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Spectrographic analyses reveal signals of individuality and kinship in the ultrasonic courtship vocalizations of wild house mice

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ABSTRACT

Male house mice produce ultrasonic vocalizations (USVs) during courtship; however, it is unclear why males produce USVs and what information their calls communicate. In laboratory mice, male USVs are attractive to females [1,2]. They appear to facilitate mating and coordinate copulation behavior [3,4] perhaps because USVs provide information about males' quality or compatibility. In our studies on wild house mice (*Mus musculus musculus*), we found that females can discriminate the USVs of unrelated males versus siblings [5]. In this study we conducted spectrographic and temporal analyses on recordings of courtship USVs of wild males. We found evidence that males' vocalizations contain signatures of individuality and kinship. The individuality of males' USVs could be due to differences in the filter function of the vocal tract or differences of the vocal apparatus, which might directly influence the temporal and spectral features of vocalizations. Further studies are needed to determine the consistency of individual USVs over longer periods of time and across contexts, and whether the familial effects we found are due to genetic relatedness, social learning (imprinting), or both.

1. Introduction

House mice (*Mus musculus*) emit ultrasonic vocalizations (USVs), which are outside the range of human hearing [1,3,6,7]. USVs of adult house mice are sexually dimorphic [8], produced mainly by males during courtship [1,2,4,9] in response to sexually mature females or their scent [laboratory mice: 10–12; wild mice: 5]. The evolutionary functions of males' USVs are not completely clear, though our studies on wild house mice support the idea that they play a role in mate choice and kin discrimination: females are attracted to playbacks of male USVs, they can discriminate the calls of unrelated males versus siblings [5]. Females' ability to discriminate males' USVs suggests that they convey reliable signals of kinship or individuality. The aims of this study were to analyze spectral and temporal features of recordings of the courtship USVs of wild male mice (F1 from wild-caught *M. musculus musculus*), determine whether these vocalizations contain features of individuality or kinship, and identify these features.

Spectrographic analyses on the courtship USVs of male laboratory mice recently discovered that they are surprisingly complex and show features of birdsong: USVs consist of different types of syllables, whose temporal sequencing includes the utterance of repeated phrases [7]. Male laboratory mice produce different call types, high- and low-frequency calls, which, besides frequency, also differ in sound intensity and call duration, and multi-element calls [3,13]. We recently found that male wild house mice (*M. musculus musculus*) also produce several distinct syllable types [14]. Spectral shape of syllables shows that the USVs from wild mice can be classified by both frequency and duration, and the most apparent distinction is between low- and high-frequency calls [14]. In total four distinct syllable types were found in wild house mice (Fig. 1): low- and high-frequency calls, as well as 1-frequency step and 2-frequency step syllables [14].

Individual and kin recognition plays a role in house mice and other species, such as for inbreeding avoidance [15,16]. Individual and kinship signatures in vocalizations have been found in a variety of species, including mammals [17–24], though to our knowledge this is the first study on house mice.

2. General methods

2.1. Subjects and housing

In this study, we used 15 adult male (>8 weeks) F1 progeny of wild caught house mice *(M. musculus musculus)*. Parental mice were trapped at three locations (<1 km apart) in Gänserndorf, Austria. We prevented possible inbreeding by crossing mice among different

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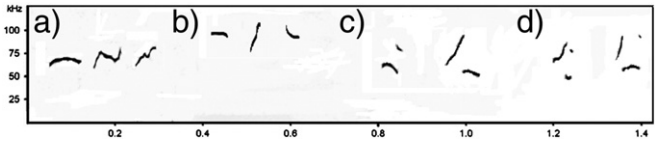

Fig. 1. Selection of spectrograms of (a) low-frequency and (b) high-frequency syllables, as well as (c) 1-frequency step syllables and (d) 2-frequency step syllables uttered by three different male wild-derived house mice (*Mus musculus musculus*).

locations. All subjects were reared in mixed-sex family groups until weaning at 21 days of age. At weaning, males (subjects) were housed individually, whereas females (urine donors) were housed in pairs with a sister in type II cages (size: 26.5 × 20.5 × 18 cm, plus high stainless steel covers, mesh width 1 cm) with bedding (Abedd). Mice were kept at a mean temperature of 20 ± 1 °C and a light: dark cycle of 12: 12 h. Food (Altromin, 1314 Forti, Lage, Germany) and water was provided ad libitum. In the colony room, mice were not acoustically isolated before or after testing periods. To standardize social experience for the adult male subjects, all individuals were exposed to adult conspecifics of both sexes before the experiment took place. The procedure followed a social experience regimen allowing repeated 10 min-interactions with individual mice over a period of two weeks, as described in [5] and [25]. At the end of the study, experimental animals were reintegrated into the breeding population. Experiments performed conform to the regulations of the Austrian Federal Ministry of Science and Research.

2.2. USV recording

Recordings were performed in an isolated wooden recording chamber situated in an isolated recording room with no other animals present [see 5, 25]. Each of the 15 individual males was recorded for one 90-min session. USVs were monitored with an UltraSoundGate CM16/CMPA condenser microphone (15–180 kHz, flat frequency response (±6 dB) between 25 and 140 kHz). The microphone was placed centrally 20 cm above the cage cover. UltraSoundGate 116, an integrated PC-based hardware and software for recording and playing back ultrasonic signals (Avisoft Bioacoustics, Berlin, Germany) and an external soundcard (Edirol UA-101, 24-Bit/192 kHz 10-in/10-out Hi-SPEED USB audio interface for multitrack computer recording) were used for USV recordings. At the beginning of each recording session, one male was placed in a type II cage containing clean bedding and a water bottle on a cage lid and food was removed to reduce sound interference. The cage was then placed in the center of the recording chamber. After a 5-min habituation period, the recording session was initiated by introducing a stimulus of freshly voided female urine. Fresh urine was used because freezing urine reduces its efficiency to elicit males' vocalizations and ability to discriminate the scent of familiar versus unfamiliar individuals [25]. Urine of donor females (n = 5), which were all unfamiliar and unrelated to the subjects, was collected on clean aluminum foil, as described by Nyby and colleagues [12]. The estrus stage of females was not controlled, as it was previously shown to have no influence on male USV responses [12]. Nevertheless, urine samples were pooled within and among females to control for estrus, individual variation, and any other potential effects from the female odor stimulus. After pooling the urine samples, 60 μl was pipetted directly from the aluminum surface onto a clean cotton swab and placed into the middle of the test cage (storage time ≤ 5 min). If a male produced any USVs during acclimatization time, the habituation time was further prolonged for another 2 min, to record only direct responses to the stimulus. Between trials, the recording chamber was cleaned with a handheld vacuum cleaner. During recording, no experimenter or other person was present in the recording room. Three of the 15 subjects did not vocalize during the 90-min session and therefore could not be included in USV analyses.

2.3. USV structure and terminology

Calls or syllables, defined as a unit of sound separated by silence from other sound units [26], consist of one or more elements, which are continuous markings on a sonogram. In this study, *general syllables* are syllables consisting of one element, whereas syllables consisting of two elements without a separation in time include a major sudden frequency jump and therefore are termed *1-frequency step syllables* [14,27]. Syllables composed of three elements without a separation in time include two major sudden frequency jumps, and are termed *2-frequency step syllables* [14,27]. In this paper, the term *frequency step syllables* is used for what we previously called *complex syllables* [5] to improve clarity and consistency in terminology among studies. A syllable type or call type is defined as a category of syllables, observed regularly in the animal's vocalization, distinct from other syllable types [7]. We define *individuality* as greater inter- versus intra-individual variability of call parameters, which is a commonly used criterion in bioacoustics [28] and other communication modalities. Similarly, we consider vocalizations that reveal signals of *kinship* for calls that show greater variation among versus within sibling groups.

2.4. USV analyses

Sound analyses were conducted using the software Sound Analysis Pro (SAP, Version 1.04) [29]. Beforehand, we extended the duration of the exceptionally short USV syllables of the mice using the time-warp feature of the software Goldwave (50% via rate, GoldWave v5.14, www.goldwave.com) and this procedure enabled us to measure the duration of these very short syllables more accurately. Sampling rate was adjusted from 250 kHz (sampling rate when recorded) to the SAP-sampling rate of 22.05 kHz. Using SAP feature batch mode, we separated USVs from non-vocalization background noise. The detection of animal sound via SAP is based on three parameters: (i) the amplitude threshold within a frequency range; (ii) Wiener entropy threshold within a frequency range (which is a measure of the width and uniformity of the power spectrum of a syllable); and (iii) noise rejection based on power in unexpected frequency ranges. Background noise, especially cage noise usually has low amplitude and high Wiener entropy compared to vocalizations. In addition to the amplitude and Wiener entropy threshold, the noise filter eliminates segments that are not syllables but noise (SAP manual). Thresholds were adjusted separately for each individual recording session by performing 30 fake trials. Parameters ranged between 22 ± 3 dB for amplitude and 4.5 ± 0.1 for Wiener entropy across all sessions. The noise rejection level was set as default with 50% below a frequency of 500 Hz. Afterwards we analyzed all USV segments using SAP feature batches. Resulting data included the following features: *duration, harmonic pitch, mean frequency*, *frequency modulation* (FM), *Wiener entropy* and *goodness of pitch*. In addition to mean frequency, we included harmonic pitch in all analyses, because wild male mice also emit general and frequency-step USVs with harmonics (Fig. 2). FM is estimated based on the slope of frequency traces in reference to the horizontal line. It is estimated using time and frequency derivatives across frequencies: if the frequency derivatives

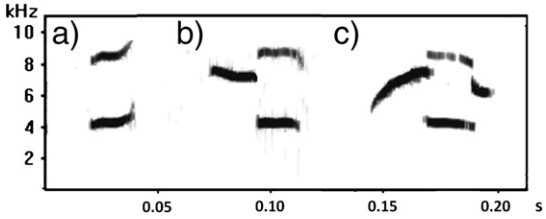

Fig. 2. Example of a) a general ultrasonic syllable with a harmonic b) a 1-frequency step syllable with a harmonic and c) a 2-frequency step syllable with a harmonic uttered by different male wild house mice (*Mus musculus musculus*).