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Vocal behaviour and intraspecific call variations of Kempholey Night Frog (*Nyctibatrachus kempholeyensis*) in the Western Ghats, India

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ABSTRACT

Signal analyses, basic call descriptions and call variations are the basis for assessing repertoires of individuals and species and also play a role in understanding social and breeding behaviour of frogs. The primary objective of this study was to describe call structure and repertoire of *Nyctibatrachus kempholeyensis* and to quantify call properties. The secondary objective was to investigate potential sources of variability of these properties, and thirdly to examine individual variations in their vocalisation both within and among individuals. The study was based on a sample of 627 calls recorded and analysed from 30 individuals in Mavingundi, Uttar Kannada, Karnataka State, India. The vocal repertoire of *Nyctibatrachus kempholeyensis* consists of two call types: a shorter and rarer Call B type and a more common and complex Call A type with two different notes in it. Temperature did not affect any of the measured call properties, neither other environmental variables, but body weight and SVL had statistically significant influence on call duration, Note 2 duration, silent interval and call rate. Silent interval, inter-note interval and dominant frequency of Note 1 were classified as dynamic call property, while all other call traits were intermediate. The ratios of among-individual to within-individual variability were greater than 1.0 which can indicate individual recognition cue. Dynamic call properties can reveal information on mate quality, however silent interval and call rate are generally dependent on motivation and disturbance. Differences among individuals in their acoustic behaviour might be important in determining their success in attracting a mate, but correlations between male mating success and call behaviour is difficult to detect.

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1. INTRODUCTION

The estimated number of species on Earth varies between 7.4 and 10 million (Mora et al. 2011). Of these, 7658 are amphibians (AmphibiaWeb 2017), but the number of new species is increasing rapidly due to an intensified effort of exploration (Aravind & Gururaja 2011). In addition, species are recognised based on the use of molecular tools in integrated taxonomic surveys (Vieites et al. 2009) and related comparative bioacoustic analyses.

At the same time, amphibian populations are declining and becoming extinct at an increasing rate (Stuart et al. 2004) – well exceeding the historical background estimates (McCallum 2007). This is due to habitat loss, degradation, fragmentation and disturbance (Wells 2010), pollution (Sparling 2010), the chytrid fungus Batrachochytrium dendrobatidis (Molur et al. 2015), global changes in climate and UV radiation (Collins & Storfer 2003) and the synergistic effects of all above mentioned reasons (Vonesh et al. 2010). In general, amphibians are sensitive to environmental change and are therefore often used as indicator taxa (Welsh & Ollivier 1998). They are vulnerable because of their permeable skin, use of multiple habitat, dependence on water, complex life cycles, habitat isolation and specialisation, population structure, limited geographic range, small body size, ectothermic metabolism, limited dispersal ability, anti-predator behaviour, and boom and bust population cycles (Wells 2010).

As a consequence, amphibian decline may have knock-on effects on other trophic levels and thereby threaten other species, ecosystem structure and function. For example, Whiles et al. (2006) demonstrated consequences for primary production, shifts in algal community and consumer structure, and organic matter dynamics. This is because amphibians play diverse roles in ecosystem functions. On the one hand by – tadpoles - feeding on algae and phytoplankton (Crump 2009; Wells 2010). On the other hand, amphibians serving as prey for several species, for example snakes, other anurans, birds, aquatic insects, snails, fishes and small mammals (Aravind & Gururaja 2011; Wells 2010). Furthermore, amphibians also have economic relevance for humans (Aravind & Gururaja 2011; Crump 2009).

The Indian subcontinent provides home for a unique array of flora and fauna because of its successive and long periods of isolation (Roelants et al. 2004). Over 300 amphibian species are currently known to occur here (Dinesh et al. 2013). These include frogs and toads, caecilians and salamanders, portraying high-level endemism particularly in the Eastern

Himalayas and the Western Ghats (Roelants et al. 2004). The Western Ghats mountains are classed as a biodiversity hotspot (Myers et al. 2000), where more than 92% of the known 225 amphibian species are endemic (Garg et al. 2017), but diversity may be severely underestimated in this region (Biju et al. 2014). The high level of endemism is perhaps a result of the discontinuity in the mountain chain that restricts dispersal (Naniwadekar & Vasudevan 2007). The endemic species are confined to the rainforests and include the genera Ghatophryne, Xanthophryne, Micrixalus, Melanobatrachus, Ramanella, Nasikabatrachus, Nyctibatrachus, Indirana, Ghatixalus and Raorchestes. The families Dicroglossidae, Rhacophoridae and Nyctibatrachidae account for 50% of the total species richness in the Western Ghats (Aravind & Gururaja 2011).

The Nyctibatrachidae family evolved between the Cretaceous and Palaeocene, coinciding with the time that the Indian sub-continent was isolated from Gondwana (Van Bocxlaer et al. 2012). It consists of the genus Lankanectes, confined to Sri Lanka and the genus Nyctibatrachus endemic to the Western Ghats (Van Bocxlaer et al. 2012). The family today contains 36 known species (Garg et al. 2017; Krutha et al. 2017). The first Nyctibatrachus species, or Night Frog species as they are commonly known (Garg et al. 2017), was described in 1882 by Boulenger and its taxonomy was revised recently by Biju et al. (2011) . The distribution of Night Frogs stretches through the Western Ghats, from northern Maharashtra to Tamil Nadu in the south (Biju 2011). They are associated with torrent mountain streams most of the time (Aravind & Gururaja 2011), but they can also be found in leaf litter (Priti et al. 2015). Their size (snout-vent length) vary between 10 and 77 mm and they can be identified by their brownish dorsal colour, glandular wrinkled skin, rhomboid pupil, notched tongue and pointed vomerine teeth (Biju 2011).

Anuran reproductive modes vary widely and exhibit unique features between species. This diversity, with forty distinctive reproductive modes, is due to environmental, evolutionary and ecological selective pressures (Aravind & Gururaja 2011). A common categorisation incorporates the different modes of egg deposition (sites) and tadpole development. In addition, there are variations in fertilisation mode (external or internal), in amplexus (inguinal, axillary, cephalic or the lack of it), in egg and clutch size and in reproductive effort (Wells 2010). In tropical regions, many anurans breed throughout the long rainy season, some even all year round, but with varying intensity depending on the rainfall (Wells 2010).

Active partner choice by females is a process where females assess and select males with the most attractive characteristics, i.e. the ones offering the most benefits. The direct benefits increase the reproductive performance of females via access to resources (e.g. high quality oviposition sites) possessed by the male, by parental care or choosing the most fertile male. They also avoid disease - or parasite - infected males, and by selecting males that can be easily located, they reduce search time, and predation risk. The indirect benefits include traits that increase the genetic quality of the offspring (Wells 2010).

Females may base their choices of partner on resource quality, size, parental care, chemical cues or calling activity. Females can also select mates on the basis of physical traits such as male size. This may indicate certain fitness-related traits in the offspring, for example higher survival or faster growth rates. In a wider context, territory quality and parental care assessment can also be considered as visual cues. When males defend oviposition sites and these vary in quality, it is possible that females chose mate on this basis, although male size and territory quality can be correlated (Wells 2010).

Calling activity or vocalisation, is a sort of information transfer that is practiced by several animals from insects to whales (Badrinath & Gururaja 2012). Amphibians produce a wide range of sounds depending on the context, so it can signal distress cause by a predator, warning, release (from the grip of another male), territory boundary, and advertisement. Calling behaviour and call characteristics of males are important in mate attraction, female mate choice and male mating success in frogs (Pröhl 2003). Each species have distinctive 'singing', and advertisement calls can happen in a chorus or individually. Reproductive calls are the most commonly encountered, mostly emitted by males during the breeding season. Advertisement calls typically have two functions: attracting mates and signalling territorial information to conspecifics (Koehler et al. 2017).

Basic call descriptions and their variations play a role in understanding the social and breeding behaviour of frogs (Hopp et al. 2012). These variations can occur at different levels, namely within individuals, between individuals in the same population, and between individuals of separate populations. Acoustic divergence within and between individuals could be influenced by male motivation, which in turn can depend upon external (e.g. environmental impacts) and internal factors. Variation can be expected to be the lowest within individuals, then between individuals of the same population, and the most between individuals of different populations (Koehler et al. 2017). In addition, bioacoustic variations among individuals might offer cues for females' choice (Gambale et al. 2014). On the within-individual level, the less variable (static) call properties might mean species recognition and individual identity, whereas the more dynamic call traits inform about mate quality and directional female preferences (Gerhardt 1991).

As above mentioned, environmental and morphologic factors often influence calling behaviour. Temperature, for instance regulates the vocal activity period and the characteristics of calls, whereas body size is generally strongly correlated with the dominant frequency of signals. This means that smaller frogs emit higher frequency calls because of their shorter vocal cords (Koehler et al. 2017).

Kempholey Night Frogs (*Nyctibatrachus kempholeyensis*) are abundant and can be found in the torrent streams of evergreen and semi-evergreen forests as well as at shaded edges of agricultural areas. This small sized nocturnal species occupy semi-aquatic and aquatic habitats where they mainly breed along the shallow stream edges and slow-flowing waters. They display nocturnal breeding activity and courtship behaviour in the form of advertisement calls (Gururaja 2012).

Although Nyctibatrachus species are relatively well-studied in terms of morphological descriptions, genetic relationships and natural history (Garg et al. 2017), research on behavioural aspects, vocal repertoires, call structure and intraspecific variation are relatively few. The basic call structure of *Nyctibatrachus kempholeyensis* was described by Gururaja et al. (2014) examining call duration and dominant frequency mainly for comparative purposes with *Nyctibatrachus kumbara* and *Nyctibatrachus jog* species. This study attempts to provide a more detailed acoustic description in other call properties, as well as examining individual differences to know more about their role as a female attractant among males.

Therefore, the primary objective of this study was to describe the call structure of *N.kempholeyensis.* I also investigate potential sources of variability of call properties, such as body size and temperature, and examine within- and among-individual variations in vocalisation.

2. MATERIALS AND METHODS

2.1 Study area

The study area was located in the Southwest coastal region of India, within the central Western Ghats mountain range in the Sharasvathi River basin (Fig.1). This wet hilly region has tropical monsoon climate coming from southwest which begins in June and lasts up to November with a mean annual rainfall of c. 7500 mm. High humidity, continuous cloud cover and mostly quiet winds characterize the wet season and the region in general. The temperature varies between 21 and 26°C during the season.

Rice paddy fields and Areca nut plantations intermit the natural vegetation of dense tropical evergreen rainforest ecosystem that provides home for diverse flora and fauna, many of which are endemic.

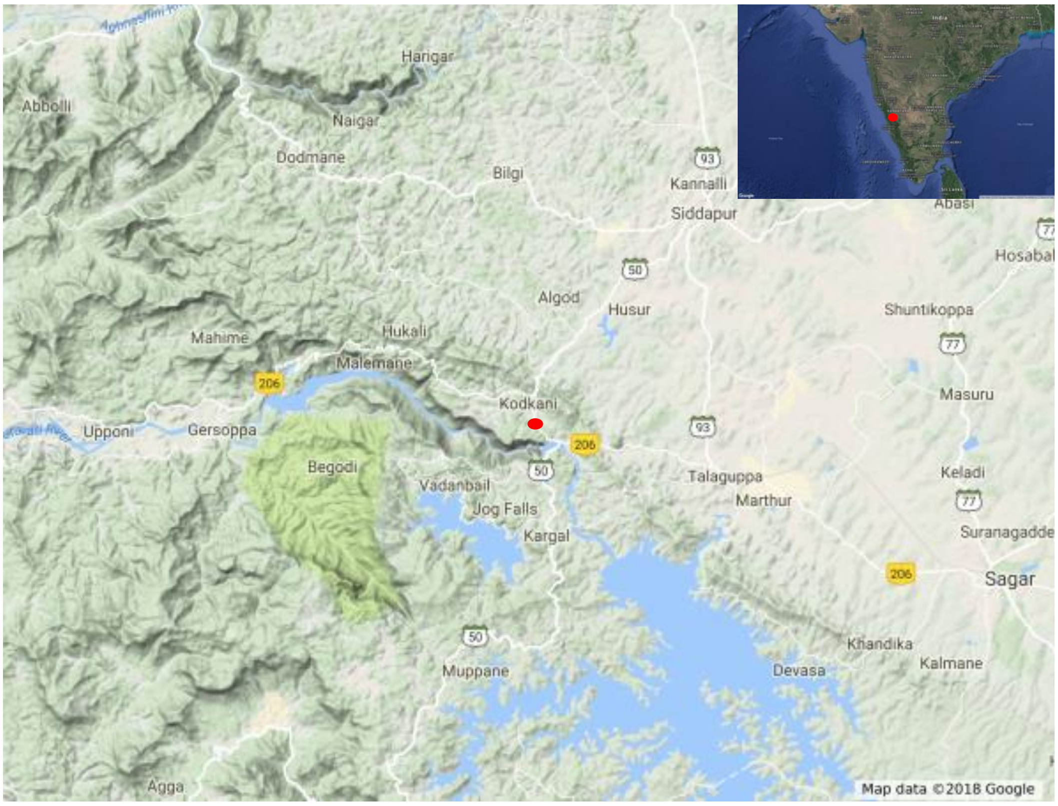

Figure 1: Location of the study site in the Western Ghats mountains on the Indian sub-continent (insert)mand the local placement of the study area within Siddapur region of Karnataka State

The study took place at two sites close to Mavingundi, which is a small hamlet of Kodkani village, in Siddapur subdivision, in Uttar Kannada District of Karnataka State, India. The study sites are part of the down streams of Sharavathi River catchment and consist of a matrix of evergreen forests, Areca palm plantations and paddy fields. The first site (14.25204-

14.25291°N, 74.80120-74.80210°E, 541 m above sea level) is located in small areas of uncultivated and cultivated Areca plantations (c. 200 m²) and uncultivated rice fields that are currently used as grazing land for cattle and buffalos (Fig.2A). Rice cultivation began about 90 years ago and lasted until 1991 (personal communication with Ashok Hegde). Kempholey Night Frogs can be found along the shaded edges of abandoned paddy fields, Areca gardens, and along the stream.

The second site (14.25541-14.25715°N, 74.80612-74.80703°E, 602 m above sea level) is located north of the Mavingundi Falls and consists of forest, streams and a power line clearing (Fig.2B). For the power line (at the north end of the site) trees were cut about 60-65 years ago and this small patch is occasionally used for grazing. There is a small dam at the southern end where the stream is diverted towards the hamlet for drinking purposes (personal communication with Ashok Hegde). The status of the forest is Reserved Forest with minor forest use such as honey and fruit (*Garcinia gummigutta*, *Artocarpus gomezianus*) extraction (personal communication with forest guards in Mavingundi).

Figure 2: Map of the study sites with the two sites at Mavingundi.

2.2 Study species

Three Night Frog species are found at Mavingundi, namely *N. jog*, *N. kumbara* and *N. kempholeyensis*. The latter were chosen for this study because they are more widely distributed in the study area compared to the two other species, however they are the least easily detectable due to their smaller size.

Nyctibatrachus kempholeyensis is distributed in streams at Jog falls, Someshwar, Kempholay, Muthodi and Kemmanagundi in Karnataka, and at Banasura and Suganthagiri in Kerala (Biju 2011). The breeding and adult habitat of the species is confined to perennial streams and shallow water - both of which are filled with leaf litter and organic mulch – in evergreen and semi-evergreen forest. These nocturnal frogs can be found on and under low vegetation, organic litter and small rocks and stones.

Figure 3: Photo of Lali (SVL: 19.67 mm; weight: 1.59 g).

Species identification was based on the following physical characteristics: average size 18-26 mm, 'square' appearance of glandular folds on dorsum, rhombus and horizontal pupil, Y shaped ridge on snout (Gururaja 2012) (Fig.3), males secondary sexual characters based on their yellowish femoral gland and pared lateral vocal sacs in whitish colour (Fig.4) and females lay relatively large pigmented eggs (1.2 ± 0.4 mm) (Biju 2011).

Figure 4: Photo of Tommy with laterally inflated vocal sacs during calling. (Measurement were not taken.)

2.3 Data collection

A preliminary study determined the peak activity period by temporal mapping of frogs with observations every half an hour from 18:00 to 6:00. Calls were occasionally heard during daytime from shaded parts of paddy, Areca plantation and forest edges, but calling generally started at 19:00 and peaked between 20:00 and 24:00. The call activity pattern is presented in Appendix 1. Similarly, spatial mapping was done in three different 100x100m quadrats. Within these quadrats, 10x10m quadrats were randomly selected and visited to assess *N. kempholeyensis* distribution. At each sample site, 10 minutes were spent detecting frogs. GPS coordinates and elevation data were provided by Garmin Etrex 30.

Figure 5: Photo of Luke with 2 clutches of eggs. (Measurements were not taken.)

Mating behaviour begins with advertisement calls of the male when the paired vocal sac inflats along their side (Fig. 4). After the female approaches the male, he mounts and grasps her by the arm pits forming axillary amplexus. They remain in amplexus for about 3-5 minutes, while they usually search for an ideal oviposition site by turning around together. Then the female slowly releases the pigmented eggs ($\bar{X}$=6.3, n=8) at average height of 8.7 cm (n=12) and leaves. Oviposition materials varies between Indian Pandanus, black pepper leaves, other shorter bushes, grass blades, higher stemmed plants (Fig.5), dead leaves (Fig.6) or sticks (Fig.7). The male stays with the eggs and sometimes starts calling soon after mating. He exhibits parental care by protecting the eggs for several days.

Figure 6: Photo of Kumar with a clutch of eggs on a dead leaf (SVL: 21.83 mm; weight: 1.57 g).

Figure 7: Photo by Amit Hegde of a male protecting his egg clutch. (Measurements were not taken.)

The subsequent study on call structure took place between 23rd of August and 9th of September 2017. *N. kempholeyensis* found using a flashlight and by tracing calling males below and on the short vegetation along the edges of streams and flowing water. Frogs typically called from grass, bushes, dry leaves or from stones and sticks at height between ground level and 20 cm. After finding the target individual, their calling was recorded from a distance not greater than 30 cm with a Zoom H1 recorder (in WAV format at a sampling rate of 96 kHz/24 Bit). After this, the males were captured, the snout-vent length (SVL) measured with a digital vernier calliper (Powerfix) and weight measured with a small digital balance (GuanHeng) for the purpose of examining the influence of these morphometric traits on call properties. Strong site tenacity of territorial males allowed reliable individual identification, but to exclude the possibility of double-sampling, dorsal photos of the frogs were also taken. This reveals individual differences in a non-invasive manner. These measurements did not last longer than 5 minutes, and frogs were immediately released. In total, thirty individual males were recorded and measured.

In addition, other measurements were also recorded in order to investigate their influence on the call properties. They are the following: weather condition (light rain/no rain); lunar cycle (waxing crescent/first quarter/waxing gibbous/full moon/waning gibbous); air temperature and relative humidity (by using Brunton ADC Pro digital thermo-hygrometer). On the following days the estimation of canopy cover (crown closure) took place at the breeding site using Glama android application.

Furthermore, individual male observations took place between the 11th and 30th of September (2017), by visiting eight individuals three consecutive days for clutch height, clutch size and behavioural activity observations.

2.4 Acoustic analysis

Call recordings were imported to the audio analysis software, Raven Pro 1.4, a commonly used tool for biological sound measuring, analysis and illustration (Koehler et al. 2017). The call structure analysis was based on the note-centred approach. From the waveform display (oscillogram), temporal measurements were quantified, such as call duration, note duration, call rate, note repetition rate, silent interval, and inter-note interval. From the spectrogram, the spectral properties such as dominant frequency of calls and notes were measured (256-point fast Fourier transform, Hann window, 50 % overlap, 539 Hz resolution) (Table 1). High signal-to-noise ratio allowed for the precise determination of the start and ending time of calls and notes. A minimum of 7 and maximum of 43 calls ($\bar{x}$=15) were recorded and analysed of each male, and each recording was limited to maximum two minutes. In the following, I present an overview of the measured call properties (Table 1).

Table 1: Detailed description of measured call traits following Koehler (2017).

Call duration	The duration of a single call, measured from the beginning to the end of the call.
Call repetition rate	The number of calls emitted in a defined period of time, usually in a calls/minute unit.
Dominant frequency of call and note	The peak frequency of the call or note, which is the highest sound energy. It is measured in Hz or kHz. (Frequency is the number of oscillations per second, simply as the pitch. Higher notes have high frequencies, i.e. more waves, and lower notes have lower frequencies, i.e. fewer waves.)
Silent/inter-call interval	The time between two consecutive calls which is measured from the end of a call to the beginning of the next call.
Note	The main subunit of a call, a 100% amplitude modulation (a change from maximum relative amplitude to full silence) with short intervals between them.
Note duration	The time measured from the beginning of a note to its end.
Inter-note interval	The time between two consecutive notes within the same call. It is measured from the end of one note to the beginning of the next note.

2.5 Statistical analysis

All statistical analyses were conducted in *R* (R Core Team 2018) with significance level set at P=0.05, and using packages *ggplot2* (Wickham 2009) and *tidyverse* (Wickham 2017). All measurements were summarized per individual and these values were used in the statistical comparisons. Data collection from two different sites was done to ensure adequate sample size and to avoid spatial autocorrelation. (Site was included in the statistical analyses as an independent variable, however comparison was not done between them.)

As calling is an energetically expensive activity, it is dependent on temperature due to the ectothermic nature of frogs. Environmental temperature can influence the characteristics of vocal signals, in particular the temporal features (Koehler et al. 2017). Similarly, body size (SVL) can highly correlate with certain call traits, particularly with dominant frequency (reviewed in Wells 2010). Therefore the association between air temperature, body size, body mass, weather condition, lunar phase, canopy cover, site and acoustic features were examined using multiple regression analysis. For each call trait, environmental and morphological factors were included as independent variables. In addition, stepwise regression analyses with ‘backward’ method were also conducted that reduced variables until only the significant ones are left in order to eliminate masking effects.

To assess call variability within and among individuals, coefficients of variations ($CV=100\%*SD/\bar{X}$) were calculated. Within-male coefficients of variation (CV_w) were based on the mean and standard deviation of each call properties for each male. To distinguish between static and dynamic traits, Gerhardt (1991) suggested that CV values less than 5% are static, and CV values more than 12% are dynamic traits. The values between 5 and 12% are considered intermediate (Gerhardt 1991). Among-individual coefficient of variation (CV_a) were calculated from the grand mean and standard deviation based on the average values of individuals (Gasser et al. 2009). The ratio of among-individual and within-individual variation was determined as CV_a /CV_w in order to see variability among males. If the CV_a /CV_w ratio is > 1 for a given call property, it is relatively more variable among individuals and it might function as individual recognition cue (Bee et al. 2001; Jouventin et al. 1999).

3. RESULTS

3.1 Call structure

Kempholey Night Frog males produced two distinctive call types during their advertisement calls: a common Call A type (Fig.9 and 10), and a rarer and much shorter Call B type (Fig.8). Call A (Fig.9 and 10) consists of multiple notes per call and is a complex call because it contains two different note types. These notes are organised into one initial note of

Note 1 followed by a series of Note 2 types in rapid succession. Note 2 could be placed between pulsed and pulsatile notes, but more towards the pulsatile part of the continuum (personal communication with Miguel Vences).

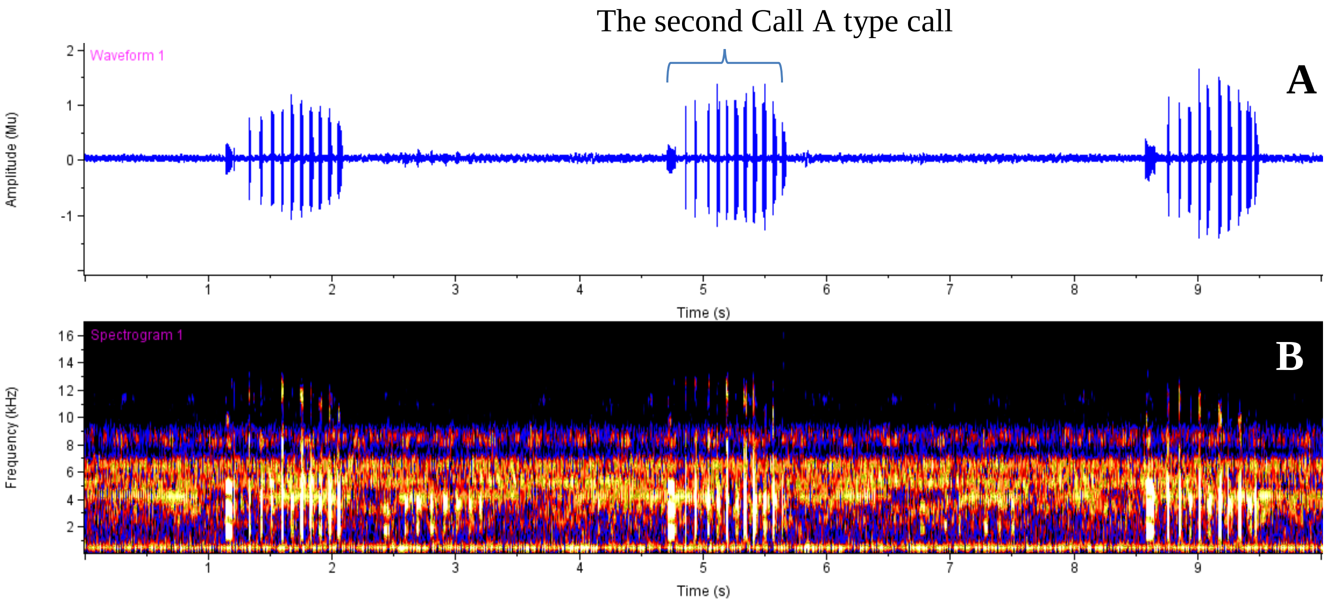

Figure 9: Oscillogram (A) and spectrogram (B) of three consecutive calls of Call A type (segment of 10 sec) of Walter (SVL=21.74 mm and body weight=1.5 g). Air temperature: 25.05ºC. Calls were emitted in general advertisement context and **s**ilent intervals are visible between the calls.

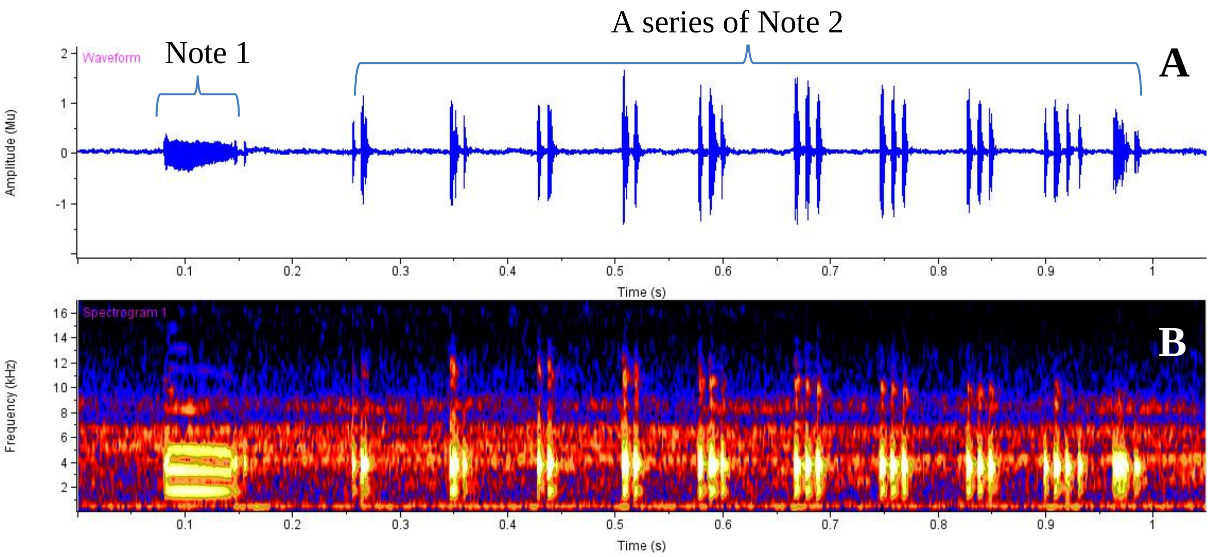

Figure 10: Oscillogram (A) and spectrogram (B) of one call of Call A type (the third call from Fig.9) (segment of 1 sec) of Walter (SVL=21.74 mm and body weight=1.5 g). Air temperature: 25.05ºC. The first, flat note is type Note 1 and the subsequent notes are Note 2 types.

Call B type is a simple call, which sounds like whining, consisting only one note type within the call. Its mean duration is 0.49 second, its average call rate is 11.5/minute and its mean dominant frequency is 3410 Hz (Table 2). They were emitted in rapid succession with average an silent interval of 1.29 seconds, but only by three males (the analysis was based on 168 calls). They were often followed by Call A types (after about 3-15 call of Call B). These calls were heard when another male was close-by. Its further examination is outside the scope of this study.

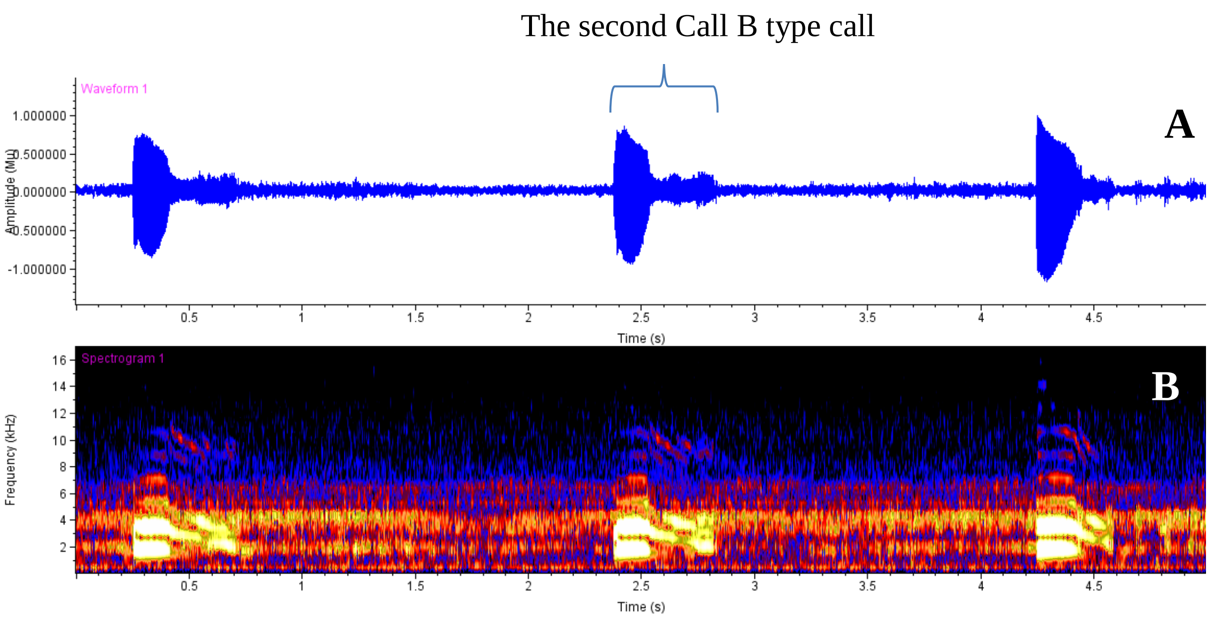

Figure 8: Oscillogram (A) and spectrogram (B) of three consecutive calls of Call B type (segment of 5 sec) of Chris (SVL=19.21 mm and body weight=1.34 g). Air temperature: 22.65ºC. Calls were emitted in general advertisement context and **s**ilent intervals are visible between the calls.

Spectral and temporal call features are given in Table 2. The two call types mainly differed in call duration and call repetition rate. The mean call duration of Call A ($\bar{X}$=0.89 s) was almost double of Call B ($\bar{X}$=0.49 s), while the mean call repetition rate (in min/call) of Call B ($\bar{X}$=11.5) was much higher than Call A ($\bar{X}$=3.9). However, dominant frequencies of the two types of calls were similar (Call A: $\bar{X}$=3489 Hz and Call B: $\bar{X}$=3410).

Table 2: Descriptive statistics of Call A and Call B properties based on mean individual values of 30 and 3 *Nyctibatrachus kempholeyensis* males at Mavingundi, Uttar Kannada, Karnataka State, India. Air temperature variation was between 20.8 and 25.6°C.

	Call A		Call B	
Call properties	$\bar{X}$ ± SD	Range (n=30)	$\bar{X}$ ± SD	Range (n=3)
Call duration (s)	0.89 ± 0.18	0.45-1.41	0.49 ± 0.003	0.491 -0.498
Note 1 duration (s)	0.06 ± 0.01	0.04 – 0.07	–	–
Note 2 duration (s)	0.71 ± 0.17	0.25 – 1.19	–	–
Silent interval (s)	11.87 ± 6.23	2.28 – 24.72	1.29 ± 0.46	0.76 – 1.58
Inter-note interval (s)	0.12 ± 0.03	0.07 – 0.21		–
Call repetition rate	3.9 ± 2.3	1.3 – 11.5	11.5 ± 5.8	4.8 – 15
Note repetition rate	7.6 ± 1.8	2.8 – 12.2	–	–
Call dominant frequency (Hz)	3489 ± 410	2150 – 4018	3410 ± 1037	2694 – 4599
Note 1 dominant frequency (Hz)	2943 ± 550	1755 – 3799	–	–
Note 2 dominant frequency (Hz)	3495 ± 382	2150 – 4018	–	–

3.2 Influence of temperature and body size on call structure

The mean temperature was 23.7 ºC across recordings and ranged between 20.8 and 25.6°C. By exploring the data, a positive relationship was found between air temperature and call duration, Note 2 duration and Note 2 rate. The relationship between temperature and dominant frequency of Note 1 was negative, while there was no linear relationship between Note 1 duration, call rate, call and Note 2 dominant frequency, silent interval and inter-note interval.

Males ranged in body size between 18.2 mm and 22.6 mm SVL ($\bar{X}$ ± SD=20.4 ± 1 mm, n=30) and from 1.1 g to 2 g ($\bar{X}$ ± SD=1.5 g ± 0.2 g, n=30). Since these two morphometric variables correlated by only 0.39, both of them were included in the statistical analyses. A positive relationship was found between SVL and call rate, while SVL exhibited a negative relationship with call duration, Note 2 duration, Note 2 rate and inter-note interval. No relationship was found between SVL and dominant frequencies, Note 1 duration and silent interval. Weight negatively related to silent interval and inter-note interval, and positively related to durations of call and notes, dominant frequencies (both of call and of the two note types), call and Note 2 rate.

In addition, canopy cover exhibited positive relationship with Note 1 duration, dominant frequency of call and Note 2 and with inter-note interval. It negatively related to dominant frequency of Note 1 and silent interval, while there was no relationship between call and Note 2 duration, call and Note 2 rate.

Table 3 shows the results of the multiple linear regression models where all variables (temperature, SVL, weight, weather condition, lunar phase, canopy cover and site) were included. Of all variables, only body weight had a significant effect on call properties of call duration (R^2= 0.6379, F= 4.69, P= 0.0481), silent interval (R^2= 0.4445, F= 8.0436, P= 0.0132), call rate (R^2= 0.3971, F= 5.4704, P= 0.0347) and Note 2 duration (R^2= 0.5557, F= 4.1964, P= 0.0597). The other call traits were statistically unaffected by all measured variables.

The multiple linear stepwise regression models showed significant effect of SVL and body mass on call duration (R^2= 0.3298, F= 5.9169, P= 0.0219, β= 0.097 for SVL and F= 7.3703, P= 0.01142, β= - 0.428 for weight)(A2), on Note 2 duration (R^2= 0.3198, F= 5.1473, P= 0.03149, β= 0.092 for SVL and F= 7.5493, P= 0.01056, β= - 0.428 for weight)(A3), and on Note 2 rate (R^2= 0.2656, F= 6.5611, P= 0.01632, β= 0.814 for SVL and F= 6.5611, P= 0.0163, β= - 4.211 for weight)(A3). Call rate (R^2= 0.2522, F= 9.4408, P= 0.00469, β= 15.61)(A5) and silent interval (R^2= 0.2331, F-value= 8.5093, P-value= 0.00689, β= - 5.969)(A6) was also significantly influenced by body mass: where there was a negative relationship between body mass and call rate, while the relationship was positive between body mass and silent interval. Of the measured environmental and morphometric factors, none of them showed significant effect on call properties. The output of these regression models is found in the Appendix (A2-A6).

Table 3: Summary of statistical tests including all variables to estimate temperature, SVL, weight, canopy cover, lunar phase, weather condition and site effects on call properties of Call A type in *Nyctibatrachus kempholeyensis.* CD: Call duration (s); SI: Silent interval (s); CR: Call rate (min/call); N1D: Note 1 duration (s); N2D: Note 2 duration (s); II: Inter-note interval (s); N2R: Note 2 repetition rate (min/note); CDF: Dominant frequency of call (Hz); N1F: Dominant frequency of Note 1 (Hz); N2F: Dominant frequency of Note 2 (Hz). Bold type indicates statistical significance.

		Temperature		SVL		Weight		Canopy cover		Lunar phase		Weather		Site	
Call properties	r^2-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
CD	0.6379	3.1386	0.0982	3.9821	0.0658	4.6900	**0.0481**	1.8927	0.1905	2.7018	0.0738	0.1258	0.7281	0.0215	0.8854
SI	0.4445	0.1356	0.7182	0.0034	0.9542	8.0436	**0.0132**	0.1127	0.7420	0.0902	0.9840	1.0732	0.3178	1.4728	0.2450
CR	0.3971	0.5647	0.4648	0.7735	0.3940	5.4704	**0.0347**	0.0001	0.9942	0.5858	0.6782	0.0673	0.7991	0.0011	0.9742
N1D	0.2183	0.0471	0.8312	0.0559	0.8166	0.3576	0.5594	0.3439	0.5669	0.5284	0.7169	0.2289	0.6397	0.7635	0.3970
N2D	0.5557	2.4826	0.1374	2.9543	0.1077	4.1964	**0.0597**	0.0014	0.9705	1.9525	0.1575	0.0492	0.8277	0.0165	0.8997
II	0.3418	0.0073	0.9330	0.1614	0.6939	0.3254	0.5774	0.8492	0.3724	1.3486	0.3010	0.4500	0.5133	0.0824	0.7782
N2R	0.4649	0.8496	0.3723	2.9244	0.1093	3.6585	0.0765	0.0100	0.9217	0.9375	0.4707	0.0487	0.8286	0.9222	0.3532
CDF	0.2362	0.0313	0.8621	0.0183	0.8942	0.3928	0.5409	1.4033	0.2559	0.2309	0.9164	1.5343	0.2358	0.0254	0.8757
N1DF	0.2560	0.0861	0.7735	0.0003	0.9857	0.2579	0.6195	0.3609	0.5576	0.8163	0.5357	0.8294	0.3779	0.0173	0.8971
N2DF	0.2465	0.0479	0.8299	0.0026	0.9604	0.3550	0.5608	1.4434	0.2495	0.3076	0.8681	1.4984	0.2411	0.0016	0.9683

3.3 Individual variation in call structure

Within-individual differences in call structure showed most variability (dynamic properties) in the silent interval (46.8 %), dominant frequency of Note 1 (18.3 %) and inter-note interval (18.1 %). Call dominant frequency (9.4 %) and Note 2 dominant frequency (9.4 %) showed least variability (more static properties) (Table 4).

Among-individual variability was highest in the call rate (58.9 %) and silent interval (52.5 %). Least variable among-individuals were the dominant frequency of Note 2 (10.9 %), call dominant frequency (11.8 %) and Note 1 duration (13.2 %) (Table 4).

The ratio of among-individual and within-individual coefficient of variation was lowest in the dominant frequency of Note 1 (1), followed by silent interval (1.1) and duration of Note 1 (1.1). Highest ratios were obtained for call duration (1.9), Note 2 duration (1.8) and Note 2 repetition rate (1.8) (Table 4).

Table 4: Coefficient variations of 10 call properties of type Call A of *Nyctibatrachus kempholeyensis.*

Call properties	Within-male (CVw)	Among-males (CVa)	Type[a]	CVa/CVw
Call duration (s)	10.5	19.9	Intermediate	1.9
Silent interval (s)	46.8	52.5	Dynamic	1.1
Call rate	—	58.9	—	—
Note 1 duration (s)	11.5	13.2	Intermediate	1.1
Note 2 duration (s)	13.4	24.3	Intermediate	1.8
Inter-note interval (s)	18.1	25.8	Dynamic	1.4
Call dominant frequency (Hz)	9.4	11.8	Intermediate	1.3
Note 1 peak frequency (Hz)	18.3	18.7	Dynamic	1
Note 2 peak frequency (Hz)	9.4	10.9	Intermediate	1.2
Note 2 repetition rate	13.2	23.2	Intermediate	1.8

[a] Classification by Gerhardt (1991) based on $\bar{x}$ within-male CV.

The analysis based on coefficient variation (CV) showed less variability of the spectral properties within-individuals compared to temporal properties with the exception of Note 1 dominant frequency (Fig.11).

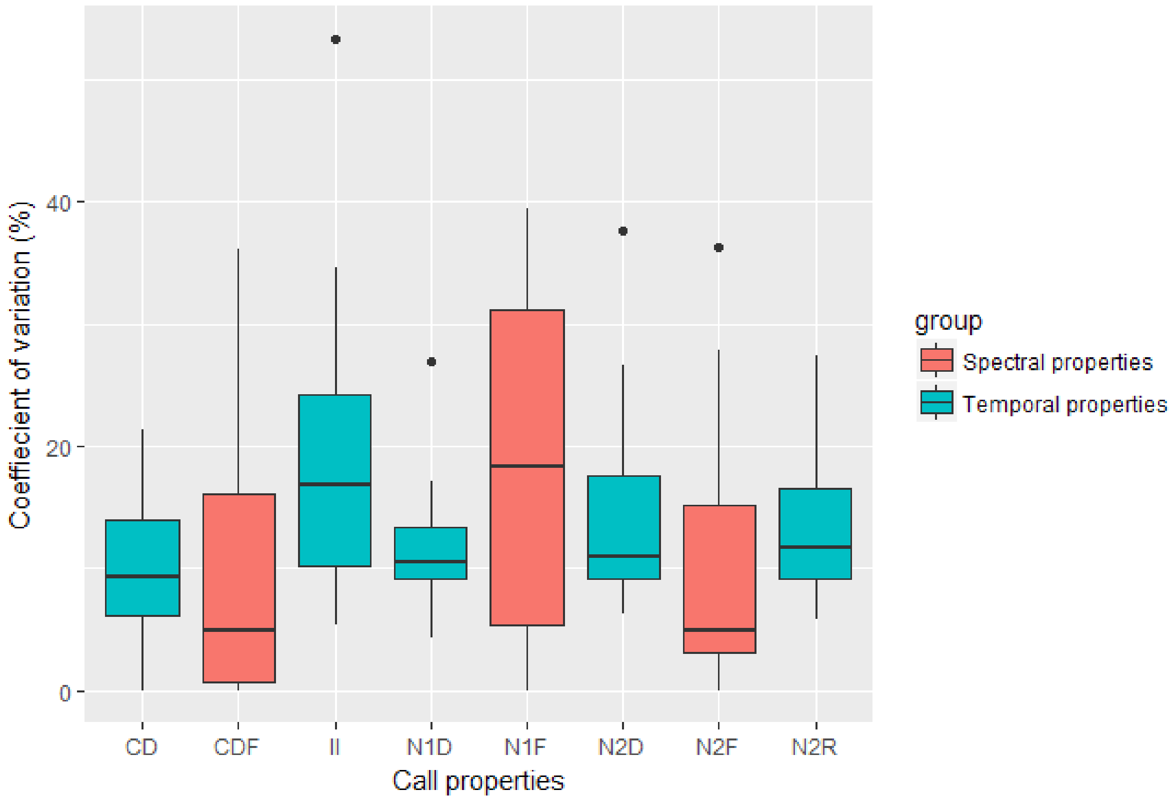

Figure 11: Boxplots showing within-male coefficients of variation (CV) for the examined properties of Call A type call in *Nyctibatrachus kempholeyensis.* CD: Call duration (s); CDF: Dominant frequency of call (Hz); II: Inter-note interval (s); N1D: Note 1 duration (s); N1F: Dominant frequency of Note 1 (Hz); N2D: Note 2 duration (s); N2F: Dominant frequency of Note 2 (Hz); N2R: Note 2 repetition rate.

4. Discussion

4.1 Call structure

Kempholey Night Frog males called from a fixed site and females approached them. This is similar to other frog families, such as in Bombina, Rana and Discoglossus (Gururaja et al. 2014; Wells 2010).

The vocal repertoire of Kempholey Night Frogs consists of two call types: a simpler, shorter and rarer Call B type, and a more complex and often heard Call A type. They mainly differed in call duration and call rate, but not in dominant frequency. However it does not mean that other call types are non-existent within the species repertoire. Comparing the results to the comparative study of Gururaja et al. (2014), the call structure regarding Call B type, was differently termed by the authors (I followed terminology and hierarchy of main units and subunits proposed by Koehler et al. (2017)) Their longer Call B duration ($\bar{X}$=11.69 sec) and lower dominant frequency ($\bar{X}$=1633.67 Hz) is due to an analysis that would comply with a call group of a series of Call B type, followed by one Call A type. Since there are silent intervals in between, the dominant frequency is consequently lower. The common Call A type was similarly described regarding the call structure, and its duration is comparable to the results here ($\bar{X}$=0.517 sec compared to $\bar{X}$=0.89 sec here), albeit a bit shorter. When comparing the call structure of *N.kempholeyensis* to other Nyctibatrachus species, *N.Athirappillyensis* produces a somewhat similar call to Call A type of *N.kempholeyensis* in having 2 parts (or notes) in it. Similarly part 1 is much shorter than part 2. The single call of *N. malanari* is similar to Call B type of *N.kempholeyensis* (Garg et al. 2017).

According to our observations, the short, whining Call B type was heard when two males were in close proximity, agreeing with the general experience that aggressive calls are directed towards male competitors during close encounters. Call A has different note types and can have multiple functions, such as attracting females and repelling males, since the receivers of advertisement calls could be potential mates or neighbouring males. Hence the different note types or calls with variable note types could serve different functions as Wells concluded (2010). In addition, the complexity of calls can also vary depending on the social environment of the male. Future playback experiments might reveal the exact functions of the two call types.

It is important to observe and report the social context in calling activity, since for example the proximity of other males and females can influence the calling behaviour. In addition, since advertisement calls can also advertise parental and resource qualities (e.g. breeding site) in order to show intent to potential partners for breeding (Wells 2010), further studies on parental care and on egg predation would be beneficial. Furthermore, in addition to acoustic signals, mechanism of mate choice in frogs can involve pheromones (Starnberger et al. 2013), visual signalling (Boeckle et al. 2009; Hödl & Amézquita 2001), inflations of vocal sacs (Hirschmann & Hödl 2006), water surface waves (Walkowiak & Münz 1985), surface vibrations (Cardoso & Heyer 1995; Hill 2001), and age (Pröhl 2003). Mate choice in frogs is most likely to be the complex and dynamic combinations of these cues (Narins et al. 2003), and examination of these cues could reveal more about this for females in *N.kempholeyensis*.

4.2 Influence of temperature and body size on call structure

In this study, the correlation of air temperature with temporal properties of call duration, Note 2 duration and Note 2 rate is in line with results from previous studies (e.g. Gasser et al. 2009) as these features are linked to temperature dependent muscular contractions (Koehler et al. 2017). According to Bee et al. (2013), call rate is often positively correlated with temperature, while call duration is negatively related to it. However, contrary to this, call duration was positively related to temperature, not negatively.

The results show that none of the call properties were significantly influenced by temperature. The reason can be that the temperature range across the recordings were relatively small (between 20.8 and 25.6°C) as in the study by Bee et al. (2013), although the range was smaller there (18.6 and 20.4°C). Perhaps a more constant temperature throughout the days and throughout the monsoon season in Tropical regions might be an explanation of why the temperature did not affect call properties. Nevertheless, the energetically expensive activity of calling and the ectothermic nature of frogs indicate that temperature can influence call properties. Therefore it is important to report relationships between temperature and call properties. In other studies where temperature played an important role, call properties were corrected for its influence by averaging the temperature across recordings (Bee et al. 2010; Gambale et al. 2014; Gasser et al. 2009; Kaefer & Lima 2012).

When other environmental variables were included in the statistical model, such as lunar phase, weather condition, canopy cover and site, they did not influence any of the call properties. The measurement of canopy cover might be less useful because of the different angles and viewpoints of frogs from the ones the photos were taken from (personal communication with KV Gururaja). In addition, regarding the lunar phase, there is no clear proof provided in this study that the moon does not influence call performance. This is because the study period was from between 23rd of August and 9th of September (with measurements of environmental factors), thus it did not make up a complete lunar cycle. Nevertheless, a literature review showed evidence that most amphibians respond to lunar phase, including reproductive behaviour and this response is highly species specific (Grant et al. 2012). Furthermore, regarding the weather conditions, on heavy rainy nights more calls were heard according to our observations in July and August. However, during the study period at the end of August and in September, rainy nights were fewer.

According to the findings, SVL did not relate to spectral properties, but to the temporal properties of call rate (positively) and to call duration, Note 2 duration, Note 2 rate and inter-note interval (negatively), while weight related to temporal (silent interval and inter-note interval negatively) and to spectral properties (dominant frequency of notes and call, duration of call and notes and rates of call and Note 2 positively) as well. Interestingly, call duration had a negative linear relationship with SVL, but positively related to weight. This might indicate the relationship between calling activity and its energy consumption, since temporal call variables usually correlate with energy expenditure (Koehler et al. 2017).

The multiple linear regression models showed that the potential sources of variability of call properties of call duration, Note 2 duration, Note 2 rate, call rate and silent interval were due to SVL and body mass. Thus, it seems that morphological variables had greater influence on some of the temporal call properties than on the spectral ones. This is in contrast to some studies, which reported strong negative correlation of dominant frequency and body size (in Wells 2010). However, other studies found that correlation between body size and call properties were not present among males of the same population, but only in geographically distant populations (in Gasser et al. 2009; in Wells 2010). For this reason, it would have been interesting to examine the difference between the two populations of the two study sites.

The effect of both SVL and weight might indicate that these variables together can give a better estimate than SVL alone, or possibly the use of body condition index. Body condition index uses of residuals from a regression of body mass on a linear measure of body size (Albrecht I. Schulte-Hostedde et al. 2005). It is important to note however, that silent intervals and call rate are often dependent on calling motivation and disturbance (Koehler et al. 2017). In addition to the influence of environmental and morphometric factors on call properties, social environment can effect these features as mentioned above, but it is more difficult to measure.

4.3 Individual variation in call structure

All examined call properties varied more among males than within males (Table 4), hence the ratios of among-individual variability to within-individual variability (CVa/CVw) were greater than 1.0. According to Jouventin (1999), if call traits exceed the ratio of 1 and are relatively more variable among individuals, they might serve as individual recognition cues. The high values of CVw-s of silent interval and of CVa-s of call rate and silent interval indicate high variability both within and among individuals in these call traits. It is not surprising since silent interval and call rate can be highly affected by disturbance and calling motivation as already mentioned.

Three properties exhibited relatively more than average variability among males than within males, namely call duration, Note 2 duration and Note 2 rate and hence got the highest CVa/CVw ratios. Spectral features had lower CVa/CVw ratios compared to temporal traits (except Note 1 duration), which indicate that temporal properties showed more variability among males than within males compared to spectral properties.

None of the call traits showed static nature in this study, however dominant frequency of call and Note 2 were least variable (both within- and among-individuals), albeit classified as intermediate type. It is possible that other call properties (e.g. call rise time, call fall time, pulse rate, which is the smallest unit of a call) might prove to be static, especially because fine temporal call traits tend to be static, as Gerhardt (1991) proposed. The classification of Gerhardt (1991) for distinguishing between static and dynamic call properties is based on the relative measure of within-individual variability. On the one hand, static properties (with less variability within individuals) are generally physically constrained features and also traits that are important in species and individual recognition and have stabilizing or weakly directional roles in female preference tests (Bee et al. 2013).

Dynamic properties on the other hand, show greater variability within individuals probably due to changing environmental and social contexts. Silent interval, inter-note interval and dominant frequency of Note 1 were classified here as dynamic traits. They might reveal information on mate quality and hence they are a target of directional female preferences. The spectral call property of Note 1 dominant frequency as dynamic type is relatively surprising since frequency is generally related to body size, a physically constrained feature, as discussed above. However, it is also possible that frogs can change ‘their pitch’ according to their social environment as *Rana catesbeiana* bullfrogs do (Bee & Bowling 2002). Some of the among-male variation of call rate can be assigned to body size and weight, that is, smaller males are calling faster than larger ones. These results are in line with some other studies, for example in *D. pumilio* described by Prohl at al. (2007). It is important to note that this classification of dynamic and static type of call traits is a continuum, rather than bimodal pattern of variation (Koehler et al. 2017). Moreover, Reinhold (2009) found that the variation increased with the duration of the recorded and analysed calls, consequently, the influential factors increased as well.

Females often respond to call intensity, call duration, calling rate and call complexity and they prefer males that invest more effort in calling performance (Wells 2010). Therefore it would be beneficial to conduct future research with acoustic playback experiments to find out the role of whining call for example and studies on territoriality.

In conclusion, basic call descriptions and intraspecific variations play a key role in understanding social and breeding behaviour of frogs (Bee et al. 2010) and the evolution of frog communication systems (Bee et al. 2013). It can also provide information for conservation efforts that rely on acoustic monitoring (Bee et al. 2010) in a non-invasive manner.

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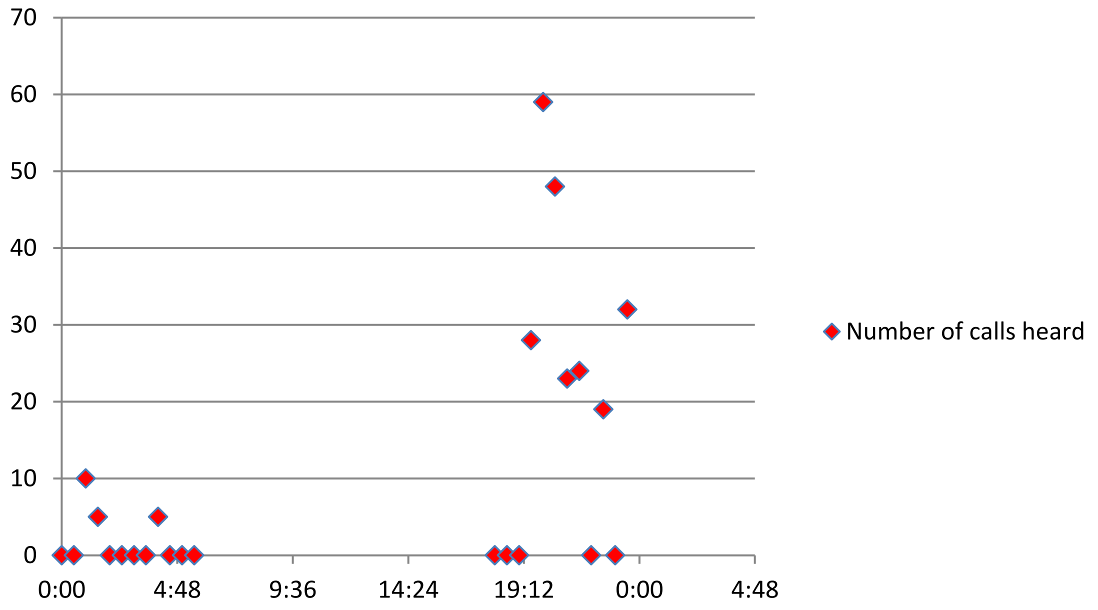
APPENDIX



A1: Pattern of calling activity. Note that that observations were made only between 18:00 and 6:00.

```
Call:
lm(formula = Dur ~ SVL + Weight, data = Study1Summm)

Residuals:
     Min       1Q   Median       3Q      Max 
-0.33054 -0.09941  0.00776  0.06668  0.41294 

Coefficients:
            Estimate Std. Error t value Pr(>|t|)   
(Intercept) -0.45476    0.55110  -0.825  0.41651   
SVL          0.09720    0.02938   3.308  0.00266 **
Weight      -0.42809    0.15769  -2.715  0.01142 * 
---

Residual standard error: 0.1503 on 27 degrees of freedom
Multiple R-squared:  0.3298,	Adjusted R-squared:  0.2802 
F-statistic: 6.644 on 2 and 27 DF,  p-value: 0.004505
```

A2: Multiple linear regression output of call duration as the function of SVL and weight

```
Call:
lm(formula = Note2Dur ~ SVL + Weight, data = Study1Summm)

Residuals:
     Min       1Q   Median       3Q      Max 
-0.35227 -0.08265 -0.01970  0.08933  0.38341 

Coefficients:
            Estimate Std. Error t value Pr(>|t|)   
(Intercept) -0.52633    0.54432  -0.967  0.34215   
SVL          0.09202    0.02902   3.171  0.00376 **
Weight      -0.42792    0.15574  -2.748  0.01056 * 
---

Residual standard error: 0.1485 on 27 degrees of freedom
Multiple R-squared:  0.3198,	Adjusted R-squared:  0.2695 
F-statistic: 6.348 on 2 and 27 DF,  p-value: 0.005499
```

A3: Multiple linear regression output of Note 2 duration as the function of SVL and weight

```
Call:
lm(formula = Note2RR ~ SVL + Weight, data = Study1Summm)

Residuals:
    Min      1Q  Median      3Q     Max 
-3.8387 -0.8815 -0.0768  0.9857  3.8091 

Coefficients:
            Estimate Std. Error t value Pr(>|t|)  
(Intercept)  -2.7261     5.7453  -0.474   0.6390  
SVL           0.8139     0.3063   2.657   0.0131 *
Weight       -4.2108     1.6439  -2.561   0.0163 *
---

Residual standard error: 1.567 on 27 degrees of freedom
Multiple R-squared:  0.2656,	Adjusted R-squared:  0.2112 
F-statistic: 4.882 on 2 and 27 DF,  p-value: 0.01549
```

A4: Multiple linear regression output of Note 2 rate as the function of SVL and weight

```
Call:
lm(formula = CallRate ~ Weight, data = Study1Summm)

Residuals:
    Min      1Q  Median      3Q     Max 
-3.1596 -1.2513  0.1124  0.6853  5.8966 

Coefficients:
            Estimate Std. Error t value Pr(>|t|)    
(Intercept)   12.766      2.912   4.384 0.000149 ***
Weight        -5.969      1.943  -3.073 0.004690 ** 
---


Residual standard error: 2.016 on 28 degrees of freedom
Multiple R-squared:  0.2522,	Adjusted R-squared:  0.2254 
F-statistic: 9.441 on 1 and 28 DF,  p-value: 0.00469
```

A5: Multiple linear regression output of call rate as the function of body weight

```
Call:
lm(formula = AvgSilInt ~ Weight, data = Study1Summm)

Residuals:
     Min       1Q   Median       3Q      Max 
-11.8257  -3.9498  -0.5762   4.1195  10.3936 

Coefficients:
            Estimate Std. Error t value Pr(>|t|)   
(Intercept)   -11.34       8.02  -1.414  0.16849   
Weight         15.61       5.35   2.917  0.00689 **
---

Residual standard error: 5.553 on 28 degrees of freedom
Multiple R-squared:  0.2331,	Adjusted R-squared:  0.2057 
F-statistic: 8.509 on 1 and 28 DF,  p-value: 0.006889
```

A6: Multiple linear regression output of silent interval as the function of body weight

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