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Behavioural context shapes vocal sequences in two anuran species with different repertoire sizes

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Acoustic signals in animals serve to convey context-dependent information to receivers. Birds and mammals combine diverse sounds into complex sequences to communicate, but the role of temporal sequencing of signals remains understudied in other taxa. Anuran vocalizations are a prominent feature of their life history, and function in defence of territories and to attract mates. However, there are few data on whether anurans pattern their calls into sequences, and whether temporal sequences convey information about context. Here, we investigated the context-dependent vocal repertoire and the use of vocal sequences by two anuran species belonging to different lineages, comparing frogs vocalizing alone and in the presence of a territorial rival. Using a robust analytical framework, we present evidence that both species modify their vocal sequence structure according to context. Specifically, one species (with a smaller repertoire, from a more basal lineage) appends notes to generate more complex sequences, whereas the other (more recently diverged and with a larger repertoire) shifts to different note types, resulting in different sequences for different contexts. Thus, despite differences in repertoire size, both frog species are capable of adjusting the temporal sequence of vocalizations to communicate in different contexts. Vocal sequences and context-dependent 'syntax' may be more common in anurans than previously thought, and our methodology presents a paradigm to study the evolution and function of these complex vocal patterns.

Acoustic communication has evolved in a variety of taxa (Bradbury & Vehrencamp, 2011; Chen & Wiens, 2020), and enables different messages to be conveyed to different receivers, often simultaneously. The production of sound is energetically costly (Gerhardt, 1994; Gillooly & Ophir, 2010) and carries the risk of detection by unintended receivers such as predators or parasites (Rand & Ryan, 1981; Zuk et al., 2006). These factors compound the challenge of conveying information effectively to intended recipients, such as prospective mates or territorial rivals, among others (Wilkins et al., 2013). Some animals employ distinct acoustic signals for a mate versus a rival, thus communicating information about context (Berglund et al., 1996; Hebets & Papaj, 2005). In addition to this, many animals change the temporal patterns in which signals are emitted, resulting in complex sequences (syntactic structure) that convey information about context (Fishbein et al., 2020; Gentner et al., 2006). For example, different types of notes may be incorporated in different contexts, or sequences may become either more or less elaborate depending on the intended recipient. The individual notes serve as modules, which are combined or elaborated to convey different messages (Engesser et al., 2019). However, studies of animal communication using complex vocal sequences are mostly restricted to birds (Engesser et al., 2019; Fishbein et al., 2020; Gentner et al., 2006; Marler, 1990) and mammals (Allen et al., 2018; Bohn et al., 2009; Kershenbaum et al., 2012; Zuberbühler, 2018). Using quantitative tools such as first-order Markov chain models and sequence similarity analyses (Kershenbaum et al., 2012, 2014), we now possess evidence of context-dependent syntactic structure in diverse birds and mammals. Vocal sequences in other vertebrates, though, remain essentially unstudied.

Anurans (frogs and toads) are a diverse amphibian order that have evolved conspicuous acoustic signals to attract females

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(Narins, 1992, 1995; Wells, 1977). These signals form one of the most prominent features of their life histories. In addition to serving as honest indicators of mate quality, anuran acoustic signals are also used to advertise position, convey information about reproductive state, defend territories and communicate fitness to rivals of the same sex (Bee et al., 2013; Bradbury & Vehrencamp, 2011; Wells, 1977). Studies have identified multinote repertoires in anurans, and ascribed call types to different functions (Toledo et al., 2015), identifying certain note types that are emitted only in territorial or mating contexts (Narins & Capranica, 1978; Rand & Ryan, 1981). However, there is still little empirical knowledge of whether anurans employ vocal sequences of different note types, and, if so, whether the sequence of notes varies according to behavioural context. For instance, do anurans with multinote repertoires emit the same notes in different temporal sequences to communicate context? Alternatively, do they use different note types to generate entirely different sequences (Engesser & Townsend, 2019)? Addressing these questions is important to inform our understanding of how complex communication in vertebrates evolved.

Here, we present evidence that two anuran species, representatives of endemic lineages from southwest India's Western Ghats, emit vocal sequences consisting of different note types and test the hypothesis that these sequences may be used to communicate behavioural context. The genera *Nyctibatrachus* and *Pseudophilautus* have both evolved in the Western Ghats–Sri Lanka biodiversity hotspot (Meegaskumbura et al., 2019; Torsekar, 2019; Van Bocxlaer et al., 2012; Vijayakumar et al., 2016), and are distantly related, having diverged about 63.8–94.7 million years ago (Van Bocxlaer et al., 2006). Members of both lineages use vocalizations to communicate and are prolonged, territorial breeders (Bee et al., 2013; Gramapurohit et al., 2011). They are thus likely to communicate both with potential mates and with territorial rivals. Specifically, we hypothesized that the presence of a territorial rival(s) should influence the sequence patterns produced, either by sequence change or by shifting to different sequences (of different note types). Our study thus sought to examine whether different note types could be combined to generate a syntactic structure, which in turn could provide information about context.

METHODS

Study Species

We studied two endemic anuran species, Humayun's night frog, *Nyctibatrachus humayuni* (Nyctibatrachidae) and Amboli bush frog, *Pseudophilautus amboli* (Rhacophoridae), in the Western Ghats hill ranges of southwest India (Fig. 1). Both species are relatively common within their geographical ranges (Meegaskumbura et al., 2019; Van Bocxlaer et al., 2012; Vijayakumar et al., 2016). *Nyctibatrachus humayuni* (Bhaduri & Kripalani, 1954) is a relatively large frog restricted to the northern Western Ghats (males measuring up to 46.8 mm and females up to 50.6 mm), found in forest streams and swamps between 560 and 1228 m above sea level. Males of this species are territorial and vocalize at night on rocks or on vegetation overhanging water. *Pseudophilautus amboli* (Biju & Bossuyt, 2009) is a medium-sized frog restricted to the northern and central Western Ghats (males measuring up to 34.1 mm and females up to 37.5 mm). Individuals vocalize at night on leaves and saplings up to 2 m from the ground in a variety of habitats, including disturbed evergreen

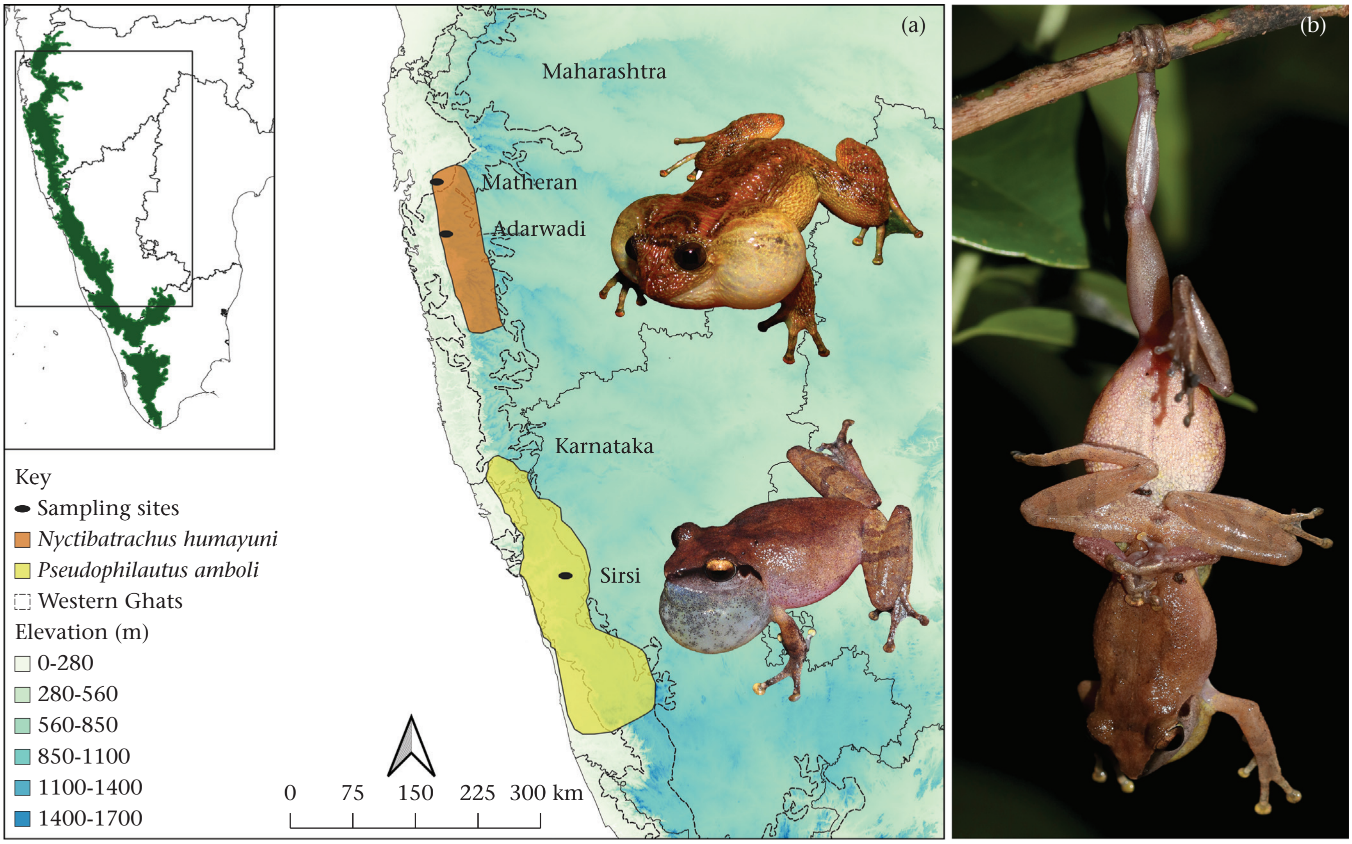


Figure 1. (a) Geographical distributions of *Nyctibatrachus humayuni* (top) and *Pseudophilautus amboli* (bottom). Inset map shows the Western Ghats in green and the box indicates the study area. (b) Two male *P. amboli* engaged in physical combat.

forests, arecanut, *Areca catechu*, plantations, and home gardens between 500 and 1000 m above sea level (Fig. 1). Both species are easy to locate in their preferred habitats and are thus relatively easily observed and recorded. They thus present an opportunity to relate vocalizations and vocal sequence patterns to behavioural context. All methods used in this study were purely observational and conducted outside protected areas without disturbance to the animals or their habitat. Thus, ethical approval was not required.

Study Sites

We studied *N. humayuni* at two locations in Maharashtra (Adarwadi village, Pune district, 18°25.838′N, 73°24.085′E, 634 m above sea level, and Matheran, Raigad district, 19°00′N, 73°17′E, 800 m above sea level) in June and July 2019. These localities comprise mid-elevation wet evergreen forest, receiving about 4073 mm rainfall annually (Sawant et al., 2020). After digitizing notes in Raven Pro 1.6 (Cornell Laboratory of Ornithology, Ithaca, NY, U.S.A.), we used a principal components analysis to examine whether notes differed between these two locations (Fig. A1, Table A1). We measured call characters from the recordings using the selection features in Raven Pro 1.6. These characters were peak frequency, note duration (using the Delta time measurement in Raven Pro), average entropy, maximum entropy, the bandwidth at 90% of the energy in the selection, interquartile range (IQR) bandwidth, interquartile range (IQR) duration (i.e. the differences between the first and third quartile frequencies and times in the selection), maximum and minimum slope of the peak frequency contour (PFC, representing peak frequencies across the duration of the selection), the minimum and maximum frequencies of the PFC and the peak time (time between when a note began to when it first attained the peak amplitude, normalized to the duration of the note). Further information may be found in the Raven Pro user manual (http://ravensoundsoftware.com/wp-content/uploads/2017/11/Raven14UsersManual.pdf). We studied *P. amboli* in Sirsi, Uttara Kannada district, Karnataka (14°44′N, 74°45′E, 611 m above sea level) in July 2020. This locality comprises a semievergreen forest (http://wgbis.ces.iisc.ernet.in/energy/water/paper/ETR24/index.htm) and receives a mean annual rainfall of 2500–3500 mm with peak rainfall during June–August (Bhat, 1992). We recorded vocalizations of *N. humayuni* for 7 nights (6 consecutive nights in Adarwadi and 1 night in Matheran), whereas *P. amboli* was sampled for 9 consecutive nights.

Recording Vocalizations

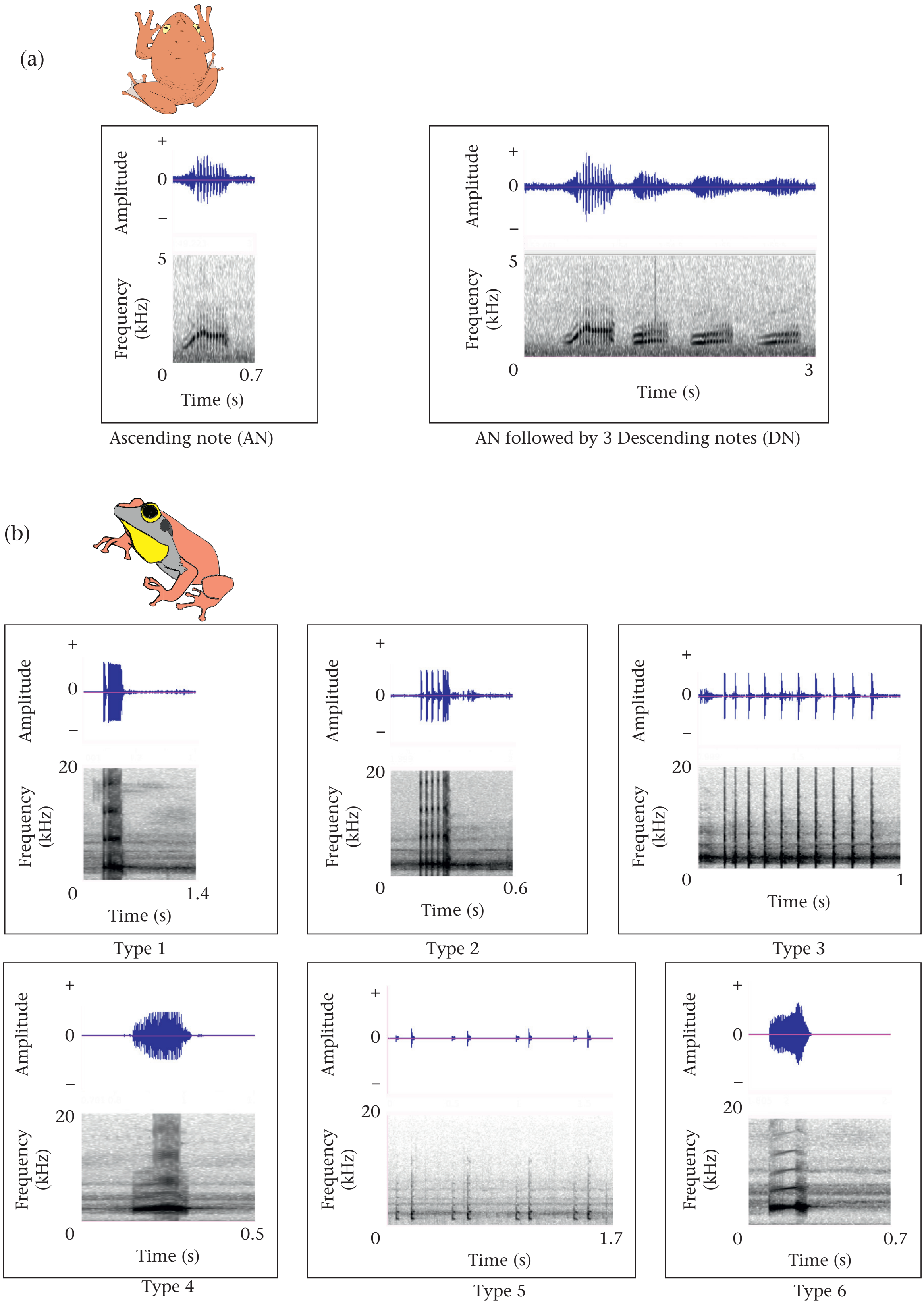
At each site, we recorded frogs in clear weather between 1830 and 2200 hours, as both species are nocturnal or crepuscular. We used a Sennheiser (Wedemark, Germany) microphone connected to a Zoom (Tokyo, Japan) H6 recorder (*N. humayuni*) or a Marantz (Kawasaki, Kanagawa, Japan) PMD-660 recorder (*P. amboli*) to record vocalizations. We recorded at least 20–30 calls per frog at a sampling rate of 44.1 kHz. Before beginning a recording session, we assigned individuals to behavioural contexts (each male was therefore sampled for a single behavioural context) as follows: alone (a single vocal frog with no vocalizing neighbours within an estimated 100 body lengths, i.e. snout–vent lengths in the horizontal plane); vocalizing with neighbours (another vocal male within 100 body lengths in the horizontal plane; we only considered cases where the vocal neighbour was visible to us, so as to be stringent in our criteria); and territorial dispute (a confrontation between vocal males, including aggressive posturing and physical contact). We used 100 body lengths in the horizontal plane as a measure based on published studies of nearest-neighbour distances (Kunte, 2004), and work on related frog genera such as *Raorchestes* (Seshadri, 2017). All social contexts presented here involve only a single neighbour, as incidents with more than one vocal neighbour within this radius were extremely rare (and have therefore not been analysed as part of this study). We only analysed vocal bouts where we could keep track of the frog's movements throughout the period of observation and took care to sample different localities or territories on different nights to avoid recording the same male twice.

Analysis

Using Raven Pro 1.6, we digitized individual call notes, and classified them into various note types based on their time–frequency properties. Three authors independently verified the note type classifications by listening to the recordings, as note types were very distinct from each other and were thus easily classified (Fig. 2). For further analysis, we used custom codes written in Python and R (R Core Team, 2020), as well as the Python packages numpy (https://numpy.org; Harris et al., 2020), seaborn (https://seaborn.pydata.org; Waskom, 2020), matplotlib (https://matplotlib.org; Hunter, 2007), pandas (https://pandas.pydata.org; McKinney, 2011) and scipy (https://scipy.org; Virtanen et al., 2020), and the R packages ggplot2 (https://ggplot2.tidyverse.org; Wickham, 2016), ggpubr (https://cran.r-project.org/web/packages/ggpubr/index.html; Kassambara, 2020), stringr (https://cran.r-project.org/web/packages/stringr/index.html; Wickham, 2019) and dplyr (https://dplyr.tidyverse.org; Wickham et al., 2021).

Initially, we calculated the number of each note type emitted by each individual as a proportion of the total notes (of all types). We compared these proportions across contexts for each note type using either a Wilcoxon rank-sum test (*N. humayuni*, where we compared two contexts), or a Kruskal–Wallis test (*P. amboli*, where there were three contexts). Our null hypothesis in either case was that the proportion of a particular note type was similar across contexts, i.e. that there were no context-dependent changes in the use of specific note types.

Subsequently, to examine context-dependent changes in the sequences of notes, we performed a series of analyses. In the first of these steps, we examined note diversity or variability in the vocal repertoire during each context using Shannon entropy. The Shannon entropy of a stochastic process with n different possible states is given by $H = -\sum_{i=1}^{n} p_i \cdot log(p_i)$, where p_i is the probability of occurrence of the i^{th} state, and if $p_i = 0$, we define $\log(p_i) = 0$. H is a positive number between 0 and $\log(n)$, and a higher value of H indicates that the process is more ‘unpredictable’. In information-theoretic terms, a higher value of H corresponds to a process that has a higher diversity of states (Kershenbaum et al., 2016; Shannon, 1948). For frog vocalizations, therefore, a higher value of H corresponds to greater note diversity, or more variation in vocal sequences in any given behavioural context. Using custom scripts, we calculated the Shannon entropy for each individual for each context and compared across contexts using either a Wilcoxon rank-sum test or a Kruskal–Wallis test (see above). We then complemented this approach using multiple analyses of sequence patterns and context-dependent variation, to examine how vocal sequences changed across contexts. We modelled vocalizations of both species as a first-order Markov chain to calculate the transition probabilities between different note types, where each note depended only on the one immediately preceding it. For a time-homogeneous, discrete-time, discrete-space Markov chain with S distinct states (in our case, the number of different note types) and the random variable X_t (corresponding to the value of note number t, or the note under consideration), the process provides an



Ascending note (AN)

AN followed by 3 Descending notes (DN)

Type 1

Type 2

Type 3

Type 4

Type 5

Type 6

Figure 2. Oscillograms and spectrograms of the vocal repertoire of (a) *N. humayuni* and (b) *P. amboli*.

$S \times S$ transition probability matrix $P = [p_{ij}]$, (where $p_{ij} = Pr\{X_{t+1} = j|X_t = i\}$). Well-known methods (Anderson & Goodman, 1957; Singer et al., 2014) calculate p_{ij} from data using maximum likelihood estimation, and utilize Lagrange multipliers to account for the fact that the transition matrix is row stochastic (i.e. each row in the transition probability matrix must sum up to 1, $\sum_{j=1}^{n} p_{ij} = 1 \, \forall i$). By definition, p_{ij} is the probability that the system will go from the i^{th} state at time step t to the j^{th} state at time step (t+1). Therefore, summing over j for a fixed value of i gives us the probability that the system will be in any one of the possible states at time (t+1). The system has to be in one of the possible states at time (t+1) (i.e. it has to go from one of the notes to one of the other possible notes), and thus this summed value must always equal 1. Lagrange multipliers are used to enforce this constraint. The maximum likelihood estimate for the transition probabilities is given by:

$$p_{ij} = \frac{n_{ij}}{\sum_{k=1}^{S} n_{ik}}$$

where n_{ij} denotes the number of times an $i \rightarrow j$ transition occurred.

This gives us the probability that each note type is followed by another note type, and we examined these to infer how the sequences of different note types changed across contexts. To incorporate information on the start and end states of vocal sequences (i.e. silent periods between notes), we also incorporated ‘Silence’ as a state (labelled as note type zero) in this and the subsequent analyses. To do this, we discretized silent bouts as periods corresponding to five times the length of the longest note for each species. This also enabled us to account for variability in the length of silent periods between notes, as a longer silence would result in more of these discrete silent units. Thus, a typical sequence would take the form ‘001230001220’ where 0 indicates a silent period of duration equal to five times the length of the longest note emitted by the focal species.

We calculated transition probabilities for each context using the above-mentioned custom scripts. A string of the sequence of calls (including silent periods) emitted by an individual was first extracted from the data, and we created strings in this way for all individuals across contexts. The transition matrix P was then calculated by estimating each n_{ij} for each context. To statistically compare transition probability matrices across contexts, we used a divergence test that computes a minimum discrimination information statistic (mdis), developed specifically for contingency tables and Markov chains (Kullback et al., 1962). This statistic tests how different the distributions of transition probabilities are, using the Kullback–Leibler divergence. This statistic is asymptotically distributed as χ^2, with S*(S-1) degrees of freedom, where S is the number of states (or notes in this case). A P value is obtained by comparing where the computed test statistic (mdis) falls with respect to the above χ^2 distribution. We additionally cross-verified the results from the Markov chain analysis by performing a different analysis of note sequences, because animal vocal sequences may not always follow Markovian dynamics (Kershenbaum et al., 2014). This latter analysis made no assumptions about the underlying stochastic process used to generate the observed vocal sequences, and thus allowed us to verify how the choice of method influenced our conclusions. For a given set of note sequences (again, including discretized silent periods as mentioned above), we defined ${}^{d}C_{ij}$ as the probability that note type j occurs within a distance of $d-1$ notes after note type i. Using a Monte Carlo sampling method, we first calculated ${}^{d}C_{ij}$ for the vocal sequence data from our recordings. We then constructed sequences for comparison, where notes were ‘randomly’ distributed according to a stationary distribution in which the probability of occurrence of each note type was equal to its proportion of occurrence (no. of focal note type/total no. of all note types combined) in the data set. We synthesized 50 such random data sets and averaged the co-occurrence across all of them to obtain a robust estimate of the expected (‘null’) co-occurrence. Comparing the ratio ${}^{d}R_{ij}$ of the observed to expected co-occurrence allowed us to quantify whether note type j occurred within a distance of $d-1$ notes after note type i more or less often than we would expect in a random sequence of notes. A value of ${}^{d}R_{ij} < 1$ means that the note type j (or a silent period) occurs within a distance of $d-1$ after note type i less often than random, and ${}^{d}R_{ij} > 1$ means that the note type j (or a silent period) occurs within a distance of $d-1$ after note type i more often than random. We carried out this analysis using different values of d for each species, to assess how the patterns depended on the length of sequence under consideration (see Fig. A2 for an illustration of the workflow).

RESULTS

Both Species Exhibit Different Repertoire Sizes

Both species of anurans emitted calls as sequences comprising two or more note types (see Supplementary Video S1). Specifically, *N. humayuni* produced vocal sequences comprising two note types (Fig. 2a). According to existing classification systems (Emmrich et al., 2020), *N. humayuni* falls within the call guild F (‘frequency-modulated call with uniform notes’). Henceforth, the first note type is referred to as an ‘ascending note’ (AN; note emission rate = 5.43 ± 2.36 notes/min; note duration = 370 ± 66 ms, range 228–619 ms; dominant frequency = 1575.2 ± 237.58 Hz, range 1033.6–2153.3 Hz; N = 489 notes from 19 males). The AN was the more commonly recorded of the two, constituting 66.2% of total notes recorded across individuals. The second note type, which we refer to as the ‘descending note’ (DN), was lower in frequency (note emission rate = 2.68 ± 2.450 notes/min; note duration = 360 ± 57 ms, range 230–553 ms; dominant frequency = 1098 ± 304.86 Hz, range 602.9–1722.7 Hz; N = 243 notes from 16 males). DNs were typically emitted in variable numbers (between 0 and 8) immediately following an AN. Notes emitted at the two different locations (Adarwadi and Matheran) overlapped in principal components ordination space, indicating that there were no striking differences in note properties between the two locations (Fig. A1). The first three principal components explained nearly 60% of total variation in the data (see Table A1 for principal component loadings).

Males of *P. amboli* fell within the call guild G (‘nonfrequency-modulated complex calls’). The vocal repertoire of this species comprised six distinct note types (henceforth referred to as note types 1–6; for note parameters see Table 1; for spectrograms see Fig. 2b). Among all calls recorded, note type 5 was most frequently encountered, comprising 28.7% of total recorded notes, whereas the rarest was note type 6, comprising only 1.02% (this note type was recorded too infrequently for statistical analysis). Individuals emitted two to six different note types in a single recording session.

Distinct Notes are Emphasized in Territorial Contexts

Vocalizing males of *N. humayuni* varied the proportion of DNs in vocal sequences, appending more DNs following an AN in the presence of a vocalizing neighbour (Wilcoxon rank-sum test: W = 6, N = 19, P < 0.01; Fig. 3a). However, they did not vary the note emission rate (measured as the emission rate of any note irrespective of note type; Wilcoxon rank-sum test: W = 34.5, N = 19, P > 0.1). When vocalizing alone (N = 11), males often emitted only the AN, and only rarely added DNs after it. In most cases where a neighbour was present (N = 8), frogs typically appended a single DN to their call (Fig. 3b), and rarely up to three DNs, thus resulting in a more complex vocal sequence. We observed only a single ‘territorial

Table 1
Properties of the note types of *P. amboli*

Note type	Peak frequency (Hz) ± SD	Note duration (ms) ± SD	Bandwidth 90% (Hz)	Sample size (of notes measured)
1	2669.9 ± 131.28	154.4 ± 40.1	2859.3	382
2	2610.7 ± 162.32	218.8 ± 44.6	2857.7	1039
3	2283.7 ± 536.02	48.7 ± 11.7	2544.1	961
4	2478.0 ± 382.11	312.6 ± 105.4	1106.7	291
5	1954.4 ± 483.61	49.4 ± 16.1	1532.0	1095
6	2557.5 ± 62.96	219.5 ± 55.1	439.5	39

dispute' involving physical contact between two males. In this encounter, the male under observation regularly emitted high numbers of DNs, ranging up to eight DNs following an AN (see Supplementary Audio S1).

In contrast, males of *P. amboli* varied the proportions of each note type in vocal sequences according to behavioural context (Fig. 3c, Table 2). Males vocalizing either alone ($N = 26$) or with a neighbour ($N = 14$) emitted note types 1, 2 and 3 most often (representative portions of sequences from our data: '…10022002333…' when alone, '…13022002200…' in the presence of a neighbour). All sequences shown here are part of longer sequences, as our total recordings were considerably longer. On the other hand, individuals engaged in territorial disputes ($N = 10$) mostly emitted note types 4 and 5 (representative portion of a sequence from our data: '…40055505544…'; again, this is a part of a longer sequence). Furthermore, males vocalizing with neighbours emitted a higher proportion of type 3 notes (Kruskal–Wallis test: $H_1 = 4.72$, $P < 0.05$) and a lower proportion of type 2 notes (Kruskal–Wallis test: $H_1 = 5.02$, $P < 0.05$) than males vocalizing alone.

Different Behavioural Contexts Result in Different Vocal Sequence Patterns

The differences in use of note types described above were accompanied by differences in Shannon entropy based on social context (*N. humayuni*: Wilcoxon rank-sum test: $W = 6$, $P < 0.01$; *P. amboli*: Kruskal–Wallis test: $H_2 = 25.38$, $P < 0.001$; Fig. 4a and b). In *N. humayuni*, males vocalizing in the presence of a neighbour exhibited an increased Shannon entropy, consistent with more elaborate vocal sequences. In *P. amboli*, we observed no difference in Shannon entropy of the sequences emitted by males when vocalizing alone as opposed to vocalizing with neighbours. However, male *P. amboli* engaging in territorial disputes exhibited a lower Shannon entropy, which is indicative of more stereotyped vocal sequences. When we modelled vocal sequences in each context as a first-order Markov chain (including silence as a measure of start and end states), we observed differences in the transition probabilities of different note types based on context for both species (Fig. 4c and d; *N. humayuni,* alone versus vocalizing with neighbours: $\chi^2_6 = 81.63$, $P < 0.001$; *P. amboli*, alone versus vocalizing with neighbours: $\chi^2_{42} = 430.31$, $P < 0.001$; alone versus territorial dispute: $\chi^2_{42} = 1250.85$, $P < 0.001$; vocalizing with neighbours versus territorial dispute: $\chi^2_{42} = 753.06$, $P < 0.001$). In *N. humayuni,* the presence of a territorial neighbour roughly doubled the probability of an AN–DN transition, and increased the probability that DNs would repeat, thus resulting in more elaborate sequences. In the more complex vocal repertoire of *P. amboli,* males vocalizing alone usually produced sequences consisting of note types 1, 2 and 3, whereas during territorial disputes repetitive sequences of note types 4 and 5 had the highest probabilities of occurrence. This supports our previous finding of more stereotyped sequences.

Using Monte Carlo methods to calculate the probability of two note types occurring within $d = 3$ (*N. humayuni*) and $d = 6$ (*P. amboli*) notes of each other (the size of the repertoire for each species), we were able to verify the conclusions from the Markov chain analysis without the assumption that the underlying stochastic processes were Markovian. Plotting the pairwise measures of the ratio of observed to expected probability of co-occurrence (see Methods) for *N. humayuni*, we found that ANs co-occurred much less often than expected by chance, and instead were highly likely to co-occur with a DN. For *P. amboli,* this analysis uncovered two note groups, one containing note types 1, 2 and 3, and the second containing note types 4 and 5 (Fig. 5). We defined these groups using the probability of co-occurrence, such that co-

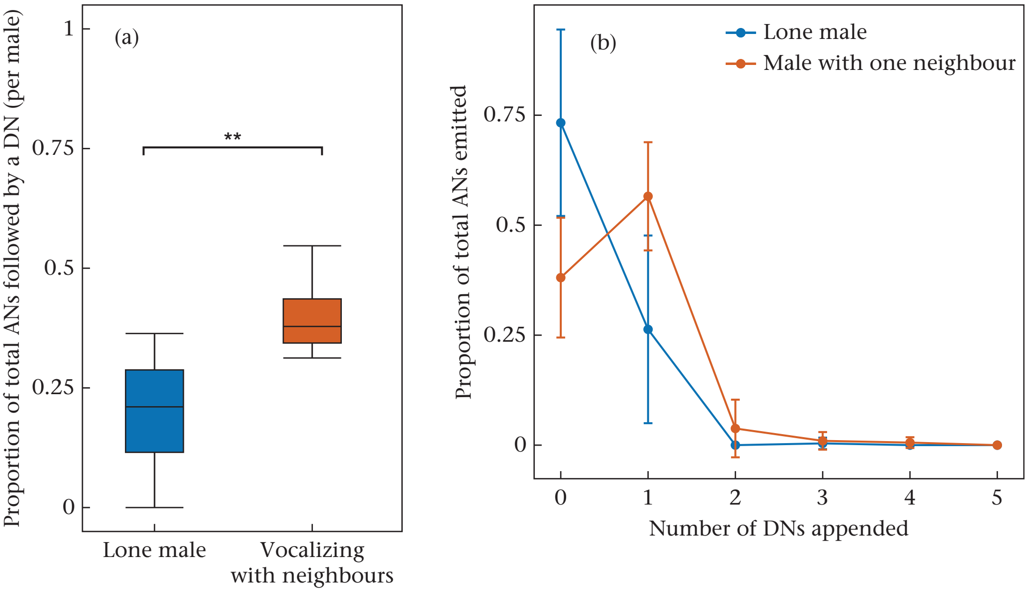


Figure 3. (a) Variation in proportion of each note type used across behavioural contexts in *N. humayuni.* AN: ascending note; DN descending note. (b) Proportion of times a male *N. humayuni* appended different numbers of DNs in each context. (c) Variation in proportion of each note type used across behavioural contexts in *P. amboli.* The upper and lower edges of the box indicate the third and first quartile, respectively, and the internal line indicates the median. Whiskers extend to 1.5 times the interquartile range, and circles above and below represent outliers beyond this limit. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

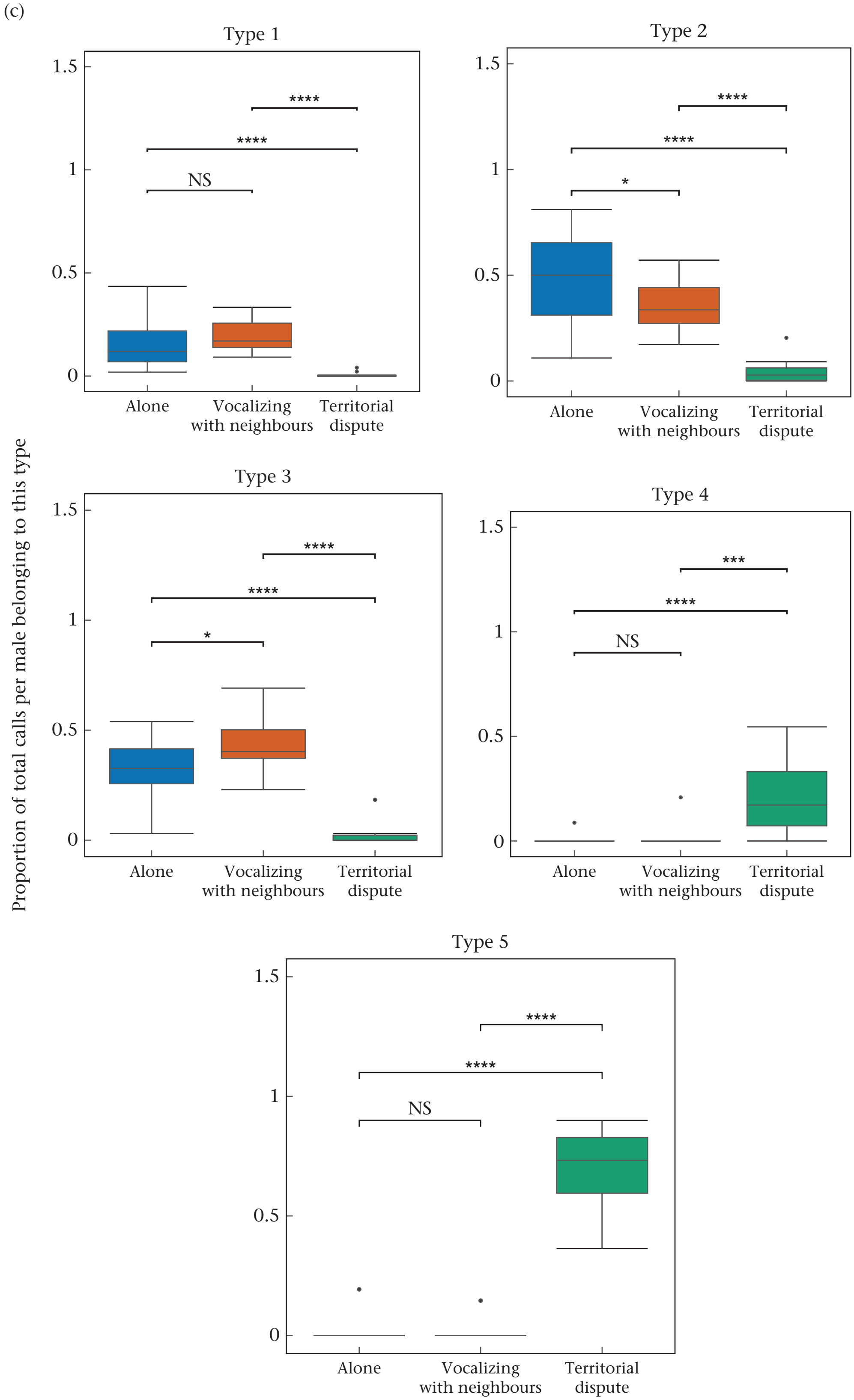


Figure 3. Continued.

Table 2
Variation in proportion of note types of *P. amboli* based on behavioural context (results of Kruskal–Wallis tests

Note type	Results of Kruskal–Wallis test
1	$H_2 = 24.55$, $P < 0.001$
2	$H_2 = 26.04$, $P < 0.001$
3	$H_2 = 26.67$, $P < 0.001$
4	$H_2 = 32.03$, $P < 0.001$
5	$H_2 = 41.11$, $P < 0.001$

occurrences of any notes within the group were higher than expected by chance, and co-occurrences of notes from different groups were lower than expected by chance. Thus, ${}^{d}R_{ij}$ was > 1 for intragroup pairings and < 1 for intergroup pairings. This trend was independent of the value of d for both species (*N. humayuni:* $2 \leq d \leq 5$, *P. amboli:* $3 \leq d \leq 12$; additional plots are shown in Figs A3–A6). The presence of note groups, together with the results of the Markov analysis, are consistent with the presence of different vocal sequences for different contexts. Male *P. amboli* thus shift from one note group to the other in a territorial dispute, resulting in more stereotyped vocal sequences, whereas male *N. humayuni* produce more elaborate sequences with more DNs in a territorial context.

DISCUSSION

Diverse sensory signals may exhibit temporal patterning, where individual components or modules of the signal are strung together and sequenced in a definite order, so as to convey complex information (Hebets & Papaj, 2005). Changing the order of these modules changes the signal itself, as does elaboration (greater complexity) or redundancy (increased repetition or stereotypy; Engesser & Townsend, 2019). From the complex multimodal displays of birds-of-paradise (Ligon et al., 2018; Scholes III, 2008) to the acoustic sequences of bats, primates and cetaceans (Kershenbaum et al., 2014), both birds and mammals use sequences of individual signal modules. Here, we uncovered evidence that members of two distantly related anuran lineages produce vocal sequences consisting of more than one note type. Both species (with different repertoire sizes) modified their vocal sequences in the presence of a territorial rival by incorporating different note types. One species appended notes in the presence of a rival, resulting in more elaborate sequences, whereas the other switched to a different group of notes in a territorial dispute, producing more stereotyped sequences of notes.

Animals direct acoustic signals to both potential mates and territorial rivals (Candolin, 2003; Hebets & Papaj, 2005; Johnstone, 1996; Wells, 1977). A number of studies have demonstrated how the sequencing (temporal pattern or syntactic structure) of these acoustic signals influences the communication of context (Arnold & Zuberbühler, 2006; Clay & Zuberbühler, 2011; Crockford & Boesch, 2005; Engesser et al., 2019; Leroux et al., 2021; Rauber et al., 2020; Suzuki et al., 2016; Taylor et al., 2009). These sequences may take a number of forms, including combining individually meaningless units into meaningful sequences (e.g. hyraxes, *Procavia capensis*, Kershenbaum et al., 2012; chestnut-crowned babblers, *Pomatostomus ruficeps*, Engesser et al., 2019), using different combinations of notes to convey different meanings (e.g.: primates, Arnold & Zuberbühler, 2006; Clay & Zuberbühler, 2011; Crockford & Boesch, 2005), or combining meaningful elements to convey entirely different contexts (e.g.: pied babblers, *Turdoides bicolor*, Engesser et al., 2016; Engesser & Townsend, 2019). However, despite the multinote repertoires of many anuran species, and the evidence that they use acoustic signals to communicate context, relatively little is known about sequencing or the presence of temporal structure in how sequences are produced. Our finding that *N. humayuni* appends additional DNs to its vocal sequences bears some similarities to the sequences of pied babblers (Engesser et al., 2016), where alert calls are typically combined with recruitment calls when mobbing a predator. Primates, on the other hand, typically employ different types of calls in combination, such that there may be a very large number of possible call combinations to convey diverse contexts (Arnold & Zuberbühler, 2006; Crockford & Boesch, 2005; Leroux et al., 2021). *Pseudophilautus amboli* bears some similarity to the latter system in possessing a larger repertoire. However, the number of combinations used is far fewer. Instead, certain note types are used in certain contexts, and thus tend to co-occur with each other more often than expected by chance. Because of the vital role that acoustic signals play in anuran life histories (Ryan, 1988; Wells, 1977), studying these will help us understand how complex forms of communication evolved.

Several anuran species across the world possess ‘compound advertisement calls’ in which one component is directed at other males and the other is directed at females (Arak, 1983; Backwell, 1988; Furtado et al., 2016; Littlejohn & Harrison, 1985; Nali & Prado, 2014; Schwartz & Wells, 1984; Wells & Greer, 1981). The two components of the call are thus ‘functionally partitioned’. For example, *Eleutherodactylus coqui* possesses a two-component ‘co-qui’ call, where the ‘co’ component is directed towards males and the ‘qui’ component is directed towards females (Narins & Capranica, 1978). However, some of the best evidence we have for sequences in frogs comes from studies of the túngara frog, *Engystomops pustulosus*, wherein the male vocal repertoire comprises two notes: a ‘whine’ and a ‘chuck’. A male may emit only a whine (a ‘simple’ call), or append a variable number of chucks to the end of a whine to produce a ‘complex’ call (Rand & Ryan, 1981). Females prefer complex calls over simple calls (Ryan, 2019), and vocal males adjust the complexity of their own call in response to other complex calls (Ryan, 2019). *Nyctibatrachus humayuni* bears some superficial similarity to the tungara frog; however, the fact that more DNs were added in encounters with other males also bears some similarity to the system of *E. coqui*. *Pseudophilautus amboli,* on the other hand, bears no resemblance to any of these anuran species, instead employing different combinations of notes in different contexts. Our data thus suggest that multinote vocal repertoires in anurans (Narins et al., 2000) present a number of avenues by which vocal sequence may be modified according to behavioural context.

In general, sensory signals may communicate context in multiple ways. In some cases, the signaller may employ entirely different signals for different receivers, or in different contexts (Andersson et al., 2002; Balakrishnan & Pollack, 1996; Baptista, 1978; Byers & Kroodsma, 2009; Centeno et al., 2021; Karubian et al., 2009; Leo, 1959; Rosenthal et al., 2018; Vanderbilt et al., 2015; Zambre & Thaker, 2017). Alternatively, signallers with limited repertoires may modify the sequence in which notes are emitted, such that similar signals may convey different messages to different receivers (Berglund et al., 1996; Dalziell & Cockburn, 2008; Moskát & Hauber, 2019; Myberg Jr, 1997; Vasconcelos et al., 2010). Studies have modelled vocal sequences as first-order Markov chains, or used Shannon entropy to characterize sequence variability (Kershenbaum et al., 2016). This provides information not just on context-dependent changes in sequence structure, but also on how labile they are (i.e. how much information is contained in the variability of sequences). However, each of these methods is prone to a number of assumptions about the underlying processes (for example, that they follow Markovian dynamics; Kershenbaum et al., 2014). To this end, our analysis of note co-occurrence patterns offers a generally applicable method to test hypotheses about

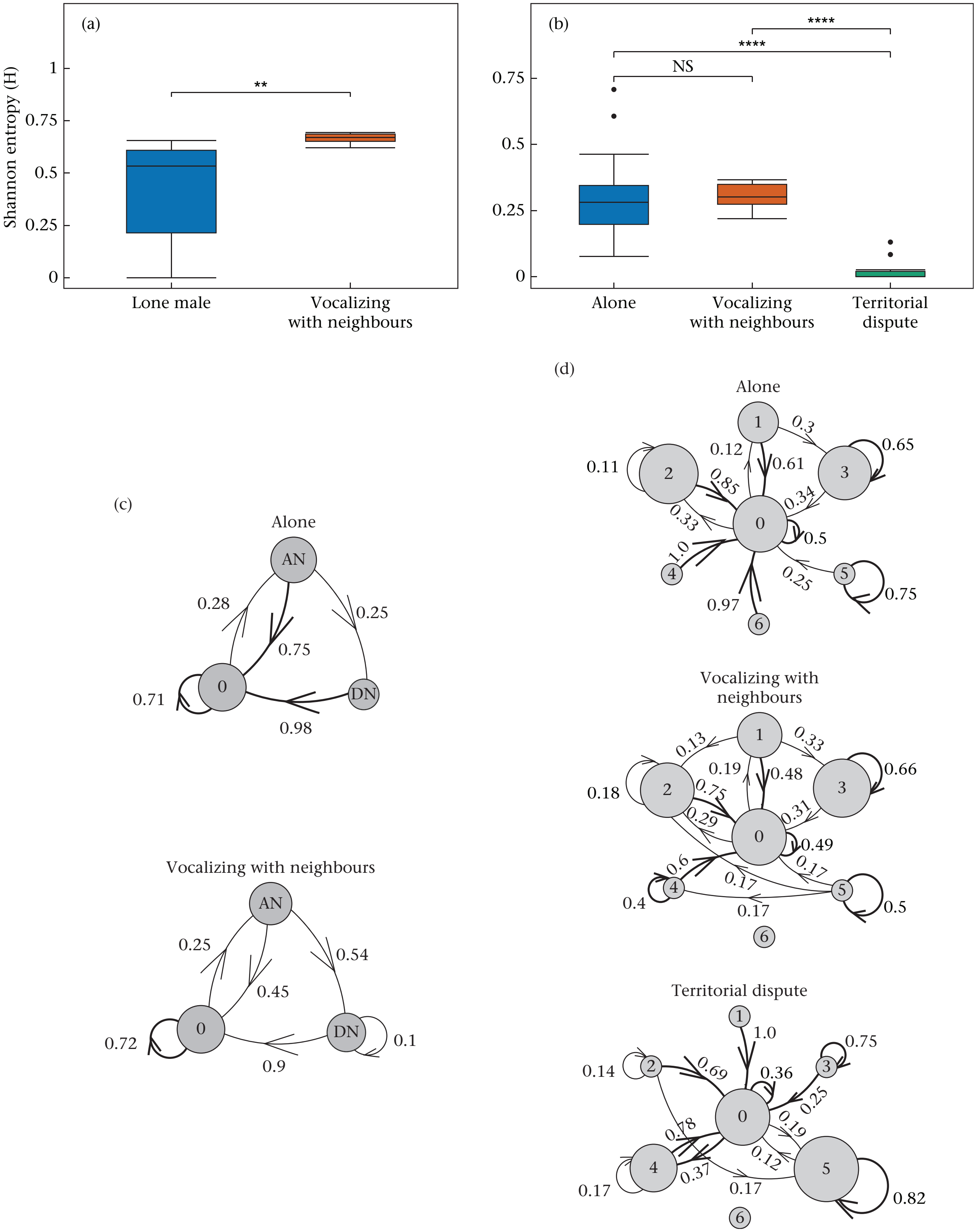


Figure 4. (a, b) Variation in Shannon entropy of vocalization with behavioural context in (a) *N. humayuni* and (b) *P. amboli*. The upper and lower edges of the box indicate the third and first quartile, respectively, and the internal line indicates the median. Whiskers extend to 1.5 times the interquartile range, and circles represent outliers beyond this limit. $^{**}P < 0.01$; $^{****}P < 0.0001$. (c, d). Transition probabilities obtained by modelling the vocal sequences of (c) *N. humayuni* and (d) *P. amboli* as first-order Markov chains for different behavioural contexts. Size of each circle represents the average proportion of that note type (relative to all notes produced) in that context, and numbers on the arrows are transition probabilities. The thickness of the arrows is scaled to the probability of that transition occurring. Transition probabilities that are less than 0.1 have been omitted for the sake of clarity. The note type 0 represents silent periods in between notes, and the silence–silence transition probability represents the probability of extending a silent interval without emitting a call.

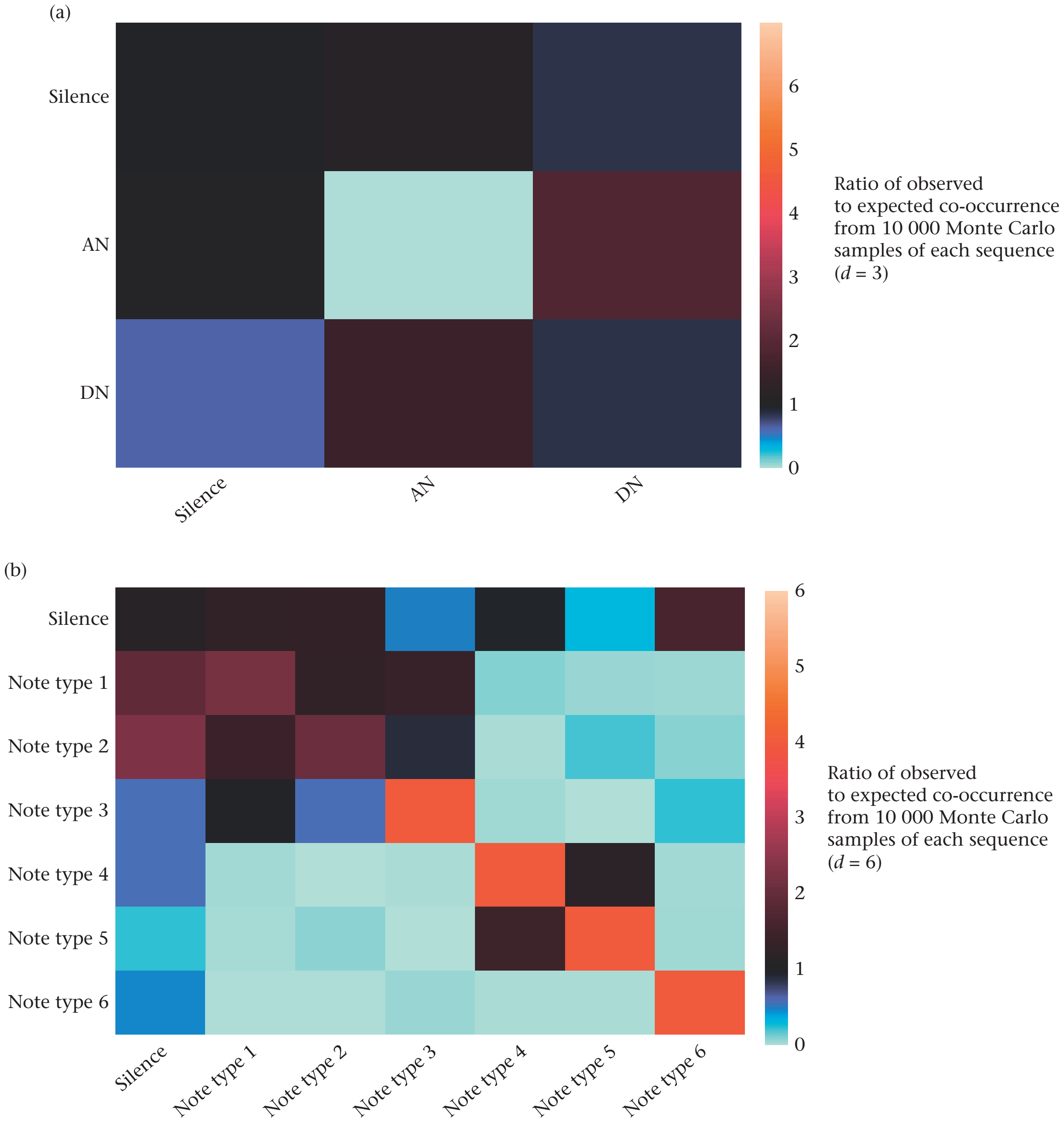


Figure 5. Ratio of observed to expected probabilities of note type co-occurrence ($^{d}R_{ij}$), for (a) *N. humayuni* and (b) *P. amboli* calculated using 10 000 runs of the Monte Carlo method for each sequence, with expected co-occurrence being averaged over 50 synthesized random data sets. Colour indicates the value of $^{d}R_{ij}$ for d values of 3 (*N. humayuni*) or 6 (*P. amboli*). Redder colours indicate greater co-occurrence than expected by chance alone, and bluer colours indicate lower co-occurrence than expected by chance (see Methods).

vocal sequences, without relying on these underlying assumptions. This method expands on analyses employed in recent studies of mammalian vocalizations (Leroux et al., 2021; Rauber et al., 2020). Taken together, these analyses provide a general analytical framework that can be compared across taxa. Our results suggest concordance of the co-occurrence analysis with other analytical tools, indicating that our combination of methods may provide a versatile tool to examine sequences and syntactic structure.

Using this method, we have demonstrated both evidence of sequence structure in frog vocalizations and that the presence of conspecific males drives specific changes in vocal sequence. In *P. amboli*, note types 4 and 5 are emitted primarily during territorial disputes, typically as repetitive, stereotyped sequences with lower variability. This suggests an ‘agonistic’ or ‘territorial’ function for sequences consisting of these notes, directed towards other males. On the other hand, males vocalizing in nondispute contexts produced more diverse sequences typically consisting of note types 1, 2 and 3. A strength of our analysis is that we accounted for variable periods of silence between vocal bouts, allowing us to estimate start and end states in these sequences. This also reduced the likelihood of notes that were emitted far apart being characterized as ‘sequences’. The co-occurrence analysis suggests that *P. amboli* forms sequences out of ‘groups’ of notes, resulting in very different vocal sequences in different contexts (Figs. 4 and 5). Future studies will investigate how properties of notes (e.g. note duration) influence their use and likelihood of repetition in sequences. *Nyctibatrachus humayuni,* with a limited vocal repertoire of only two note types, emits the same call types when alone as well as in the presence of other males. However, in the presence of a neighbour, males appended more DNs to the end of their call, resulting in more elaborate vocal sequences with a higher Shannon entropy. Thus, using the same signals, males of this species modified the sequence of notes to signal to both potential mates and territorial rivals. Playback experiments are necessary to reveal how females and rival males respond to sequences of different complexity. In addition, our study did not investigate the effects of morphology (for example, size) of individual frogs on their vocal sequences, as well as the responses of other males. The effect of sequence complexity and individual traits on the outcome of territorial disputes should also prove fertile ground for future studies.

Acoustic signals and vocal repertoires are structured by evolutionary history, physiology and the effects of body size (Ryan, 1988). By presenting evidence that anurans (1) produce complex vocal sequences irrespective of repertoire size, and (2) modify these sequences to communicate information about context, we highlight the need for further research on the evolution of complex communication in vertebrates. The two species we have studied modify their sequences in different ways, thus providing a starting point to understand how diverse anuran vocal sequences are. The Western Ghats is a hyperdiverse region for amphibians (Aravind & Gururaja, 2011), and studies such as ours underscore the possibilities for uncovering novel communication strategies among these understudied taxa. Our analytical workflow, including an analysis of note co-occurrence that does not assume any underlying processes, also provides a means of comparing sequences and syntactic structure to obtain more general insights into the role of sequence structure in conveying information.

Author Contributions

Conceptualization: A.S.B., S.K.S., A.K. Investigation, Methodology: A.S.B., V.A.S. Data curation, Formal analysis: A.S.B. Funding acquisition, Resources, Project administration, Supervision: S.K.S., A.K. Writing-original draft, Writing-review and editing: A.S.B., S.K.S., A.K.

Data Availability

Custom scripts used in the analyses reported in this article may be accessed at the following GitHub repository: https://github.com/ThePandalorian/frog_vocal_sequences/

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Supplementary Material

Supplementary material associated with this article can be found online at https://doi.org/10.1016/j.anbehav.2021.12.004.

Appendix

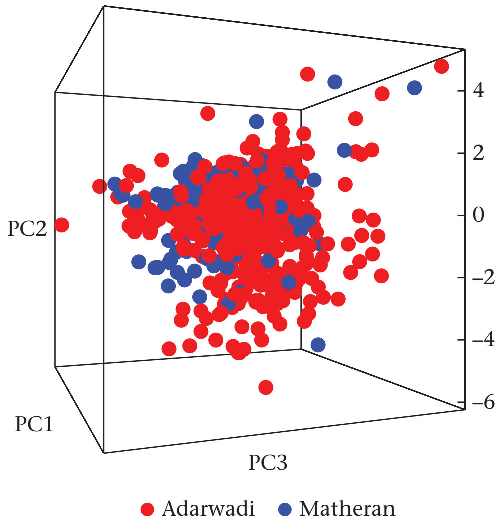

Figure A1. Principal components (PC) analysis of the notes of *N. humayuni* by location. Blue points are sampled from Matheran and red points from Adarwadi.

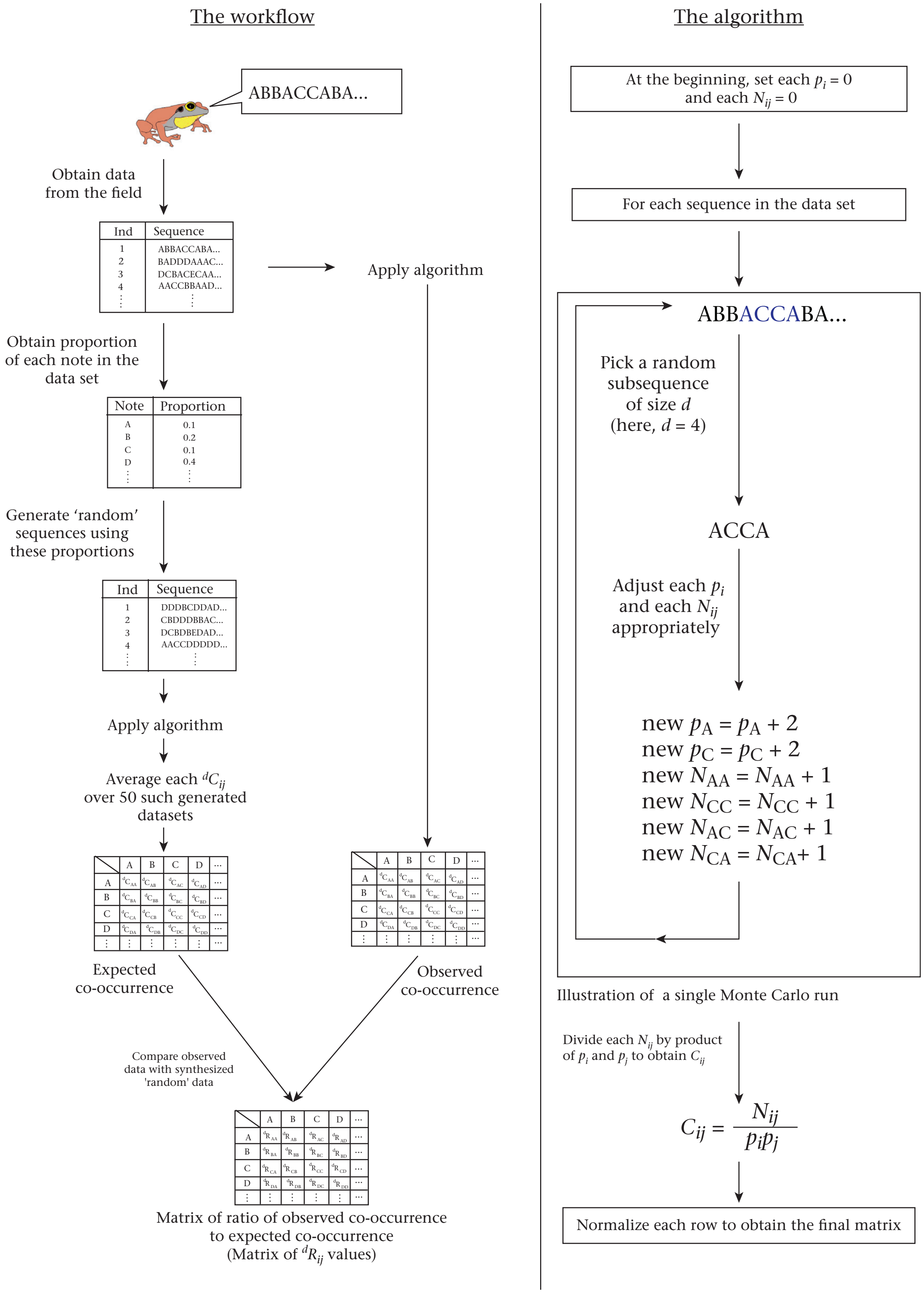

Figure A2. Workflow used to obtain the co-occurrence matrices. In the algorithm on the right, p_i denotes the number of times the i^{th} note type was found in a subsequence, and N_{ij} denotes the number of times the j^{th} note type was found after the i^{th} note type in the same subsequence. For the expected co-occurrence, the average co-occurrence obtained over 50 synthesized ('random') data sets was used.

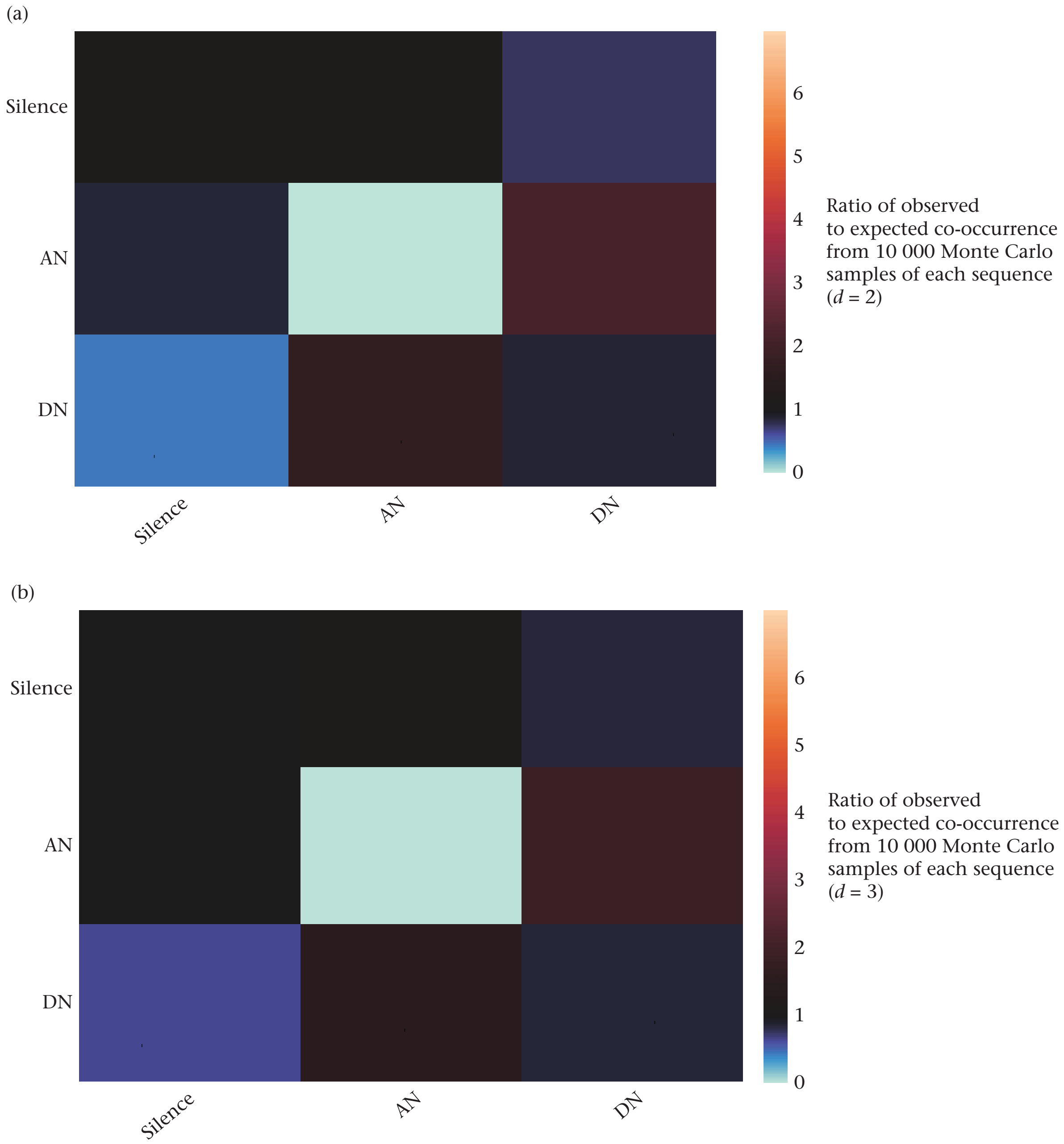

Figure A3. R_{ij} matrices for *N. humayuni* for *d* values of (a) 2 and (b) 3, from 10 000 Monte Carlo samples of each sequence. Redder colours indicate that the corresponding note types co-occur more often than expected by chance alone.

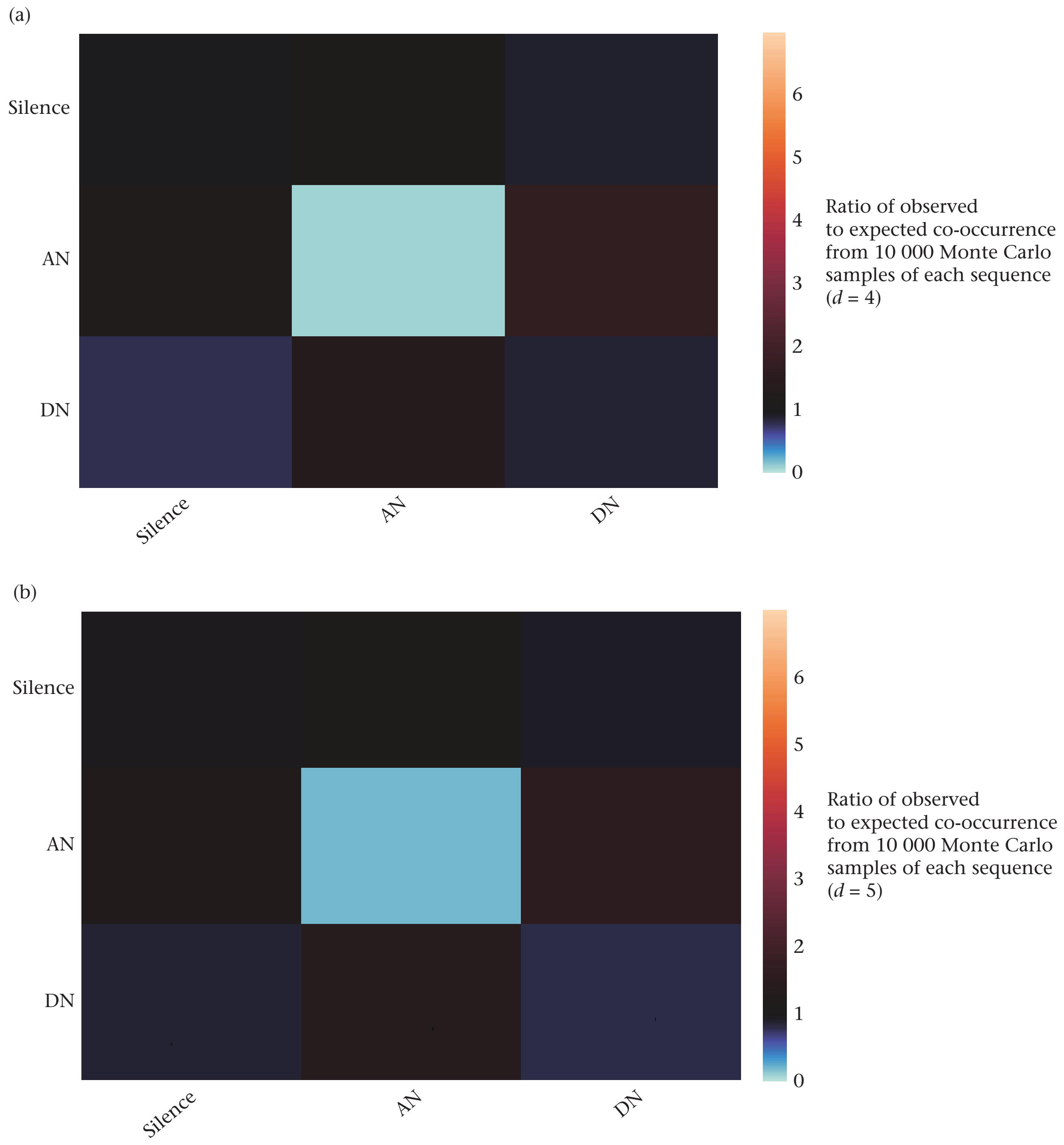

Figure A4. R_{ij} matrices for *N. humayuni* for d values of (a) 4 and (b) 5, from 10 000 Monte Carlo samples of each sequence. Redder colours indicate that the corresponding note types co-occur more often than expected by chance alone.

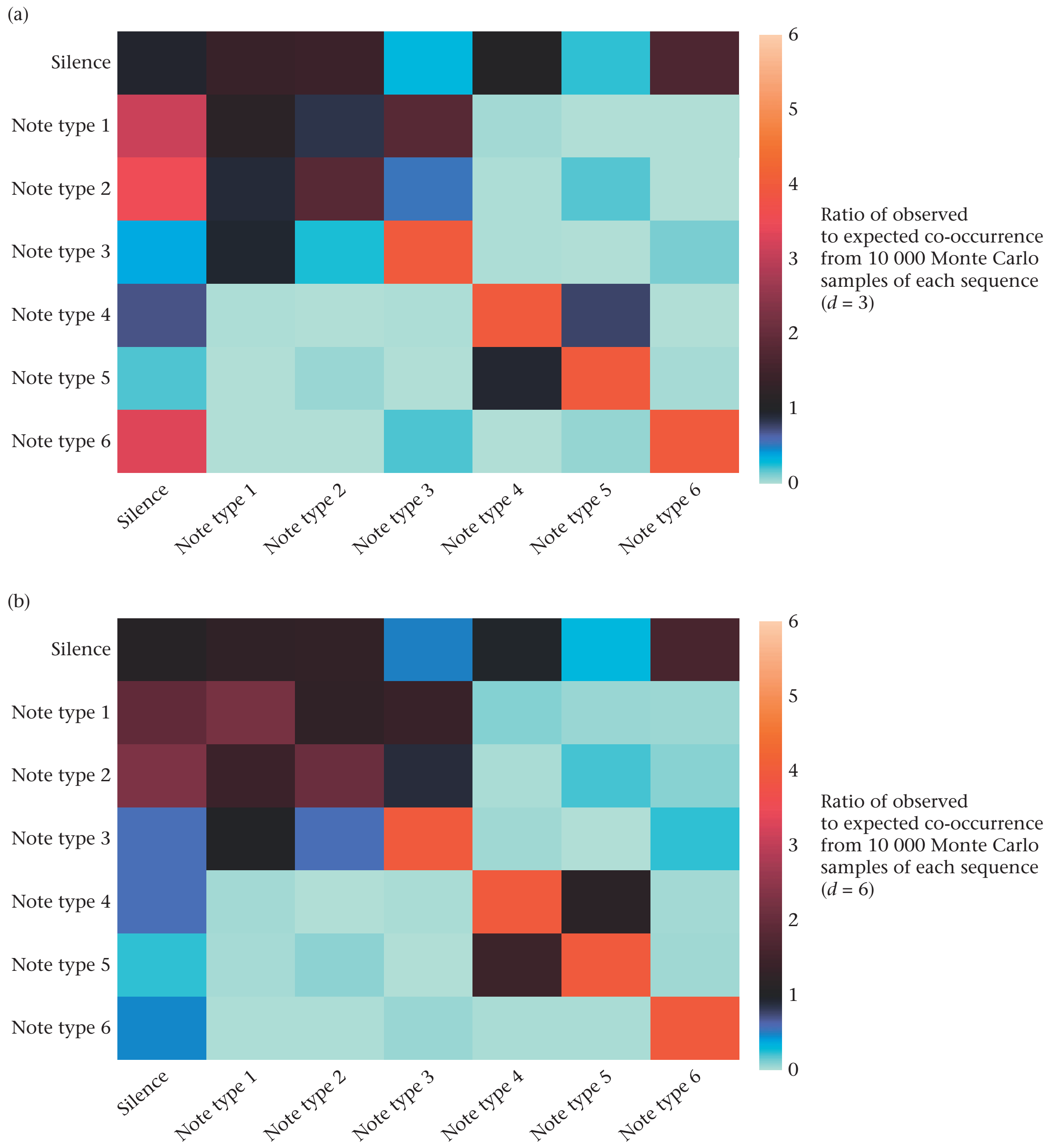

Figure A5. R_{ij} matrices for *P.amboli* for *d* values of (a) 3 and (b) 6, from 10 000 Monte Carlo samples of each sequence. Redder colours indicate that the corresponding note types co-occur more often than expected by chance alone.

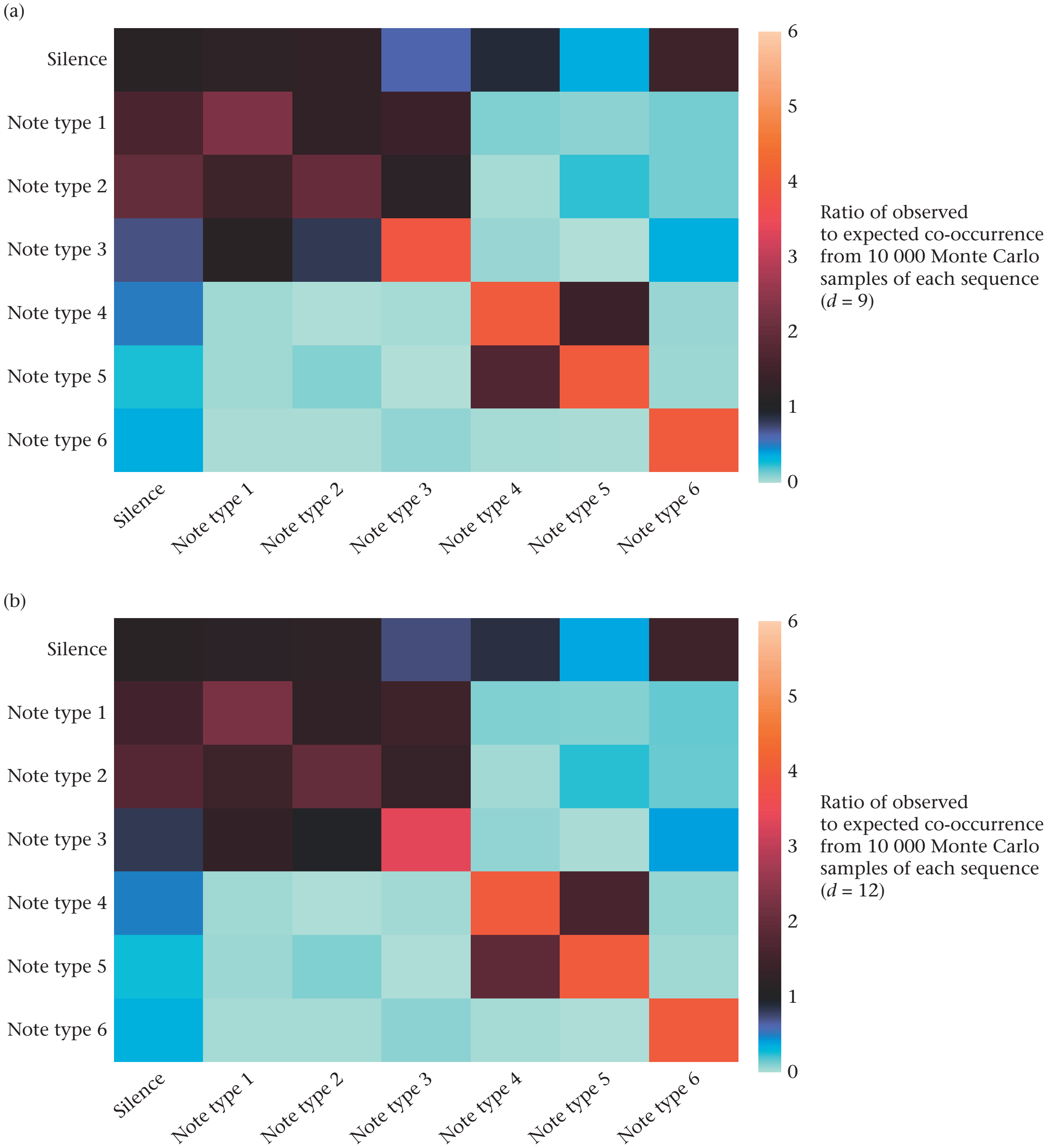

Figure A6. R_{ij} matrices for *P.amboli* for *d* values of (a) 9 and (b) 12, from 10 000 Monte Carlo samples of each sequence. Redder colours indicate that the corresponding note types co-occur more often than expected by chance alone.

Table A1

Principal component loadings for the comparison of *N. humayuni* notes by location

	PC1	PC2	PC3
Delta time (s)	0.26	0.01	0.36
Average entropy (bits)	−0.43	−0.32	−0.21
Maximum entropy (bits)	−0.29	0.03	−0.37
Bandwidth 90% (Hz)	−0.34	−0.43	−0.05
IQR bandwidth (Hz)	−0.23	−0.46	0.07
IQR duration (s)	0.04	−0.31	0.3
PFC maximum slope (Hz/ms)	−0.41	0.22	0.33
PFC minimum slope (Hz/ms)	0.39	−0.26	−0.35
Peak frequency (Hz)	−0.17	0.44	−0.15
PFC maximum frequency (Hz)	−0.37	0.29	0.03
PFC minimum frequency (Hz)	0.09	0.08	−0.54
Peak time relative	0	−0.06	0.22
Cumulative proportion of variance explained	0.24	0.46	0.6

IQR: interquartile range; PFC: peak frequency contour.