria for choosing their partner among many males and also known to function in male spacing with some species (Castellano and Giacoma, 1998; Gerhardt and Brooks, 2009; Hernández *et al*., 2010; Wei *et al*., 2012). Most species of anurans rely on one

or more unique properties of their call (*i.e.,* fundamental frequency, dominant frequency, note duration, *etc*.) to facilitate conspecific recognition (Bickford *et al*., 2007; Kohler *et al*., 2017). Several factors can influence the properties of an anuran's call, including air temperature, body size and geographic variation (Márquez, 1995; Márquez and Bosch, 1997; Cui *et al*., 2011; Baraquet *et al*., 2015). However some anurans may alter their calling patterns and the temporal properties of their calls to meet the female preference. Many researchers suggest that the female preference may affect the male call behaviour (Gerhardt and Huber, 2002), and that it is related to the female's reproductive condition (Lynch *et al*., 2005).

Preference functions describe how female preferences vary as a function of male call trait values, and define a form of selection for male signals (Gerhardt, 1991; Wagner, 1998; Murphy and Gerhardt, 2000; Rosso *et al*., 2006). These functions take several shapes, and the overall shapes of preference functions have important consequences (Murphy and Gerhardt, 2000). Ample evidence supports the notion that female anurans prefer certain advertisement call properties over others and use these calls to evaluate prospective mates (Ryan and Keddy-Hector, 1992;

Gerhardt and Huber, 2002; Tárano and Herrera, 2003; Martinez-Rivera and Gerhardt, 2008; Fang *et al*., 2014; Velásquez *et al*., 2015). Female anurans show preferences based on call duration, call rate, and dominant frequency (Tárano and Herrera, 2003).

Call rate was found to affect female response and male mating success in anurans (Andersson, 1994; Sullivan *et al*., 1995). Pröhl (2003) used behavioural observations on strawberry poison frogs, *Oophaga pumilio*, to relate male calling behavior, particularly call rate, to mating success. Tárano and Herrera (2003) demonstrated through a series of experiments on *Physalaemus enesefae* that call rate maybe important in short-range mate choice. The frog *P. enesefae* showed strongly directional preferences for call rate. Call duration is another property that potentially influences patterns of female mate choice in several anurans (Bee, 2008). The majority of female gray tree frogs (*Hyla versicolor*) were found to choose mates on the basis of advertisement calls and prefer long calls to short calls (Klump and Gerhardt, 1987; Gerhardt *et al*., 1996) and the preference function for call duration was directional (Gerhardt *et al*., 2000). On the other hand, for its putative diploid ancestor, *H. chrysoscelis*, females were shown to prefer an average-length call over shorter-than-average calls (0.5–2.0 standard deviations below average) (Bee, 2008). The pattern of preference observed on *H. versicolor* suggests that increasing call duration beyond a certain point diminishes mating success, which has practical implication for field studies looking at the effect of variations in call duration on male mating success (Gerhardt *et al*., 2000). Welch *et al*. (1998) compared growth-rate, size at metamorphosis, larval period, and larval fitness traits, and found significant differences between the half-sibling offspring of long and short callers (Welch *et al*., 1998; Doty and Welch, 2001), demonstrating that female preferences for call duration, which is a reliable indicator of energy costs to the male (Wells and Taigen, 1986), can act as a selective force. The female preference for call rate and call duration in anurans may be a potential response to evolution (Gerhardt *et al*., 2000).

Female preference through male call response has been the subject of much study in anurans. However, few studies have dealt with the mating behaviour of the giant spiny frog, *Quasipaa spinosa* (Kong *et al*., 2016). The giant spiny frog is most distinctly characterized by keratinized skin spines on the chest, and is currently distributed across ten Chinese provinces and parts of northern Vietnam (Zhao, 1998; Zheng and Liu, 2010). Our previous studies identified three divergent lineages within *Q. spinosa* (Ye *et al*., 2013; Zhang *et al*., 2018), across which geographic variations in body size and sexual dimorphism were observed (Yu *et al*., 2010). Temporal

and spectral call parameters were analysed in our previous acoustic research; calls consisted of three to seven notes, and the dominant frequency ranged from 411–1534Hz (Yu *et al*., 2009). Very significant geographic variation was observed among the calls of *Q. spinosa*, and the variation was approximate to the species level (Shen *et al*., 2015). The detection parameter of call duration and note duration in male *Q. spinosa* are negatively related to environmental temperature, but not related to body size (Chen *et al*., 2012).

The giant spiny frog, a giant spiny frog with an extensive latitudinal distribution in south China and northern Vietnam, relies on calling to communicate with each other in breeding season, and calling plays a vital role in the entire mating process. The choice of mating made by a female is not random, but based on the male vocalization of call properties (Pröhl, 2003). The present mitochondrial DNA data showed that the frog can be divided into three lineages, and suggested that the frog existed cryptic species. Further research is needed to test whether the variation in the call can cause difficulty on the inter-lineage recognition. On the other hand, the male have acoustic signals to convey information and female use the signal to evaluate prospective mates. Whether it exists preferences on female choose the mates, and whether the female preferences would affect the male's vocal behaviour is the fundamental issue on the vocal behaviour research. In this paper, the call of different stages of the giant spiny frog will be recorded and analysed, and the trend of males during the breeding period will be explored. On the other hand, the preference of females for different calling parameters will be tested by the call playback experiment, it explores the information that may be expressed by the female preferences for call properties, and make a useful exploration for the study of the call behaviour of the giant spiny frog.

MATERIALS AND METHODS

Recording and analysis of the acoustic signals

The study was conducted from April 2014 to September 2014 collected giant spiny frogs sampled from Jinhua (29°08′ N, 119°65′ E), Zhejiang Province, China. All the frogs were released into an artificial pond, which is located near a slow-flowing stream and kept in artificial imitation of the frog's natural ecological environment. We recorded the advertisement calls of 40 male frogs that were bred in an artificial pond at five reproductive stages (mid-April, early May, late June, mid-July, and early September) during the night at 19:00–21:00. Recordings were made using a Sony PCM-D50 recorder and a Sony ECM-77B condenser lavalier microphone. Recording lasted 1–2 days

for each period, in which each calling frog was recorded for at least 30 min by placing the microphone at an approximate distance of 1 m. The air temperature varied between 17–26°C.

Noises existed in the artificial ecological environment, and we used the software Adobe Audition to reduce the noise in the recorded calls before performing the analysis (Xu *et al.*, 2005). Calls were analysed using Praat version 4.2.23 (Boersma and Wecnink, 2006) at a sampling frequency of 10 kHz and sample size of 16 bits. Eight call properties were measured, the terminologies of which were as follows (Pröhl, 2003): call duration(s), note number (n), pulse rate (pulses/s), dominant frequency (Hz), fundamental frequency (Hz), and the formant (F1, F2, and F3) (Hz).

Synthesis of the acoustic stimuli

We focused on two call properties: call duration and call rate. We used the advertisement call of the giant spiny frog as a model to study the relationship between the variation in the acoustic properties of a call and female preference. Among all the advertisement calls analysed, 4-note calls were the most common; no obvious silent period that between one note and the subsequent note were observed between the notes of a call (Yu *et al.*, 2009; Shen *et al.*, 2015). To test the effects of different call durations on female choice, we edited the natural exemplar of a 4-note call that recording in mid-April and early May with Adobe Audition. We extracted a note from the middle of a male call and repeatedly inserted it to construct a new call. Thus, the new call durations (3-note, 4-note, 5-note, 6-note, and 7-note call) were identical in call properties. To assess the significance of a distinctive call rate, we used Adobe Audition to alter the call rate of representative calls of males, and then edited the duration of the silent intercall period to make new calls of varying call rates (10n/min, 12n/min, 15n/min, 20n/min, and 30n/min call).

Experimental protocol

Using phonotaxis as a behavioral assay, we conducted no-choice experiments (Bush *et al.*, 2002) with females (n=10) collected from the Jinhua frog farm. Females were returned to the lab and placed into a professional frog-feeding bucket. Because females do not respond to calls unless they are in breeding condition, we injected captive females with HCG (1ml; 250U/ml) based on the protocol of Gordon and Gerhardt (2009). All the frogs used for behavioral experiments were returned to the farm-of-capture within five days. Phonotaxis experiments were conducted in a temperature-controlled (23±1°C), soundproof chamber. The phonotaxis arena was rectangular with interior dimensions of 160×60×20 cm.

Stimuli were delivered from a Lenovo Z475 computer using Adobe Audition 3.0 interfaced with a loudspeaker (Edifier R18T). The signals were amplified and their amplitude was controlled with the aid of the computer. We monitored the amplitude of the signals with a sound-level meter (BiaoZhi GM1351) and adjusted the fast root mean square values to 85 dB SPL.

At the start of each trial, we placed the female in an acoustically transparent release box in the other side of the arena. After 2 min stimulus, we removed the lid of the release box and measured the time until the female touched the wall of the arena, directly in front of the speaker. We recorded a score of "No Response" if the female remained in the release box for 3 min, left the release box but did not reach the speaker within 5 min, or arrived at the wall of the arena in the opposite of the speaker. The time it took a female to respond to stimulus was used as preference time in the data analysis. We presented the test trials in random order until each test stimulus was presented to 10 different females. To eliminate possible memory effects between trials, we adjusted the location of the loudspeaker every day. Because the tests measured the response strength of a single stimulus rather than the preference for different stimuli, we used the term "response function" to describe the data from single-stimulus tests. The response strength can then be used to predict female preferences (Bush *et al.*, 2002).

Statistical analyses

We calculated a phonotaxis score for each trial completed by each female, based on the relative time required to reach the loudspeaker during each trial. Relative time was calculated as the ratio of the females' response time during the control trials to her response time during the test trial ($T_{control}/T_{test}$). Statistical analyses were performed with SPSS 18.0. We employed the Kruskal–Wallis test to compare the variation in advertisement calls across the five breeding periods (Sokal and Rohlf, 1981), analyzed the trend in the advertisement call variation, and used Pearson's correlation test to calculate the correlation between different acoustic parameters (Zar, 1996). Graphic plots were generated using Origin8 Pro SR4. All values were presented as mean±SD, and the significant level was set at 0.05. We regarded the 4-note and 15n/min call set-up as the control trial. The time was the average time among the trials. A phonotaxis score of 1 therefore indicated that the female approached the test stimulus with the same response time as the average of the control stimuli; a score of less than 1 indicated that the female approached the test stimulus more slowly than she approached the control. A phonotaxis score of zero was assigned to all trials in which the female received a "No Response".

Table I.- Parameters of advertisement calls of *Q. spinosa* recorded across different reproductive states (mid-April, early May, late June, mid-July, and early September).

Call property	Mid-April (n=68)	Early May (n=177)	Late June (n=194)	Mid-July (n=142)	Early September (n=175)
Call duration (s)	1.48±0.32	1.54±0.29	1.18±0.23	0.99±0.18	1.02±0.21
Note number (n)	4.31±0.79	4.63±1.01	4.25±0.84	3.99±0.86	3.97±0.73
Dominant frequency (Hz)	926.63±303.69	1015.72±205.71	973.21±258.98	1009.11±238.51	868.48±265.26
F1 (Hz)	774.82±86.01	874.06±106.49	763.42±134.69	850.61±106.47	786.56±137.26
F2 (Hz)	1231.12±77.96	1185.19±76.39	1121.43±99.39	1155.83±52.43	1214.39±107.16
F3 (Hz)	2028.63±140.58	2022.16±231.42	1689.61±255.66	2040.05±269.55	2454.26±256.22
Fundamental frequency (Hz)	387.62±76.93	423.83±67.07	420.19±62.43	474.91±47.48	438.81±65.32
Pulse rate (p/s)	136.72±59.11	181.01±63.47	235.24±71.66	238.71±68.11	229.76±82.48
Pulse (p)	197.31±82.12	277.26±103.77	277.95±95.51	237.58±82.98	234.90±98.99

A total of 756 calls were recorded. Data was expressed as mean±standard deviation (mean±SD). Significant level was set at 0.05. *Based on calls recorded at an approximate distance of 1 m.

Conversely, the phonotaxis score can exceed 1 if the female approached the test stimulus more rapidly than the control stimulus. Although there was considerable variation in the control values of the individual females, no tendencies were observed for response times to change over the course of any of the experimental series. Even if gradual changes of response times were to occur (*e.g.* due to fatigue), our analysis would be unaffected because we normalized the test trials using the control trials.
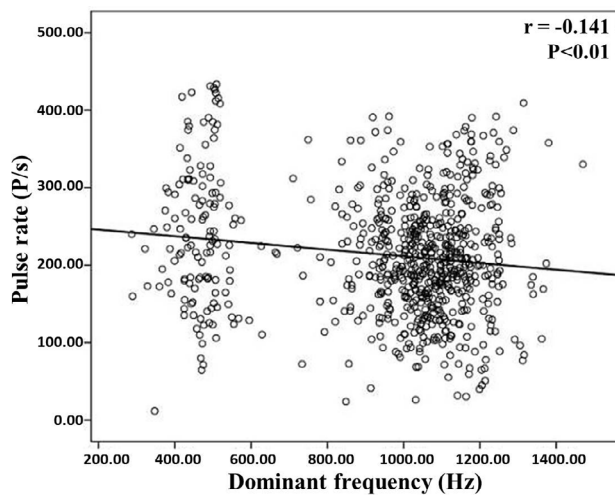

Fig. 1. Linear relationship between pulse rate and dominant frequency for *Q. spinosa*.

RESULT

Call analysis

Breeding started from the beginning of April to late September. The calling behaviour of male *Q. spinosa* always took place at dusk and lasted for the whole night during the breeding and mating season. A total of 756 calls from 40 males were recorded during the five reproductive stages. Recorded calls had mean call duration of 1.22s (SD=0.328s), a mean dominant frequency of 961.47Hz (SD=255.539Hz), and a mean fundamental frequency of 432.71Hz (SD=67.526Hz) (Table I). The pulse rate of male calls was negatively correlated with the dominant frequency (r=-0.141, P<0.01, Fig. 1) and positively correlated with fundamental frequency (r=0.151, P<0.01, Fig. 2). No significant correlation was found between the dominant frequency and fundamental frequency (r=0.036, P>0.05).
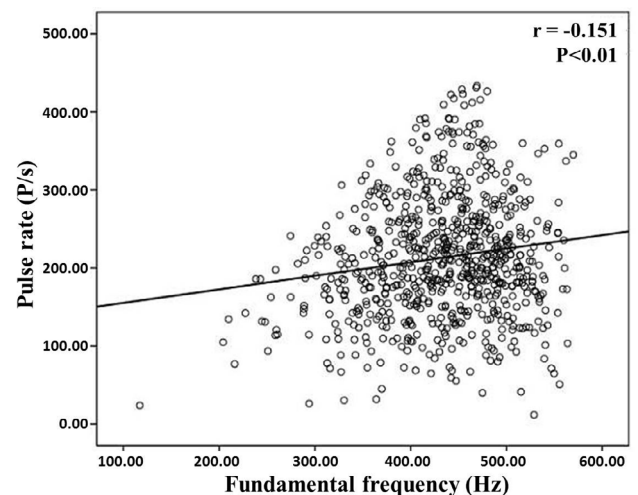

Fig. 2. Linear relationship between pulse rate and fundamental frequency for *Q. spinosa*.

Descriptive statistics for call characteristics of different reproductive stages are given in Table I. In these periods, Kruskal–Wallis tests showed highly significant

differences in dominant frequency ($\chi^2_{4,771}$=44.599, P<0.01), fundamental frequency ($\chi^2_{4,771}$=102.216, P<0.01), F1 ($\chi^2_{4,771}$=96.439, P<0.01), F2 ($\chi^2_{4,771}$=101.978, P<0.01), and F3 ($\chi^2_{4,771}$=411.065, P<0.01). Several call properties, such as dominant frequency, fundamental frequency, F1, and F2, presented different variation tendencies in the five breeding periods. A broken line graph shown in Figure 3 illustrates

these changes. Dominant frequency and F1 showed an "M" type of variation, and the values of the periods of early May and mid-July were similar, which were higher than the other periods. F2 first decreased then increased over the periods, with a minimum value in late June. Fundamental frequency first increased and then decreased over the periods, with a maximum value in mid-July.
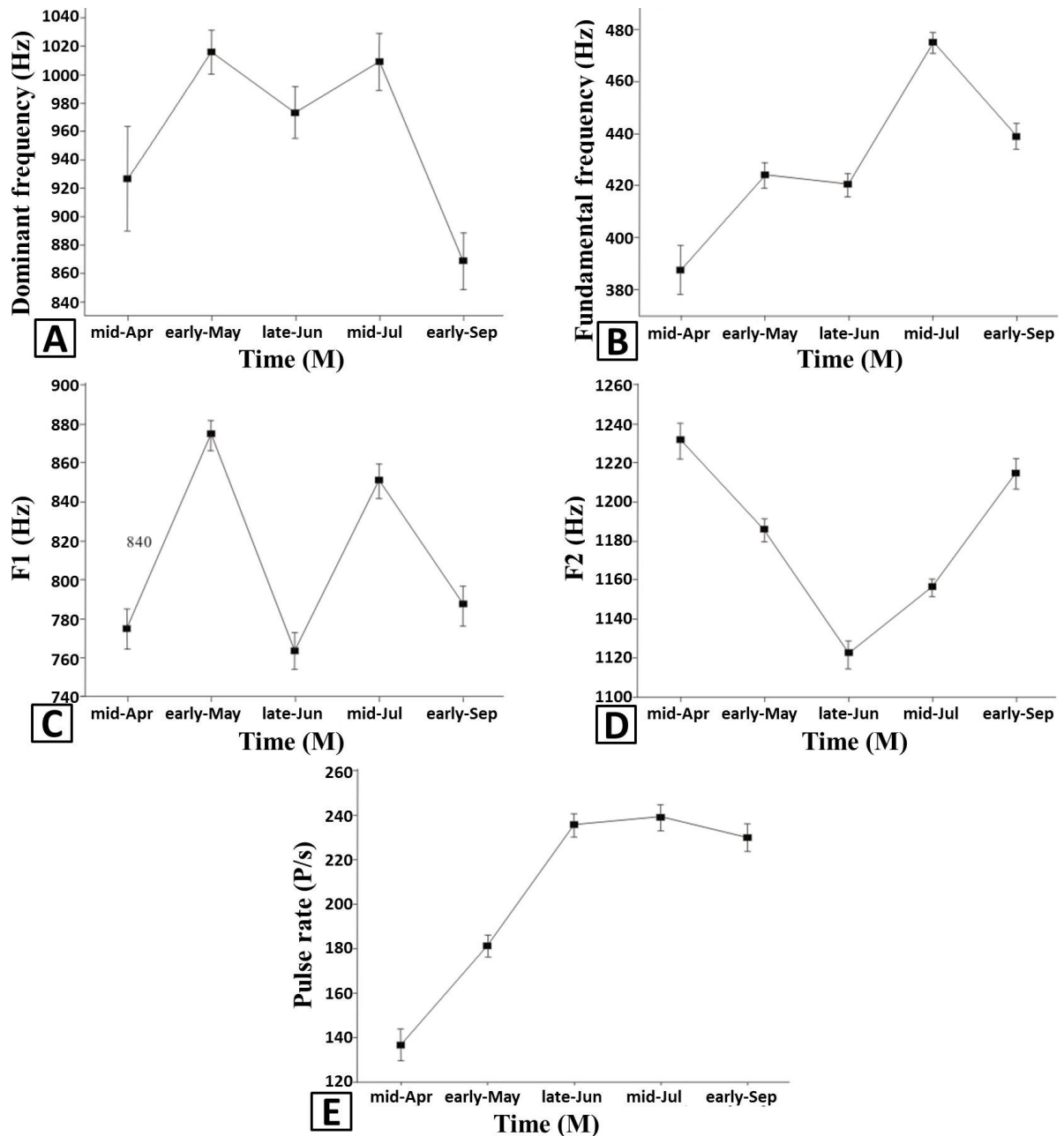

Fig. 3. Different variation tendencies in dominant frequency (A), fundamental frequency (B), the first formant (F1, C), the second formant (F2, D), and pulse rate (E) across five breeding periods (mid-April, early May, late June, mid-July, and early September). Each point represents the mean (±SE) acoustic signals of 40 males.
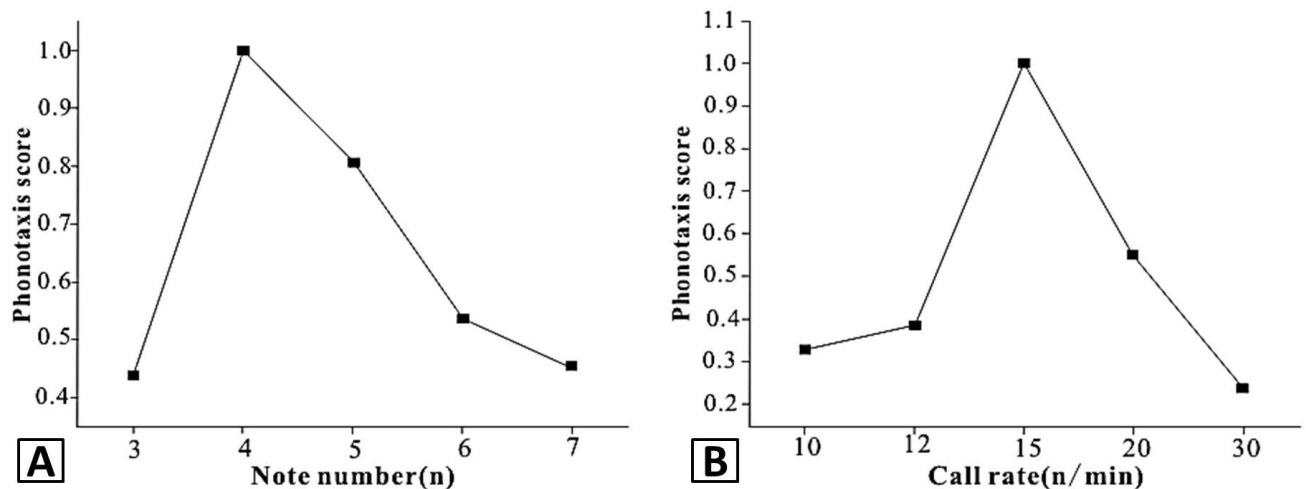
Fig. 4. Phonotactic scores of females to call models varying in call duration (A). Scores were calculated by dividing each control trial by the mean control trial of that female. Each point represents the mean (±SE) of 10 females. The number of notes used in the control trials was 4. Graphs illustrating the phonotactic scores of females to call models varying in call rate (B). Each point represents the mean (±SE) of 10 females. The call rate used in the control trials was 15n/min.

Female preference

In the first no-choice experiments, we measured the female phonotaxis preference based on call duration (Fig. 4A). In the test, high scores occurred only with the 4-note call duration (which approaches the mean call duration for mid-April and early-May). Whereas longer and shorter durations resulted in lower scores. For longer call durations, phonotactic scores were close to 0.45, whereas for shorter durations, the scores remained well within 0.40. Thus, the calls with longer duration were not attractive and the female approached them slowly. The influence of call rate on female response in our no-choice tests is shown in Figure 4B. Phonotactic scores were high (>0.9) for call rates close to 15n/min. For higher and lower call rates, the response values dropped, with a mean score of 0.37.

DISCUSSION

The preferences of females for phonotaxis have been studied extensively in insects and frogs in the context of mate attraction (Bush *et al*., 2002; Klymus *et al*., 2012; Reichert and Ronacher, 2014). Two methods were regularly used to assess such preferences: (i) choice tests, in which responses to one of two or more alternative signals are tabulated, (ii) single-stimulus tests, in which female responses to one signal are measured. The choice tests were used frequently in studies on anuran female preference. In a choice test, the shape of the preference function can only be determined if multiple stimulus pairs are presented or the stimuli cover a broad range of signal values (Gerhardt, 1991; Wagner *et al*., 1995). The choice test needs to compare female choices under different call signals which will detect the preference of females, and the more call signals to be detect, the more choice tests to be done. In addition, more choice tests may cause females to show weak preference, which may affect the accuracy of the experiment (Bush *et al*., 2002). Single-stimulus tests are less affected by this constraint, and prevents females that are engaged in repeated sampling from displaying weaker preferences. For the above reasons we used the single-stimulus test to investigate the shapes of female preference functions for acoustic signals.

For the study of most anurans, the acoustic signals are used to evaluate prospective mates, and the different call properties are taken to mean different energetic investments. We have analysed the eight call properties of advertisement call variation among populations of *Q. spinosa* across the five reproductive stages. Our results demonstrated that male *Q. spinosa* calls vary at different reproductive stages, and the call properties show different trends. The trends of some call properties, including fundamental frequency, dominant frequency, and formant, which are not affected by the external environment and reflect the male's own state, or calling behaviour strategy during a breeding period. The trends in dominant frequency and F1 were the "M" type, and the higher values appeared in early May and mid-July, which was the peak period of female breeding. According to field observations, the male often showed a more positive vocal behaviour during those periods. The higher values of the dominant frequency and FI may be a calling strategy for improving the identification

of calls. The change in male vocal characteristics is not blind, perhaps to cater to female preferences. The female's choice preference is flexible and related to the reproductive stage of female frog in *Q. spinosa*.

According to the diagram of constructed preference functions, we found the females displayed the shortest response time to a 4-note call duration and with a call rate of 15n/min. As the values of call duration and call rate changed, the response time increased significantly. The female preference for these two call properties was subject to stabilizing preferences, which means that the female phonotactic scores was highest when the values of call characteristics achieve a 4-note call duration and a 15n/min call rate. Our study suggests selection by female preference for males producing calls of average duration and rate. In anurans, calling has a high energetic cost, such that call and call rate are assumed to be under strong energetic constraints (Wells and Taigen, 1992). The call duration and call rate reflect energy investment, and long call durations and high call rate always mean high energetic costs. However, the advantage of mating with extreme conspecifics may be diminished by the risk of mating with heterospecifics (Wollerman, 1998; Welch *et al*., 1998; Richardson *et al*., 2010). Males with calls of long duration or high rate may be at a high predation risk, because their calls not only attract the attention of the female but also the predator (Sun and Narins, 2005). Bonachea and Ryan (2011) suggested that predation risk not only increases thresholds of mate attractiveness or even reverses preferences for normally attractive traits, but also reduces evaluation time and mate sampling. Hence, the preference of female *Q. spinosa* around the mean call duration and call rate may be a trade-off, it may decrease the risk of mating with heterospecifics, reduce the predation risk, and ensure that the females gain moderate fitness from choosing a mate with mean call properties. The female choice may be more complicated than originally assumed for natural conditions, because in the present study the male call and female choice were limited by many factors such as the acoustic environment and living environment resources. For future studies these factors need to be considered, as well as the combination of field observation and experimental research, to accurately recognize the female choice behaviour of *Q. spinosa* and provide a theoretical basis for further research on calling behaviour.

CONCLUSION

In conclusion, our data support the hypothesis that female *Q. spinosa* have noticeable mating preferences on the call qualities of call duration and call rates. Our findings also show that male call properties have seasonal variations and males may adjust their call properties to attract a female in different stages of reproduction. However, this evidence should be supplemented with studies of other characters such as environmental factors. More insight should be gained on the meaning and function of every call property to assert whether the female preference affects the male call and to examine the female preference across all male call properties.

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