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Variation in ultrasonic courtship vocalization in male wild house mice (Genus *Mus)*

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Abstract

Adult male laboratory mice typically emit ultrasonic vocalizations (USVs) when presented with a female conspecific or the odor of female urine. These vocalizations exhibit a number of characteristics comparable to bird songs. USVs appear to function as a courtship signal of males to attract females and may provide information about males' group or species identity. To better understand the functions I studied male USVs in wild house mice. I recorded and analyzed courtship USVs from wild- derived male mice of different species (F1 offspring of wild-caught *Mus musculus*, *Mus domesticus,* hybrids *musculus x domesticus* crosses and *Mus spicilegus*) to examine individual divergences in male USVs among wild *Mus* populations. I found significant variation in tendency of males to emit courtship USVs, when exposed to female urine, as well as variation in features of emitted USVs among *Mus musculus* populations and between *Mus* species. Male vocalizations contained 7 different syllable types and emission rates varied among populations and species. Repertoire estimation revealed significant differences in the emission rate of different syllable types among populations and species. Multivariate discriminant function analysis of one common emitted syllable type revealed that populations can be classified on the basis of spectrographic patterns suggesting that USVs are individually distinctive on the level of species. Additionally I investigated females' response to males' USVs by performing choice tests. Playback experiments indicated that females were attracted to male USVs and they could discriminate between USV of distinct *Mus* populations. My study is the first which compares USVs in wild mice. My results indicate species - specific vocal patterns in USVs which enables species discrimination on the basis of spectrographic characteristics hypothesising that male USVs potentially serve as sex and species recognition signal. Further research is needed to clarify if USVs transmit information about males' individuality or group identity.

1. Introduction

Acoustic signals are an important sensory modality for communication in variety of taxa (primates: Rendall, Owren et al. 1998; anurans: Ryan and Rand 1993; mammals: Smadja, Catalan et al. 2004; Blumstein and Munos 2005; birds: Gil and Gahr 2002; bats: Behr and von Helversen 2004; myomorph rodents: Sales 1972; D'Amato 1991). Vocalizations are used in intra - and inter - specific interactions and have evolved to communicate information about individuals' condition, social state and quality to potential rivals and mates as well as to inform listeners about objects (e.g. predators) and events in the environment (e.g. group movement) (Bradbury and Vehrencamp 1998; Owings and Morton 1998; Ryan and Rand 1993). Natural selection favors callers and their signals who are able to affect the behavior of recipients and thereby receive benefits increasing their reproductive success (Alcock 1989; Krebs and Davies 1993). The meaning and function of signals from listener's perspective may be fundamentally different from that of a signaler's (Bradbury and Vehrencamp 1998; Seyfarth and Cheney 2003).

The most complex stereotypic acoustic signals are *songs* which are characterized as a 'nonrandom temporal sequence of distinct syllable types' (Holy and Guo 2005). A syllable is defined as unit of sound separated by silence of other sound units (Holy and Guo 2005). The abundance of different syllable types within a song estimates the song repertoire size of an individual. The complexity of males' song repertoire influences female mate choice, as they can potentially provide information about males' quality, individuality and genetic compatibility (e.g. birds: Searcy and Nowicki 2000; frogs: Ryan 1990). Songs are distinguished by how they are used in social communication. Songs as a mechanism of individual recognition function in a wide range of taxa in a number of social interaction ranging from mate - attraction and courtship (e.g. songbirds: Nowicki and Searcy 2004; primates: Rendall, Owren et al. 1998; myomorph rodents Sales 1972; anurans: Littlejohn 1977; Smith and Roberts 2003), mother – infant interaction (e.g. mammals: Sousa-Lima, Paglia et al. 2002), during

intra – sexual encounters (e.g. territorial defense vocalizations of bats: Behr, von Helversen et al. 2006, elephant seals: Sanvito, Galimberti et al. 2007) and in alarm behavior (birds: Searcy and Nowicki 2000; Nowicki and Searcy 2004). Investigating vocal identity and recognition may help us to understand the social interactions in which songs are used and might provide further insight in signal evolution (Gwilliam, Charrier et al. 2008; Miller and Engstrom 2007).

In house mice few studies have focused on vocal individuality. A recent study indicated that laboratory male ultrasonic courtship vocalizations (USVs) exhibit vocal characteristics compareable to bird songs (Holy and Guo 2005) putting new light on these vocalizations. Mice are able to emit and receive USVs like many other small rodents, with frequency range between 40 and 80 kHz undetectable to human ears (Sales 1972). The laboratory strains of house mouse (Inbred lines of *Mus musculus musculus, Mus musculus domesticus* and *Mus musculus castaneus)* became an important model species investigating acoustic communication (Shu, Cho et al. 2005) as the knowledge of the genome sequence provides opportunity for testing and manipulating genetic influences on vocalizations (Brunelli and Hofer 1996; van Zutphen, Baumans et al. 1995; Wasserman, Palumbo et al. 2000). Studies in laboratory mice strains have shown that USVs are emitted by both sexes during different social context and at different age (D'Amato 1991; Hahn and Lavooy 2005; Nyby 1983; Sales 1972). Pups utter ‘distress / isolation calls’ when they are cold or separated from their nest or siblings to elicit maternal retrieval behavior (D’Amato, Scalera et al. 2005; Hahn, Hewitt et al. 1987; Smotherman, Bell et al. 1974). These calls are developed 5 - 12 days postnatal and located in a frequency band between 40 and 70 kHz (Ehret and Haack 1982; Nitschke, Bell et al. 1972). They become more stereotyped with age and vary with genotype (Hahn, Hewitt et al. 1987). Most of the calls disappear at postnatal day 12 and occur again when mature (Hahn, Karkowski et al. 1998; Zippelius and Schleidt 1956). Adult females vocalize during aggressive encounters between females or males (Maggio and Whitney 1985; Moles and D'Amato 2000), but much less often and thus hard to investigate. Some studies

suggest that females' USVs function to establish female social dominance hierarchy (Maggio and Whitney 1985) and could be used to assess social memory in ageing females (Moles, Costantini et al. 2007).

USVs occur during courtship behavior emitted by males (Whitney, Coble et al. 1973; D'Amato 1991; Sales 1972). Male vocalize in presence of a female or female olfactory cues like urine and urinary pheromones, saliva, vaginal fluids (Hoffmann 2008; Nyby, Wysocki et al. 1977; Nyby and Whitney 1978; Nyby, Wysocki et al. 1979; Whitney, Alpern et al. 1974). Previous studies in male USVs suggest that these vocalizations serve an important function in male sexual behavior. USVs appear correlated with male's level of sexual arousal (Nyby 1983). It is assumed that USVs function to show males intrest in female, keep females in close proximity (Pomerantz, Nunez et al. 1983) and facilitate female's copulation behavior (Nyby 2009). Whitney (Whitney, Coble et al. 1973) hypothesized that males' courtship USVs may be ritualized courtship display in which the male is mimicking ultrasounds emitted by infant mice which are attractive to females and reduce aggressive behavior. Females might use USVs to obtain information about males' individuality (Nyby, Dizinno et al. 1976; Warburton, Sales et al. 1989; D'Amato 1991) what might prevent females to mate with genetically different mates (Bradbury and Vehrencamp 1998). Females who mate assortatively or avoid inbreeding can increase the genetic compatibility of their mates and the viability of their progeny (Penn and Potts 1999; Drickamer, Gowaty et al. 2000). Attempts to investigate USV role during copulation behavior using devocalized laboratory male mice resulted that males reproductive behavior is not limited thus suggesting that males' ability or disability to vocalize does not influence males' reproductive success (Nunez, Pomerantz et al. 1985; White, Matochik et al. 1998). But inbreeding in laboratory strains may have alter USVs and behavioral traits e.g. it has been found that inbred strains (C57BL/6J) suffer of a loss of hearing (Nyby and Whitney 1978; Willot 2001). Male courtship USVs might be more important for wild mice under naturalistic conditions. Consequently, it is important to examine features of USVs and their behavioral components in wild mice (Kalcounis-Rueppell, Metheny et al. 2006; Keller 2002).

Only few studies focused on the production mechanism and function of USVs in rodents (e.g. Nunez, Pomerantz et al. 1985). In general mate - attraction signals require large range, good sender localization and species - specifity (Bradbury and Vehrencamp 1998). Transmission of USVs in different environments might underly selection to maintain effiecient transmission (Bradbury and Vehrencamp 1998; Ryan 1990) and avoid eavesdropping by potential predators (e.g. cats, owls; Nyby and Whitney 1978; Whitney, Stockton et al. 1979). It has been investigated that the production mechanism of USVs differs from the one of audible sound which is produced via vibrating structures in the larynx (Roberts 1975). In mice, like in many other mammals, the copulation behavior depends on androgenic activation (Kerchner 2004; Nyby 2009). Androgenic implant work evidenced that androgens and androgenic activating brain areas, like the medial preoptic area (MPOA), are also necessary in production of ultrasonic calls (Matuszczyk and Larsson 1994; Sipos and Nyby 1998) by affecting the amount of emitted USVs (Lumley, Sipos et al. 1999; Matuszczyk and Larsson 1994). Further, males' age and social experience, associated with changes in testosterone levels, is suggested to affect USV performance like other reproductive behaviors (D'Amato 1991; Dizinno and Whitney 1977; Kerchner 2004; Lumley, Sipos et al. 1999; Nunez, Nyby et al. 1978; Nunez and Tan 1984; Nyby, Dizinno et al. 1976; Warburton, Sales et al. 1989). In contrast, other studies using different laboratory strain and wild strains found no support for the influence of age and social experience on USVs (Hoffmann 2008; Sipos, Nyby et al. 1993). It is unclear if courtship vocalizations of males inform females about males' social status and physiological condition (D'Amato 1991; Warburton, Sales et al. 1989). Previous genetic studies of USV in infant laboratory mice and rats investigated that rate of USV emission and latency to call show a dominance mode of inheritance (Maggio and Whitney 1986; Thornton, Hahn et al. 2005) and suggested it as an heritable behavioral phenotype (Brunelli and Hofer 1996; Brunelli, Vinocour et al. 1997; Hahn, Hewitt et al. 1987; Sales 1979). Variability in genotype among species can generate parallel vocalization features facilitating species - specific recognition (Rendall, Owren et al. 1998), whereas inbreeding affects individual and population

performance (Keller 2002). Inbreeding of laboratory mice (Guénet and Bonhomme 2003; Van Zutphen, Baumans et al. 1995) may have reduced variability in USVs and study of wild house mice vocalizations could reveal more complexity in USVs (Hahn, Hewitt et al. 1987; Roubertoux, Martin et al. 1996; Panksepp, Jochman et al. 2007; Sales 1979) and ought to be used for comparison with acoustic communication of other species (Kalcounis-Rueppell, Metheny et al. 2006).

Stereotypic vocalizations can be taxonomical informative and serve as useful tools to identify phylogenic relationship (Geissmann and Nijman 2006; McCracken and Sheldon 1997; Packert, Martens et al. 2003). Most studies, using subtle spectral patterns of vocalizations as phylogenic traits, have been conducted with birds (McCracken and Sheldon 1997; Packert, Martens et al. 2003) and mammals (e.g. ungulates: Geissmann and Nijman 2006). Vocal signals are thought to evolve from senders' preexisting behavioral, physiological or morphological traits that can cause vocal characters to be similiar by convergent evolution (Bradbury and Vehrencamp 1998). To describe a certain trait as species – specific, variation within species should be minimal and differences between species should be large to enhance distinctiveness (Boughman and Moss 2003). Thus, a precursor to vocal recognition is that vocalizations transmit sufficient unique information of a species to be identified as species – specific (Gwilliam, Charrier et al. 2008). Additionally, playback experiments are necessary to verify existence of individual / species – specific calls and vocal recognition (Balcombe 1990; Catchpole 1979; Rendall, Owren et al. 1998; Seyfarth and Cheney 2003). A variety of morphologic and biochemical methods has been used to examine phylogenetic relationship among genus *Mus*. But taxonomic structure based on molecular data is unresolved (Box 1). The phylogenic relationship between *Mus musculus musculus* and *domesticus* remain unclear as they show similarities as well as dissimilarities in genetics, behavior and morphology (Heth, Todrank et al. 2001; Tucker, Sandstedt et al. 2005; Smadja and Ganem 2002) (Box 1). They are treated as biological distinct species (Heth, Todrank et al. 2003; Sage and Atchley 1993) but also as subspecies based on molecular

phylogeny (Smadja and Ganem 2002; Tucker, Sandstedt et al. 2005). Ultrasonic vocalizations are suggested to be stereotypic like avian vocalizations (Holy and Guo 2005) hence could function as a further phylogenic trait in taxonomic studies as mice may pay attention to. Mate choice, relying on inter - individual recognition, could limit gene flow between populations and hence play an important role in speciation (Smadja and Ganem 2002). It is necessary to identify the traits that are involved in the reduction of gene flow among populations and understand the evolutionary constraints that have acted on these traits (Smadja and Butlin 2009). Vocal recognition could avoid mating, which may lead to genetic inferior hybrids, and unnecessary male – male competition in closely related species (Gwilliam, Charrier et al. 2008; Vannoni and McElligott 2008). Hybridization occurs between *Mus musculus musculus* and *domesticus* (Box 1). Hybrids exhibit reduced fitness than pure parental crosses, hence natural selection can lead to the evolution of premating isolating mechanism that would reduce hybridization (Smadja, Catalan et al. 2004; Teeter, Payseur et al. 2008). Studies within this contact zone show patterns of reproductive character displacement affecting mating signals (urinary cues) and females' mate preference (Smadja, Catalan et al. 2004). Besides olfactory cues, USVs might be a further trait involved in mating preference systems. Differences in male courtship USVs could abet females assortative mating to increase her direct and indirect fitness.

To better understand the functions of USVs, I examined wild-derived house mice populations and species (F1 offspring from wild caught *Mus musculus musculus, Mus musculus domesticus* considered here to be subspecies after (Tucker, Sandstedt et al. 2005)*, Mus spicilegus* as well as two reared hybrid strains of *M. m. domesticus* and *M. m. musculus* to address questions concerning individuality of male USVs / species - specific differences and females' attraction to these calls. Recording trials of wild adult male mice were conducted presenting fresh female urine (Hoffmann 2008; Sipos, Alterman et al. 1995; Whitney and Nyby 1979) to test for species - specific vocal traits and variation of courtship USVs of mature males of local *Mus musculus* populations and distinct *Mus* species. In

addition to the rate of USVs and latency, spectrographic parameters of syllables could also be taxonomic informative (Bradbury and Vehrencamp 1998; McCracken and Sheldon 1997; Miller and Engstrom 2007). Further spectrographic analysis were made to detect inter - specific variability in vocal patterns (Holy and Guo 2005; Panksepp, Jochman et al. 2007; Sousa-Lima, Paglia et al. 2002; Vannoni and McElligott 2008). Prior studies suggested that females are able to obtain information about male social status, condition (D'Amato 1991; Warburton, Sales et al. 1989) and kinship (Hoffmann 2008). But the meaning and function of vocalizations from the reciever's perspective may be fundamentally different from that of a signaler's (Seyfarth and Cheney 2003). Thus, playback experiments were performed to test if females are attracted to male USV (Pomerantz, Nunez et al. 1983) and assess if females could discriminate between different *Mus musculus* populations or *Mus* species based exclusively on male courtship USVs (Dizinno and Whitney 1977; Nyby 1983; Whitney, Coble et al. 1973). If there exist habitat / population dependent USV variability among wild house mice, which could be obtained by females, female should prefer own population when presented male USV of own versus strange population or species.

In summary, this study reveals for the first time inter - and intra specific variability in ultrasonic courtship vocalizations in wild mice species. Additionally, it supports the idea that male courtship USV attracts females. Male USVs may have the potential to convey information about male's species or population identity enabling vocal recognition affected by natural selection. USVs could function as a further phylogenic signal.

Box 1: Evolution, ecology and behavior of *Mus musculus*

The genus *Mus* (family *Muridae,* subfamiliy *Murinae*) compromises 30 - 40 species (Figure A). The Palearctic clade includes *M. musculus musculus* and *M. musculus domesticus* and the sister taxa *M. spicilegus.* Classification is based on recently reported investigation, of several mitochondrial and nuclear DNA markers (Smadja and Ganem 2002; Tucker, Sandstedt et al. 2005) considering *Mus musculus musculus* and *Mus musculus domesticus* as subspecies. *M. m. domesticus* is sometimes referred to as a distinct species because it appears genetically and morphologically distinct away from the hybrid zones based on more global genetic criteria (Heth, Todrank et al. 2001; Sage and Atchley 1993). In contrast, the phylogenic relationship between species *Mus musculus* and *Mus spicilegus* is clear. Though sympatric, *Mus spicilegus* differ in genetics, behavior and morphology from the *Mus musculus* strains (Guénet and Bonhomme 2003; Tucker, Sandstedt et al. 2005).

The emergence of the genus *Mus* was ca. 5 Myr ago (Guénet and Bonhomme 2003) with the split between *Mus spicilegus* and *Mus muscuslus* less than 1 Myr ago. *Mus musculus* has its evolutionary origins in Asia and has now spread over the whole planet. *Mus musculus domesticus* is found in western and *Mus musculus musculus* is found in eastern Europe. They have differentiated in allopatry, but show interbreeding along contact zones (Hybrid zone in Europe, along north - south axis from Denmark to Caucasus (Christophe and Baudoin 1998). However, the hybrids suffer from a loss of fertility (Teeter, Payseur et al. 2008) and it is suspected that there exists reproductive character displacement in the border of hybrid zone as premating isolations mechanism against hybridization (Smadja and Ganem 2005). *M. m. domesticus* males show aggressive dominance against *M. m. musculus* (Heth, Todrank et al. 2001; Sage 1981).

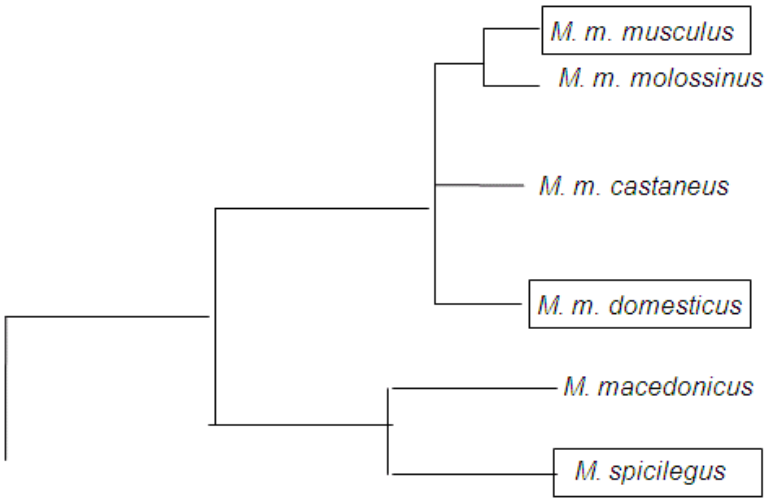

Figure A: Partial phylogenetic tree of the *Mus* species complex (after Tucker, Sandstedt et al. 2005).

Wild *M. musculus* populations consist of a dominant male that defends territories. Females mate with the dominant male and sometimes extra – pair copulations occurs.

Mus spicilegus mainly inhabit the steppes of eastern Europe (Sokolov, Kotenkova et al. 1998). A distinct character is the mound - building behavior which occurs in autumn. Generally a group of 4 - 14 juvenile, presumably members of the same family, construct special mounds to store food and where they overwinter without breeding. Laboratory studies in mound - building mice indicate a strong pair bond in this species between the familiar male and female (Baudoin, Busquet et al. 2005; Patris and Baudion 2000). These results could be interpreted as an indication of monogamy as described in several species of mammals (Kleiman 1977).

2. General Methods

2.1. Subjects and housing

All experimental animals were laboratory-bred offspring (F1) of wild adult mice. I used offspring (F1) of the mound-building mouse *(Mus spicilegus)* caught at two different location in Slovakia (henceforth M.s.). I used F1 - F3 *Mus musculus musculus* (M.m.m.) offspring of wild mice caught at three different locations in Vienna, Austria (henceforth M.m.m.K, M.m.m.V, M.m.m.R) and one location in Gänserndorf, Austria (henceforth M.m.m.S) maintained as breeding stocks in our colony. Microsatellite analyses data revealed that within M.m.m.K and M.m.m.S naturally occurs higher genetic variability (heterozygote) thus can be compromised as outbred breeding colony (‘Outbred’). Whereas M.m.m.R and M.m.m.V exhibit lower variability (homozygote) and can be described as inbred breeding colony (‘Inbred’) based on analysis of 6 polymorphic microsatellite loci (unpublished data Musolf et al.).

The *Mus musculus domesticus* population was represented by F3 offspring of wild mice caught in the Massif Central, France (henceforth M.m.d.). Hybrids were F1 crosses between *M. m. domesticus* females and *M. m. musculus* males from 2 of our stock populations (henceforth Hybrid 1, Hybrid 2).

All subjects were raised in mixed - sex family groups until weaning at 21 days of age. At weaning, males were housed individually to prevent fighting, whereas females were kept as sister pairs in type II cages (size: 26.5 x 20.5 x 18 cm, plus high stainless steel covers, mesh width 1 cm) with bedding and nesting material (Abedd). Mound - building mice were kept in same-sex as well as in mixed - sex litter groups in type II cages (size: 36.5 x 20.5 x 18 cm, plus high stainless steel covers, mesh width 1 cm) with bedding and nesting material (Abedd).

Home cages were kept in standard conditions (mean temperature 20 ± 1°C and 12:12 h light: dark cycle; lights on at 07:00 a.m.). Food (Altromin, Germany) and water were provided *ad libitum*. All experimental mice were sexually mature (> 8 weeks). Animals were not acoustically isolated (except for M.s. which were kept in a different colony room) and were kept within the same

colony room before, during and after testing periods. At the conclusion of the study, experimental animals were reintegrated into the breeding population.

2.2. Social experience

Previous studies with laboratory mice indicated that fresh female urine elicits only few or even no USVs from males when they are socially or sexually inexperienced (Dizinno and Whitney 1977; Maggio, Maggio et al. 1983), whereas a recent study found no evidence for this in wild mice (Musolf et al., submitted). Nevertheless social experience treatments during adulthood were conducted to be conservative and ecologically natural. Male mice either had been involved in a mate choice experiment allowing direct interaction with a female over a time span of four weeks (M.m.m.) or males (M.m.d.; Hybrid 1 & 2) received social experience by being systematically exposed to both male and females through a fence when sexually mature (Hoffmann 2008). As males of M.s. continued to live in mixed sex family groups until adulthood, no further social experience treatment was performed. In summary, all experimental males had contact with females and female odors before testing.

2.3. Urinary stimulus collection

Only fresh rather than frozen urine was used as stimulus to trigger male's USVs because freezing reduces its effectiveness (Hoffmann 2008). To obtain urine, the donor female mice were placed on a surface covered with clean aluminum foil while holding them by the nape. Handling was usually sufficient to induce urination, but if not I gently stroked the ventral area in an anterior-to-posterior direction, which was usually effective (Nyby, Wysocki et al. 1979). The urine on the foil was collected immediately and at least 60 µl was pipetted directly from the aluminum surface onto a clean cotton swab (storage ≤ 5 min). In addition, soiled bedding (12 g) of the same female was collected from the home cage and presented simultaneously to the test animal. For acoustic playbacks, male urine was collected from different males to create urine mixed pools. Equal amounts of freshly voided urine (5 µl per male) were mixed in a 0.2 ml PCR tube and stored at -20°C. A urinary pool consisted of aliquots of 10 unfamiliar male mice of each population or species (50 µl each). For tests with M.s., the urine pool for both (M.s. & M.m.m.S) was reduced to 12.5 µl (1.25 µl per male)

as the urine harvest was much less than in the other *Mus* species (personal observation). The experimenter was wearing gloves at all times and the metal foil was exchanged immediately after every single urine collection.

2.4. Apparatus, calibration and recording procedure

For recording USVs, an individual male mouse was placed within its home cage into a recording chamber, which was a wooden box (65 cm x 65 cm) lined inside with acoustic foam (SH 003 - Absorptionsplatten / Pyramiden, 5 cm). The lid of the box was also lined with acoustic foam, and placed loosely on the opening of the box to allow air and light supply. A condenser microphone (UltraSoundGate CM16/CMPA, 15 – 180 kHz, flat frequency response (± 6 dB) between 25 and 140 kHz) was fixed 20 cm above a hole [Ø = 20 cm] in the middle of the lid of the box. For monitoring USVs, we used an UltraSoundGate 116 (Avisoft Bioacoustics, Berlin, Germany) and an external soundcard (Edirol UA - 101, 24 – Bit / 192 kHz 10 – in / 10 - out Hi - SPEED USB (USB 2.0) audio interface for multitrack computer recording). Settings included sampling rate at 250 kHz and a format of 16 bit. For spectrogram generation, recordings were transferred to Avisoft-SASLab (Version 4.40) and a fast Fourier transformation (FFT) was conducted for signal analysis. Spectrograms were generated with an FFT-length of 512 points and a time window overlap of 50% (100% Frame, FlatTop window). A noise reduction by 90 dB below – 52 dB was used to reduce background noise. We calibrated the recording equipment by recording a pure tone of 110 kHz (commercially available tuning fork) and comparing the frequency in the spectrogram with the actual frequency recorded.

Each male was recorded to analyze their ultrasound emission in the presence of urine from an unfamiliar female. At the beginning of each recording session, a new cage lid was put on male's homcage (type II; food and water bottle were removed to reduce sound interference). The cage with the mouse was placed in the centre of the recording chamber. Test trials began after introduction of the urine and soiled - bedding stimuli. Urine and bedding originated from different unfamiliar randomly chosen adult females from our stock regardless of their oestrus stage as this has little if any effect on males' USV performance (Nyby, Wysocki et al. 1979). Each recording trial lasted for 30

minutes after stimuli introduction. After each trial, the recording chamber was cleaned using a handheld vacuum cleaner. Recordings were made in an isolated recording room with no other animals present. Temperature of the testing room was held constant at 20.4° ± 0.9°C. During recording, no experimenter or other persons were present in the recording room.

2.5. Statistical Analyses

All statistical analyses were performed using statistical program SPSS (version 15.01 for Windows). Results are reported as mean ± SE, unless specified. In all cases $p < 0.05$ was considered to be statistically significant. All data was checked for normal distribution. To control for Type I errors from conducting multiple tests, False Discovery Rate (FDR) was used (Benjamini and Hochberg 1995).

2.6. Experimental designs

2.6.1. Experiment 1 - USV features: variability among populations of *Mus musculus* or between *Mus* species

In this experiment I compared male ultrasonic courtship vocalization characteristics among different species and populations. In addition to the presence or absence of vocalizers within populations, distinct USV syllables and vocal repertoire size of individual male mice from populations of M.m.m. (M.m.m.V, M.m.m.R, M.m.mS, M.m.m.K), M.m.d, and M.s. as well as two hybrid lines (Hybrid 1 & 2) were compared. I assessed spectrographic features of the USVs and compared the repertoires among individuals to detect variations. Socially experienced, mature males (N = 134; 275.3 ± 14.3 d of age) were used and I aimed to achieve at least 1 recording per male of 10 males per population and species. When possible, a sample size of 10 males per population was analyzed except for the two hybrid strains (Hybrid 1: N = 5; Hybrid 2: N = 7) and M.m.d. (N = 4). A subset of males were recorded multiple times and only vocalizers were used for further analysis (N = 66; 269.3 ± 12.3 d of age).

2.6.1.1. Data analyses and statistical tests

To compare different frequency of vocalizing / non- vocalizing male among populations and species chi- square tests with FDR control were conducted. A binominal test was applied with setting the expected proportion to 0.5 (50%) to figure out vocalizers / non - vocalizers frequency within populations. Within several populations male mice were recorded repeatedly. A binominal test was used to test for general consistency in vocalizing behavior among these populations and following chi- square tests for population dependence of consistency.

Despite prior noise reduction (by 90 dB below – 52 dB), recordings still contained a considerable amount of 'non - USV' signals that compromised the exactness of the automated parameter-measurement functions available within the SASLab Pro software format. Thus, extraneous noise was identified and removed manually from spectrograms. Then parameters were determined automatically, including minimum and maximum frequency, peak frequency, peak amplitude, entropy and bandwidth which were derived from start, center and end spectrum of the entire syllable. Peak frequency was defined as the frequency at the location of maximum amplitude. Peak amplitude was defined as the point with the highest intensity within the spectrum. Entropy is a measure of the bandwidth and uniformity of the spectrum of a sound (SAP manual); tonal (whistle-like sounds) usually have low entropy (< 0.3), while broad-band (noisy) sounds have higher entropies (> 0.4). Bandwidth is seen as the frequency difference between maximum and minimum frequency (Avisoft SASLab Pro; version 4.40). Mean call duration was measured as temporal parameter of syllables. Latency until first ultrasonic call occurred was measured as temporal feature of vocalizations to detect differences between USV responses among populations and between species. Due to high inter - individual variance and as data could not be transformed, a nonparametric Kruskall - Wallis - H test was conducted for latency. If significance occurred, further Mann - Whitney – U tests were used for comparisons between latencies of the different populations. In total 27 Mann- Whitney -U tests with FDR control were performed. The total numbers of uttered syllables by the different individuals in 30 minutes recording were counted by visually inspecting the respective spectrograms. Due to high

variability in USV rates among males of all populations, nonparametric Kruskall - Wallis - H tests were used. An effect of age on latency and / or USV emission rate was tested with Spearman rank correlation for nonparametric data.

Only the first 100 emitted syllables per each male were taken for further detailed analysis. On the basis of their distinct spectrographic shape and parameters, distinct ultrasonic syllables were classified into 7 particular subtypes (Panksepp, Jochman et al. 2007; Portfors 2007) (Figure 1 A –G).

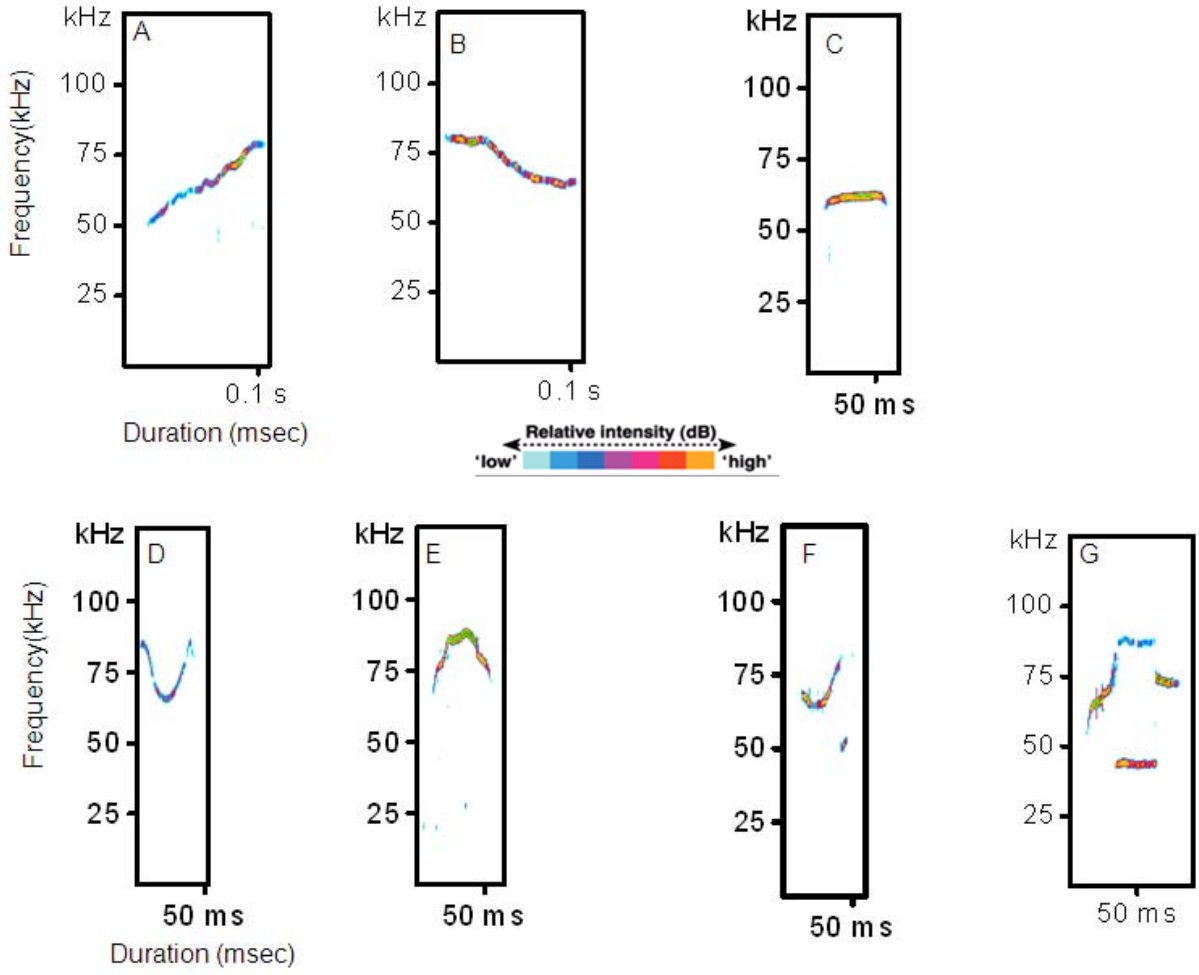

Figure 1: Spectrograms of ultrasonic syllables. Types are classified according to spectrographic parameters, e.g. start frequency, end frequency, centre frequency, frequency at peak energy and duration. (A) Frequency upsweep (B) Frequency downsweep (C) Constant modulated (D) U shaped (E) U shaped inverted (F) Complex 2 (G) Complex 3

The total number of distinct syllable types was used to estimate a males' repertoire size. The data were non – normal and could not be transformed so a nonparametric Kruskal – Wallis - H test was used to test repertoire differences of groups. Further Mann-Whitney - U tests (in sum 28) with FDR were used when an overall significance appeared to detect inter - specific variance of syllable type production.

Conveyance of information through vocalization may also depend on specific frequency and temporal properties of syllables (Geissler and Ehret 2001; Rendall, Owren et al. 1998). Inter - individual variation was explored using discriminant function analysis (DFA). This analysis detects group differences regarding to the mean of a variable and then uses this variable to predict group-membership. It computes discriminant functions which compare variation among individuals across several levels simultaneously (Tabachnick and Fidell 2001). Analysis was conducted for syllable type 'frequency upsweep' with 4 vocal variables set as classification factors. Wilk's lambda was computed to estimate discrimination among individuals and an F - test was used to determine its significance at the 0.05 level (Tabachnick and Fidell 2001). Multiway analysis of variance (MANOVA) was used to test for differences in the vocal patterns among populations. Populations were set as classification factor.

2.6.2. Experiment 2 - Females' attraction to male ultrasonic vocalizations: discrimination between different populations based on USV playbacks

A choice experiment was performed to test females' discrimination ability between populations and species and preference for males' courtship USVs. Prior experiments showed that female can discriminate between kin and non-kin USVs may influence recognition on familiar kin to other individuals (Musolf et al. submitted). For simplicity all females originated from *Mus musculus* population M.m.m.S. All females were tested during oestrus [determined by vagina smears collected 5 – 7 h before the trial; examined under a light microscope (100 x magnifications) and the cycle stage was determined according to the occurrence and proportion of leukocytes, nucleated and cornified epithelial cells (Flowerdew 1987; Snell 1941)]. Usually, females come into oestrus every four

days (Bronson 1979). To synchronize oestrus 12 g of male soiled bedding were introduced to the females' home cages (Cheetham, Thom et al. 2007).

As control two tests were conducted. To confirm that females are attracted by male USVs, 10 females (M.m.m.S; 209 ± 22.8 d of age at trial) were given the choice between male USV playbacks versus playbacks without USV. I used 310 sec of recording without USV or noise to examine females' fundamental response when presented to male USV pool (M.m.m.S) versus no USV. A USV pool was a sequence of alternating different male's calls which were not superimposed. Females (M.m.m.S; N = 10) spent more time at the fence in front of the USV playback speaker and more time in the zones on the sides with USV playback compared to the side with no USV (Wilcoxon signed rank test: Fence : 36.9 ± 5.5 vs. 24.0 ± 5.6, *Z*= 15.5, p = 0.131; Fence + Speaker zone: 96.8 ± 19.8 vs. 45.0 ± 10.8, *Z* = 19.5, p = 0.049; Fence + Speaker + Middle zone: 120.7 ± 15.7 vs. 62.0 ± 13.2, *Z* = 22.5, p = 0.020) (Fig.2). Further I tested 6 females (M.m.m.S; 184 ± 56.2 d of age at trial) for preference between playbacks of pooled USVs of 10 males (M.m.m.S) versus USV of a single male (M.m.m.S) to ensure that females do not differ between pooled USV playbacks and USV playback of a single male. USV playback of a single male consisted of five 61-sec repetitions of uttered syllable bouts recorded from 1 male (M.m.m.S). Females (M.m.m.S; N = 6) did not significantly differ in preference for playback of pooled USVs (M.m.m.S) versus single male USV (M.m.m.S) (Wilcoxon signed rank test: Fence: 31.1 ± 12.7 *vs.* 12.7 ± 4.6, test Z = - 0.674, p = 0.500; Fence + Speaker zone: 52.4 ± 22.8 *vs.* 77.5 ± 46.6 *Z* = - 0.105, p = 0.917; Fence + Speaker + Middle zone: 65.8 ± 26.9 *vs.* 83.7 ± 46.1 Z = - 0.105, p = 0.917) (Fig.2)**.**

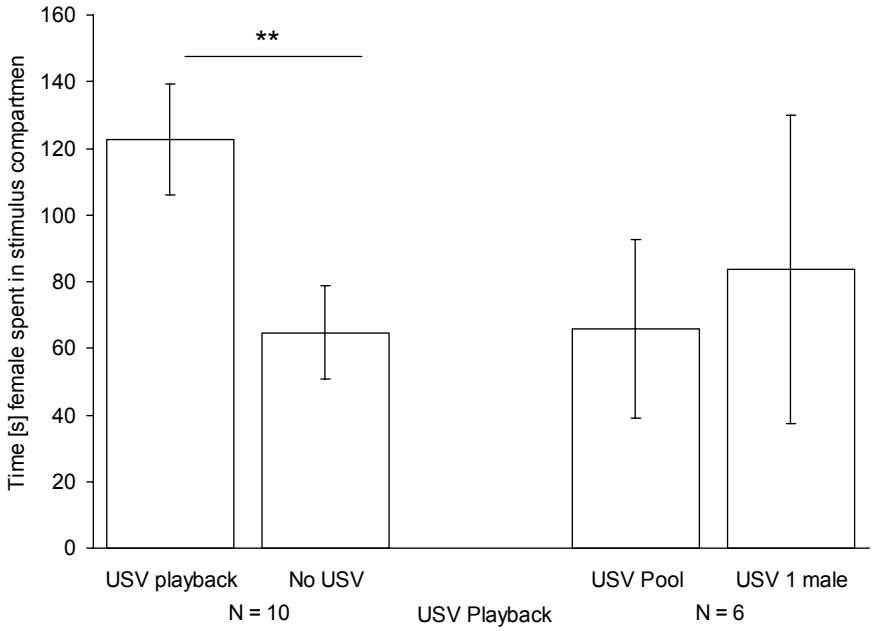

Figure 2: Time (mean ± SE) females spent in zones (Fence + Speaker zone + Middle zone) in proximity to pooled male USV playback versus no USV and pooled male USV playback versus USV of 1 male. Double asterisks represent significance at a level of $p \leq 0.01$.

As controls were positive regarding preference for male USVs, and females did not differ between pooled USVs (M.m.m.S) and USV of single male (M.m.m.S) further experiments were performed. To examine females' response to male USV's from different populations and species I used 20 female mice (M.m.m.S; 172.2 ± 11.7 day of age on the first trial). Females were tested for their preference for USV playback of males their own population (Playback of M.m.m.S males) compared to strange population, who they never were exposed to before (Playback of M.m.m.V, M.m.m.R, M.m.d., Hybrids and M.s. males). For testing, simultaneously male USV playbacks of respective population were composed. Playbacks lasted 310 sec and consisted of 10 different 30 sec segments of uttered syllable bouts per individual that were previously recorded in Experiment 1. USV playbacks of M.d. were generated of four 76.5 seconds segments of uttered syllable bouts due to the corresponding minor number of vocalizers (N = 4) in Experiment 1. Playbacks were standardized concerning the number of syllables to avoid preferences on the basis of performance-related traits (Gil and Gahr 2002). Inter - syllable durations were the same as those made by mice (< 1 sec), and duration

between each individual phrases was 1 sec. Each female was presented with 5 different playback combinations in separate trials, with the order of playback presentation balanced within subjects and switched in following trials (Table1).

Table 1: Male USV Playbacks combinations presented to females (M.m.m.S) to test for females' ability to discriminate

Comparison	USV Playback 1	USV Playback 2
Between M.m.m. Populations	M.m.m.S	M.m.m.V
	M.m.m.S	M.m.m.R
Between Subspecies (M.m.m. vs. M.m.d.)	M.m.m.S	M.m.d.
Between hybrids (M.m.m. x M.m.d.)	M.m.m S	Hybrids
Between species (M.m.m. vs. M.s.)	M.m.m.S	M.s.

Playbacks were presented using an external soundcard (Edirol UA-101, 24-Bit/192 kHz 10 in/10-out Hi-SPEED USB (USB 2.0) audio interface for multitrack computer recording) covering ultrasonic frequencies. For calibrating the ultrasonic loudspeakers, 15 previously prepared playbacks of 30 sec were played via the speakers. The sounds were recorded with the prior calibrated microphone (section 4). A comparison of the played sound and its appropriate generated playback was done by inspecting spectrograms visually.

Females were individually placed at one end ('Neutral zone') of a clean type III cage (42.5 x 27 x 20 cm), specially modified for assessing females attraction to playbacks through one of two speakers at the other end ('Speaker zone') (Fig. 2). Females could move into two equal compartments, separated by acoustic foam (Pur Skin, Sonartech, 10 mm), and at the other end with two speakers (each placed through holes, Ø = 6 cm and covered with fencing, mesh width 0.5 cm), for playing ultrasound recordings (Ultrasonic Dynamic Speaker

Magant, Frequency range: 1 - 55 kHz, Impedance: 4 ohm). The sides and tops of the speakers were insulated with acoustic foam (SH 003 - Absorptionsplatten / Pyramiden, 5 cm), which, like the partition, helped to ensure that playbacks were only heard in the corresponding arm. In the undivided section of the cage (Neutral zone), females could hear playbacks in both compartments. For analyzing females' relative attraction, the compartments were further divided into different proximity zones: 'Fence' (mice contacting the fence in front of the speakers); as well as 'Speaker zone' (area 0 – 9 cm apart from the speaker/fence); 'Middle zone' (area 9 – 18 cm apart); and 'Neutral zone' (Fig. 2).

After females were placed in the 'Neutral zone' from their home cages, habituation lasted as long as the animal explored both compartments and went back to the 'Neutral zone'. Then playback was initiated for 310 sec. To control for potential confounding side biases, the playbacks were counterbalanced between trials. The experiments were digitally videotaped (Sony Handycam DCR-SR30).

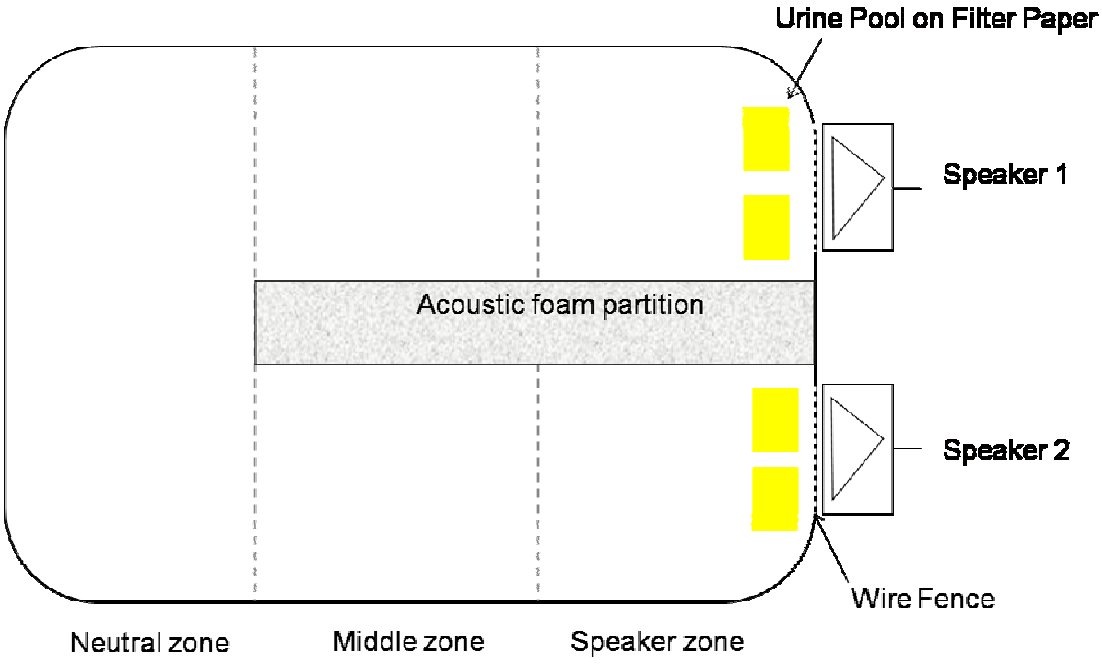

Figure 3: Playback preference box. Dashed lines indicate different proximity zones. If mice were located within the speaker zone but contacted the fence, it was valued as 'Fence'.

Since prior pilot tests showed that females had a short period of interest if playback served as the only stimulus (Hoffmann, 2008), males' urinary cues were used as enhancing stimuli. This enhancing stimulus was the same in each compartment and unbiased concerning kinship, familiarity and population membership. The cue consisted of two pools of unfamiliar non- kin urine of the two respective playback populations. Four mixtures, two of each population, were immediately pipetted on four 4x4 cm size filter paper (Whatman 3MM Chr, 0.34 mm thick) and placed in the test cage in front of the two speakers (Fig. 3).

2.6.2.1. Data analyses and statistical tests

Videotapes were analyzed in a 'blind fashion' regarding female identity and trial number using 'The Observer 7.0' (Noldus). Retention times in the designated areas of the apparatus (Neutral zone, Middle zone, Speaker zone and Fence) were measured and compared. As behavioral parameters, self-grooming and sniffing at the enhancing urinary stimulus were measured, providing further indicators for mating preferences (Ferkin and Leonard 2005). To ensure that females spent more time self - grooming in proximity of a playback stimuli, measured self - grooming time was conducted relative to entire time of a stimulus compartment and compared. All trials were checked for a general bias towards the right or left side of the y - maze box by using nonparametric Wilcoxon signed-rank tests. The initial preference (side of first entry), latencies until one arm was entered first and the number of visits in each arm were also registered. Initial preferences were checked for significances using a binomial test. The expected proportion was set to 0.5 (50%). For latency comparisons the time for a not chosen arm was set to 310 s (total time of a trial). When the distribution of means of retention times proximity zones were not normal, Wilcoxon signed-rank tests were used for statistical analysis for comparison. Time females spent sniffing at enhancing urine stimulus depending on respective USV playback of the different populations and species was determined with repeated measurement ANOVAs with speaker as the between subject factor and female as covariate. I looked also at the effect of individual variation in numbers of complex syllables within playbacks using Linear mixed Models for repeated measures entering individual number of complex syllables as fixed effect and trial number as random effect.

3. Results

3.1. Experiment 1 - USV features: variability among populations of *Mus musculus* or between *Mus* species

3.1.1. Frequency of vocalizing versus non-vocalizing mice

The frequency of males that vocalized varied significantly among *M. musculus* populations as well as between *Mus* species (N = 134, χ^2 = 29.534, df = 7, p ≤ 0.001) (Fig.4). Particularly the proportion of non - vocalizing males appeared population dependent and differed enormously among populations (13% - 79%) with M.m.d mice showing the highest proportion of non - vocalizers (79% non - vocalizing males; Binominal test: N = 19, p = 0.019). Whereas within M.m.m.K and M.m.m.S the ratio of vocalizing males was significantly higher (M.m.m.K: 87% of vocalizing males; Binominal test: N = 15 , p = 0.007; M.m.m.S : 88% of vocalizing males; Binominal test: N = 14 , p = 0.004).

Levene test for equality of variance indicated significant variation between M.m.m. populations (N = 40, $F_{3,80}$ = 16.218, p ≤ 0.001). Further comparison of variance indicated homogeneity of variance between M.m.m.K & M.m.m.S (N = 31; $F_{1,29}$ = 0.018 p = 0.894) and M.m.m.V & M.m.m.R (N = 53; $F_{1,51}$ =1.045, p = 0.312). Hence they were merged for inter-specific comparison in 'Outbred' (M.m.m.K & M.m.m.S) and 'Inbred' (M.m.m.V & M.m.m.R) based on microsatellite analyses (unpublished data, Musolf et al.). The hybrid mice (Hybrid 1 & Hybrid 2) were also checked for equality of variance and merged (Hybrids) (Levene test for equality of variance: N = 18, $F_{1,16}$ = 0.3114, p = 0.097).

An overall significant difference was found among all species (N = 134, χ^2 = 28.218, df = 4, p ≤ 0.001). In particular significance appeared in Inbred vs. Outbred (N = 84, χ^2 = 15.504, df = 1, p ≤ 0.001; significant after FDR control), Inbred vs. M.s. (N = 66, χ^2 = 4.694, df = 1, p = 0.03; significant after FDR control), Outbred vs. M.m.d. N = 50, χ^2 = 21.809, df = 1, p ≤ 0.001; significant after FDR control), Hybrids vs. M.m.d. (N = 37, χ^2 = 7.836, df = 1, p = 0.005; significant after FDR control), M.m.d. vs. M.s. (N = 32, χ^2 = 9.791, df = 1, p = 0.002; significant after FDR control) (Fig. 4). Within Inbred and Outbred

musculus pool significant variation in vocalizer / non - vocalizer frequency was found (N = 84, χ^2 = 15.922, df = 3, p = 0.001) (Fig.5): M.m.m.V vs. M.m.m.S (N = 44, χ^2 = 9.647, df = 1, p = 0.002; significant after FDR control); M.m.m.V vs. M.m.m.K (N = 43, χ^2 = 8.891, df= 1, p = 0.003; significant after FDR control); M.m.m.R vs. M.m.m.S (N = 41, χ^2 = 6.561,df = 1,p = 0.01; significant after FDR control); M.m.m.R vs. M.m.m.K (N = 40, χ^2 = 5.980, df = 1, p = 0.014; significant after FDR control).

Several males of different populations were recorded repeatedly (M.m.m.K: N = 4; M.m.m.V: N = 14; M.m.m.R: N = 10; Hybrid 1: N = 9; Hybrid 2: N = 9; M.m.d.: N = 10). The majority showed a significant consistency in their vocalization behavior (Binominal test: N = 56, p = 0.01) independent of population of origin of male mice (N = 56, χ^2 = 6.586, df = 4, p = 0.159). 60% of males of different populations did not vocalize despite repeated recording attempts (50% of M.m.m.K; 60% of M.m.m.V; 56% of M.m.m.R; 33% of Hybrids; 80% of M.m.d; Binominal test: N = 34, p = 0.024). Absence of callers was independent of the population (N = 34, χ^2 = 6.317, df = 4, p = 0.177). When vocalizing (40% of males of all populations and species), males did not change their behavior (50% of M.m.m.K ; 40% of M.m.m.V; 44% of M.m.m.R; 67% of Hybrids; 20% of M.m.d.; Binominal test: N = 23, p = 0.405) except males of the hybrid strains. Eigth of twelve males stopped vocalizing after 2 - 3 exposures (N = 23, χ^2 = 9.705, df = 4, p = 0.046).

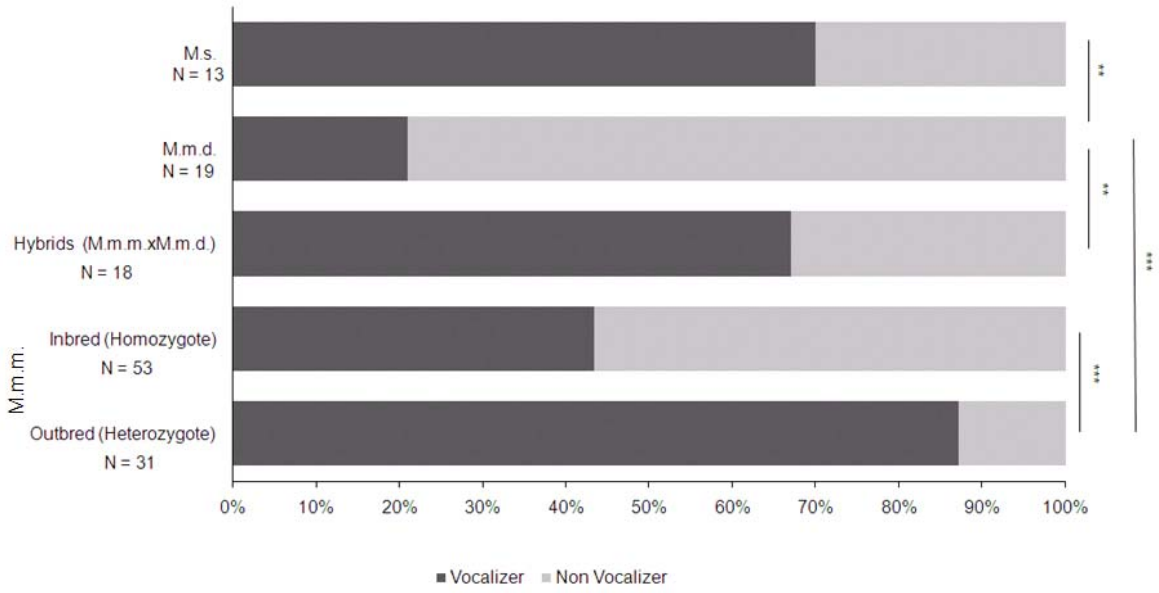

Figure 4: Frequency (%) of vocalizing / non-vocalizing male per species / group. Single asterisks represent significance at a level of $p < 0.05$, double asterisks represent significance at a level of $p \leq 0.01$ and triple asterisks represent significance at a level of $p \leq 0.001$.

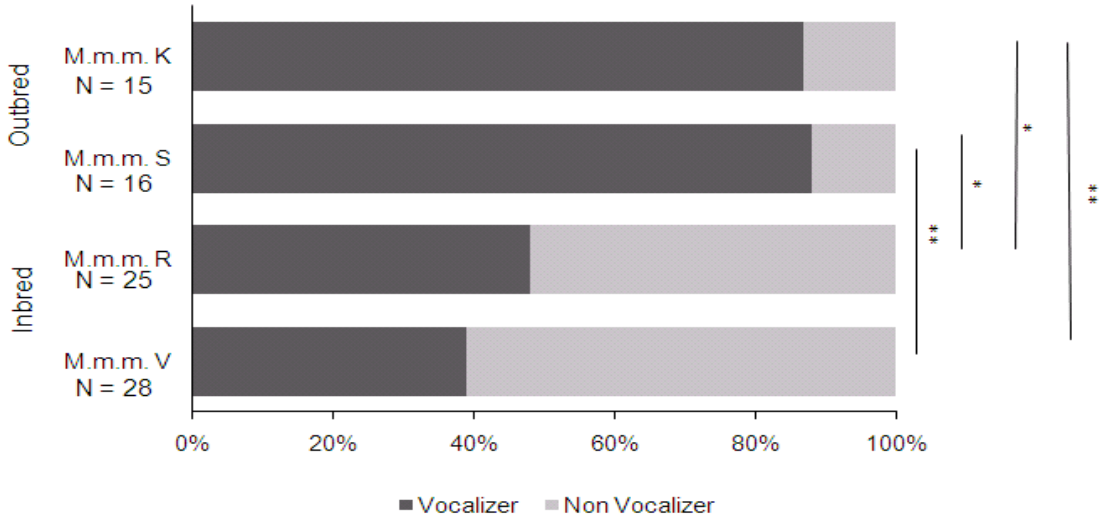

Figure 5: Frequency (%) of vocalizing / non-vocalizing male mice among M.m.m. populations (M.m.m.V, M.m.m.R, M.m.m.S, M.m.m.K). Single asterisks represent significance at a level of $p < 0.05$ and double asterisks represent significance at a level of $p \leq 0.01$.

3.1.2. Variation in USV behavior

I found variance in latency to vocalize among different populations ranging from 0.5 to 1437 sec. Levene test for equality of variances was conducted to check for intra - species variation. Mean latency did not differ among species but significant variance occurred between M.m.m. populations (range from 1.32 sec to 1437.54 sec; mean latency range: from 86.1 ± 113.9 SD to 245.2 ± 294.8 SD; Levene test of equality of variance: N = 30, $F_{3,36}$ = 9.720, p ≤ 0.001; Fig. 7) thus they were not merged for statistical analyses but for illustration of inter –specific comparison (Fig. 6). Whereas variability within hybrid strains was low (range from 0.5 sec to 981.2 sec; mean latency range: from 84.5 ± 77.3 SD to 265.1 ± 403.3 SD; N = 12, $F_{1,10}$ = 1.263, p = 0.287) hence they were combined for statistical analyses of inter- specific comparison. Across species mean latency varied between males from 48.3 ± 33.9 SD to 646.0 ± 544.7 SD (Fig. 6). Significant differences between latencies of Hybrids, M.m.d. and M.s. species was not found (Kruskall – Wallis test: N = 66, χ^2 = 4.909, df = 3, p = 0.179). Testing latencies with populations lead to a significant differences (Kruskall – Wallis test: N = 66, χ^2 = 15.94, df = 7, p = 0.026). Thus further nonparametric Mann-Whitney - U tests were used as post - hocs to determine differences between individual groups. Significant differences were found in the following comparisons (all Mann- Whitney U tests; all p - values significant after FDR control): M.s. vs. M.m.m.K : N = 20, U = 23.0, p = 0.041; M.s. vs. M.m.m.S : N = 20, U = 17.0, p = 0.013; M.s. vs. Hybrid 2: N = 17, U = 14.0, p = 0.04; M.m.m.K vs. M.m.m.R : N = 20, U = 17.0, p=0.013; M.m.m.S vs. M.m.m.R : N = 20, U = 11, p = 0.003; M.m.m.R vs. Hybrid 2: N = 17, U = 13, p = 0.032) (Fig. 6 & Fig.7).

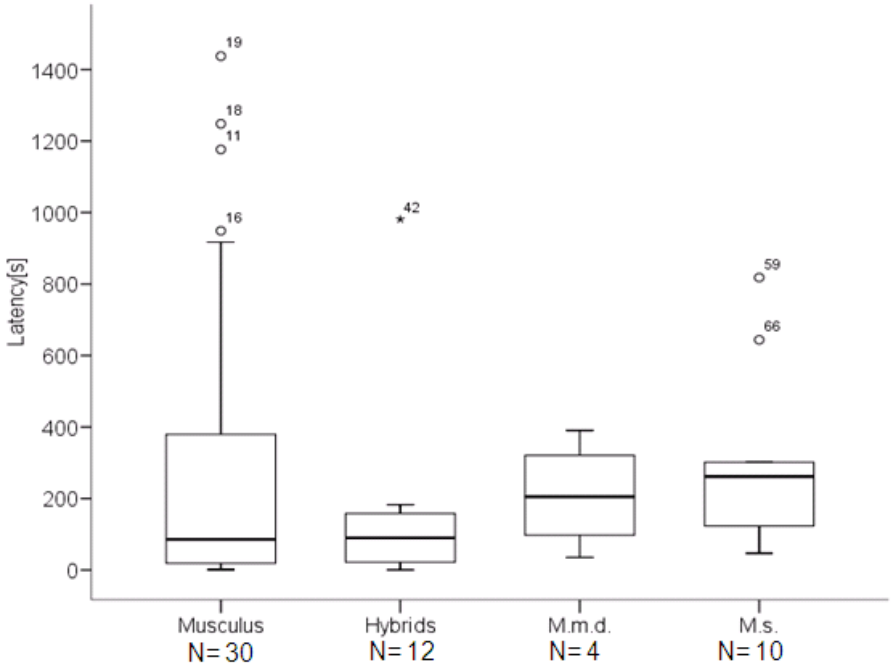

Figure 6: Latency (s) ± SD variability of different M.m.m. populations and *Mus* species (N = 66). Musculus include all four populations (M.m.m.V, M.m.m.R, M.m.m.S, M.m.m.K) and Hybrids include two lines (Hybrid 1, Hybrid 2).

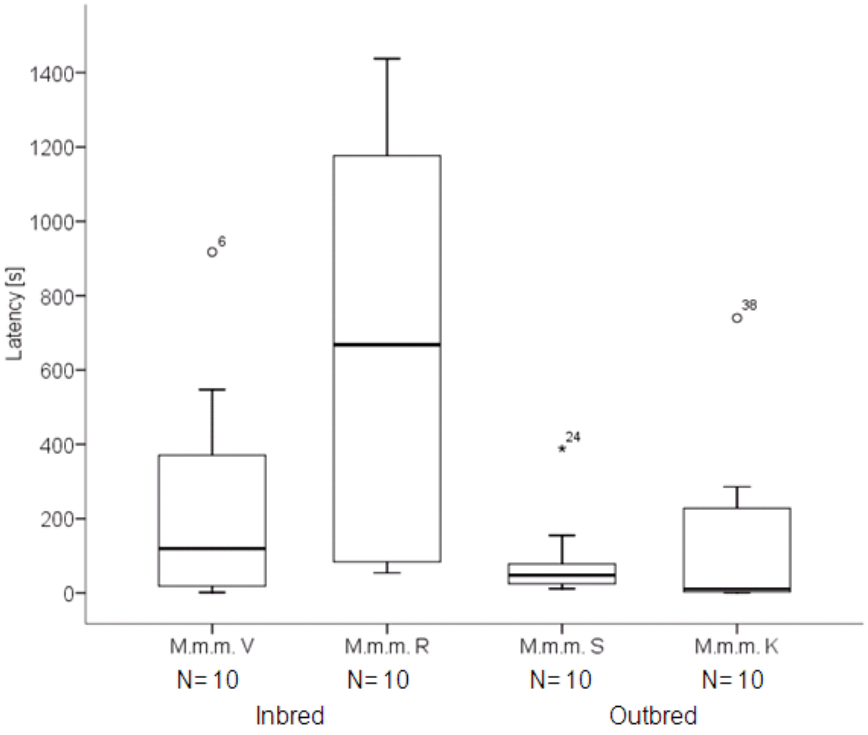

Figure 7: Latency (s) ± SD variability of M.m.m. populations.

Total number of uttered syllables ranged from 31 up to 2661 syllables in 30 min recording with a mean syllable count across species from 256 ± 347.4

SD to 1057 ± 1023.8 SD. For inter – specific comparison of syllable rate, M.m.m. populations as well as hybrid strains were merged as intra – specific variance was low (M.m.m. populations: mean syllable count from 407 ± 363.9 SD to 1056 ± 886.1 SD, Levene test of equality of variance : N = 40, $F_{3,36}$ = 2.846, p = 0.051; Hybrids: mean syllable count from 570 ± 442.9 SD to 586 ± 477.1 SD; Levene test of equality of variance: N = 12; $F_{1,10}$ = 0.504, p = 0.494). However, despite this high variability, USV emission rate among species were none significant (Kruskal – Wallis test: N = 66, χ^2 = 7.423, df = 4, p = 0.115) (Fig. 8). Call rate during 30 min recording also differed among individual of M.m.m. populations varied (from 49 to 2661 uttered syllables) but this is not significant (Kruskal – Wallis test: N = 66, χ^2 = 12.286, df = 7, p = 0.92) (Fig. 9).

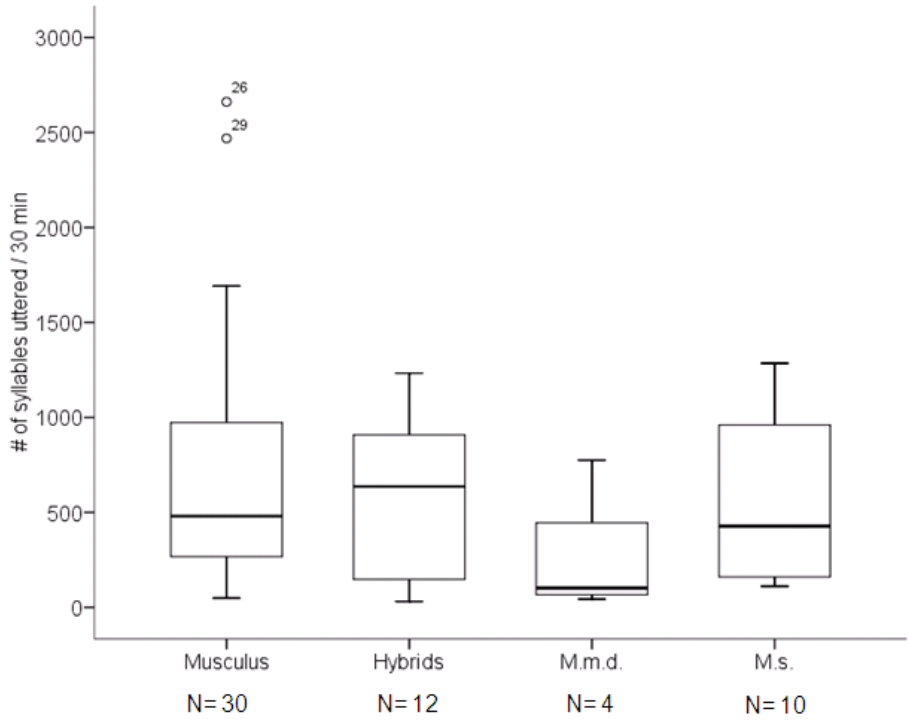

Figure 8: Number of syllables uttered by male mice mice of different M.m.m. populations and *Mus* species (N = 66) during 30 min recording (± SD). Musculus include all four populations (M.m.m.V, M.m.m.R, M.m.m.S, M.m.m.K) and Hybrids include two cross lines (Hybrid 1, Hybrid 2).

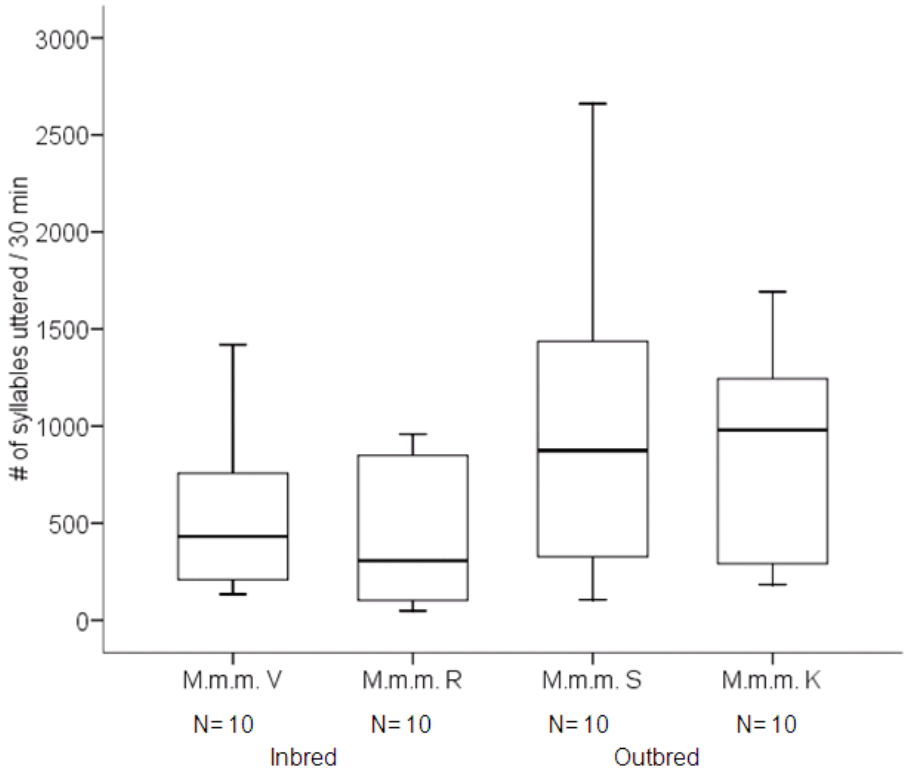

Figure 9: Number of syllables uttered per 30 min recording (± SD) of M.m.m. populations.

Age did not have an effect neither on latency nor on USVs emission rate among all populations and species (Latency: Spearman rank correlation: N = 66, r_s = 0.024, p = 0.849; Number of uttered syllables: Spearman rank correlation: N = 66, r_s = - 0.025, p = 0.843) (data not shown).

3.1.3. Repertoire variation of different *Mus* populations

I categorized the first 100 emitted USVs into distinct syllable types based on their spectrographic shape to estimate differences in repertoire size among individuals. Male mice produced different simple and complex syllables.

Classification patterns of USVs were as follows: ‘Frequency upsweep’ showed a continuous increase in peak frequency that was about 11.9 kHz higher than the peak frequency at the beginning. ‘Frequency downsweep’ exhibit a continuous decrease in peak frequency that was 8.67 kHz lower than the peak frequency at the beginning. In 'Constant modulated' calls slight increase of peak frequency was never higher than 0.94 kHz. ‘U shaped’ possessed a decrease of peak frequency that was 6.55 kHz followed by an

increase that was 7.75 kHz. In syllable type ‘U shaped inverted’ the peak frequency increased about 8.15 kHz from the beginning followed by a decrease that was 7.42 kHz. Additionally two more syllable types occurred during vocalizations which were composed of two or three elements (Fig. 1). Depending on the number of elements I classified these syllables into 'Complex 2' and 'Complex 3'.

I detected population - dependent effects on the proportion of different syllable types uttered (Fig. 10; Appendix Tab. 3). ‘Frequency upsweep’ (56.6 ± 21.4 SD) was the most common syllable type among all population. The utterance of other syllable types varied among populations. Complex syllable type with 3 elements ('Complex 3') did not occur in USVs of all individuals (e.g. M.m.m. R, M.m.m.S). Syllable type ‘u shaped inverted’ was absent in repertoire of Hybrid 2. For nearly all syllable types the differences in the population means were significant (Kruskall – Wallis test: N = 66, df = 7: 'Frequency upsweep': $\chi^2 = 34.722$, $p \leq 0.001$; 'Frequency downsweep' : $\chi^2 = 35.606$, $p \leq 0.001$; ‘Constant modulated’: $\chi^2 = 20.447$,$p = 0.005$; ‘U shaped’: $\chi^2 = 40.931$, $p \leq 0.001$; ‘U shaped inverted’: $\chi^2 = 41.919$, $p \leq 0.001$; ‘Complex 3’: $\chi^2 = 20.414$, $p = 0.005$; for details Appendix Tab. 4 - 9). In sum 28 Mann - Whitney - U tests per syllable type were computed with FDR control. Syllable type ‘Complex 2’ did not vary significantly ($\chi^2 = 10.874$, $p = 0.144$).

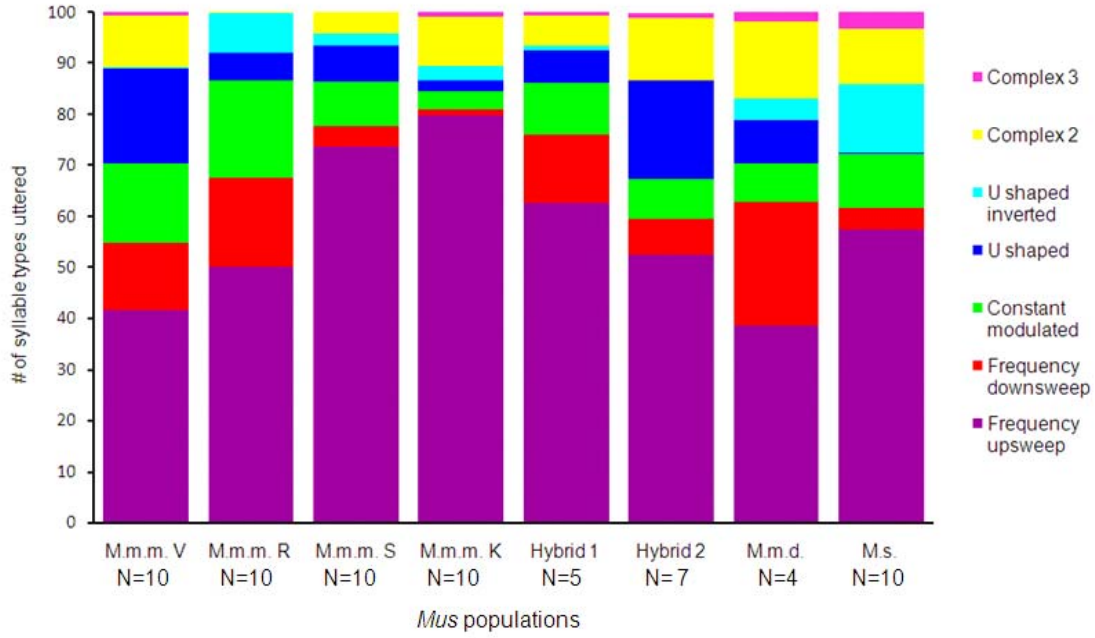

Figure 10: Repertoire composition. Distribution of syllable types uttered in first 100 syllables among different *Mus* populations. Syllable type 'Frequency upsweep' is the most common produced type (56.6 ± 21.35 SD). Syllable types 'U shaped inverted' and Complex 3 were not emitted by all strains (e.g. not M.m.m.R, M.m.m.S).

3.1.4. Population discrimination based on spectrographic properties of syllable type

Determination of repertoire composition among populations and species demonstrated significant variability in types of syllables emitted. Due to this high variance in syllable type utterance (Fig. 10), analysis based on spectrographic parameters could only be conducted with the most common type 'Frequency upsweep'. Univariate analysis of variance of 19 measured spectrographic data (duration, peak frequency , peak amplitude, minimum frequency , maximum frequency , bandwidth ,entropy at start, center and end point of a syllable) indicated 4 variables as highly significant thus showing greatest variance among populations: peak frequency ($F = 10.5$, $p \leq 0.001$) and maximum frequency at start ($F = 10.5$, $p \leq 0.001$), peak amplitude ($F = 7.6717$, $p \leq 0.001$) and entropy ($F = 2.93$, $p \leq 0.001$) at end (for details see Fig. 18 - 21; Tab. 11 - 14 in Appendix). Discriminant function analysis computed four functions to separate each *musculus* population (WILKS' $\Lambda = 0.07974$, $F = 8.71943$, $p < 0.00001$) with peak frequency (start) as the most influencing factor for categorization. First two axes covered 83% of the whole variance with the first

function providing the most overall discrimination between populations. The first discriminant factor accounted for 58.7% of the variation, the second for 24.2 %. Figure 10 illustrates the group positions plotted using the first two discriminant functions. The first function is spanned with the weights in the peak and maximum frequency (start) and equal weights of peak amplitude and entropy (end) (Tab. 2). Whereas the second function differs in the strength of peak and maximum frequency (start) and peak amplitude (end) but almost neglects the entropy (Tab. 2). This allowed discriminating the population of M.m.m.V and M.s. with the first function. The second discriminant function showed a trend to separate Hybrids versus M.m.m.S (Fig. 11). M.m.m.V varied enormously in all 4 vocal patterns and could obviously not be clearly discriminated from other groups (Fig. 11). Mainly males of M.s. are separated from other groups mainly because of higher peak and maximum frequency values (for details see Appendix Tab. 14).

Table 2: Factor loading of each variable derived from discriminant function analysis.

VARIABLE	Factor 1	Factor 2	Factor 3	Factor 4
Peak frequency (start)	-0.70010	0.74722	0.72660	-0.70492
Maximum frequency (start)	0.71329	- 066419	-0.67740	0.70890
Peak Amplitude (end)	-0.02142	0.02239	-0.08373	0.01600
Entropy (end)	-0.02472	0.00260	0.07853	0.01725
Proportion of variance	58.7%	24.2%	14.2%	2.9%
χ^2	75.2	42.5	28.3	7.1
d.f.	9	7	5	3
p value	$p<0.001$	$p<0.001$	$p<0.005$	$p<0.1$

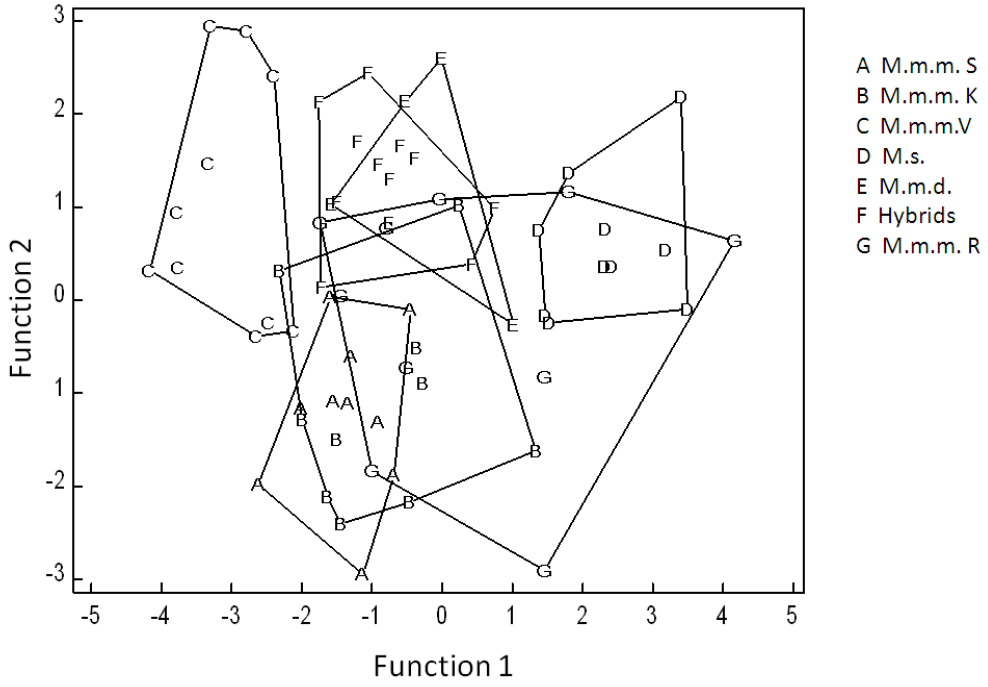

Figure 11: Plot of scores of the first two discriminant functions using 4 of 19 spectrographic variables for discrimination (peak frequency, maximum frequency at start; peak amplitude and entropy at end).

3.2. Experiment 2 - Females' attraction to male ultrasonic vocalizations: discrimination between different populations based on USV playbacks

To asses females' discrimination ability and USV preferences, females (M.m.m.S; N = 20) were presented with simultaneous playbacks of pooled USVs recorded from 10 unfamiliar males of their own population (M.m.m.S) versus different populations or species. In one trial one female did not leave the neutral zone, therefore the sample size was reduced to 19 in the statistical analyses of this test series. I examined potential differences in females' behavior between USV paybacks from own versus strange M.m.m. population or *Mus* species (e.g. Initial preference, latency to enter one stimulus compartment, number of visits -data not shown) and did not detect any statistically significant preference.

Females showed no preferences among male USV playbacks from different M.m.m. populations (all statistical tests using Wilcoxon signed rank): Test series M.m.m.S vs. M.m.m.V: Mean (s) ± SE: Fence: 26.2 ± 3.3 *vs.* 29.3± 5.6, Z = -0.443, p = 0.658 (Fig. 14); Fence + Speaker zone: 74.9 ± 17.7 *vs.* 66.7

± 12.8, Z = - 0.448, p = 0.654 (Fig. 13); Fence + Speaker + Middle zone: 91.2 ± 17.3 vs. 84.7 ± 14.9, Z = - 0.149, p = 0.881 (Fig. 12); Self - grooming: 0.45 ± 0.09 vs. 0.57 ± 0.09, Z = - 0.384, p = 0.701 (Fig. 15). For test between M.m.m.S vs. M.m.m.R (N = 19): Mean (s) ± SE: Fence: 23.5 ± 2.7 vs. 39.4 ± 9.2, Z = - 0.201, p = 0.841 (Fig. 14); Fence + Speaker zone: 62.7 ± 14.7 vs. 64.4 ± 12.2, Z= -0.201, p = 0.841 (Fig. 13); Fence + Speaker + Middle zone: 74.2 ± 14.5 vs. 74.9 ± 12.7, Z = - 0.201, p = 0.841 (Fig. 12); Self - grooming: 0.691 ± 0.08 vs. 0.69 ± 0.07, Z = - 0.652, p = 0.515 (Fig.15).

Females' preference for M.m.d. and Hybrids male USV playback was not significant but showed a slight trend: Test series M.m.d. : Mean (s) ± SE: Fence: 33.2± 11.0 vs. 21.3± 3.3, Z= - 0.201, p= 0.841 (Fig. 14); Fence + Speaker zone: 66.6 ± 16.0 vs. 48.5 ± 10.3, Z= - 0.993, p = 0.351 (Fig. 13); Fence + Speaker + Middle zone: 81.4 ± 16.2 vs. 59.54 ± 11.4, Z = - 1.083, p = 0.279 (Fig. 12); Self - grooming: 0.35 ± 0.1 vs. 0.5 ± 0.1, Z = - 0.157, p = 0.875 (Fig. 15). Test series Hybrids: Mean (s) ± SE: Fence: 29.9 ± 5.1 vs. 30.6 ± 4.3, Wilcoxon signed rank test: Z= - 0.579, p = 0.550 (Fig. 14); Fence + Speaker zone: 70.2 ± 15.1 vs. 53.5 ± 7.5, Z= - 0.299, p = 0.765 (Fig. 13); Fence + Speaker + Middle zone: 83.9 ± 15.2 vs. 63.2 ± 7.9, Z = - 0.261, p= 0.794 (Fig. 12); Self-grooming: 0.08 ± 0.01 vs. 0.09 ± 0.01, Z = - 1.260, p = 0.208 (Fig. 15).

When given the choice between USV playback of a distinct species (M.s.) and their own population (M.m.m.S) females (N = 20) stayed significant longer in all three zones together of the stimulus compartment with male USV playback of own population (M.m.m.S) than strange species (M.s.) (Mean (s) ± SE: Fence + Speaker + Middle zone: 83.2 ± 12.1 vs. 48.6 ± 8.1, Wilcoxon signed rank test: Z = - 2.277, p = 0.023; Fig. 12). Females spent significantly more time at fence in front the speaker which played USVs of own population (Mean (s) ± SE: Fence: 24.8 ± 5.7 vs. 34.1 ± 4.0, Wilcoxon signed rank test: Z = - 2.415, p = 0.016, Fig. 14) and remained also significantly longer in speaker zone of USV playback of their own population (M.m.m.S) (Mean (s) ± SE: Speaker zone: 33.4 ± 9.7 vs. 13.9 ± 2.1, Wilcoxon signed rank test: Z = - 2.203, p = 0.028). Comparing retention time of speaker & fence zone between the different stimuli compartments females stayed significantly longer in proximity to male USV playback of own population (Mean (s) ± SE : Fence +Speaker zone :

64.6 ± 11.1 vs. 38.7 ± 6.9, Wilcoxon signed rank test: Z = - 1.979, p = 0.048, Fig. 13). Additionally females self - groomed longer in proximity to the speaker which played own populations USV (M.m.m.S) (Mean (s) ± SE: 0.14 ± 0.06 vs. 0.01 ± 0.01, Wilcoxon signed rank test: Z = - 2.231, p = 0.021; Fig. 15).

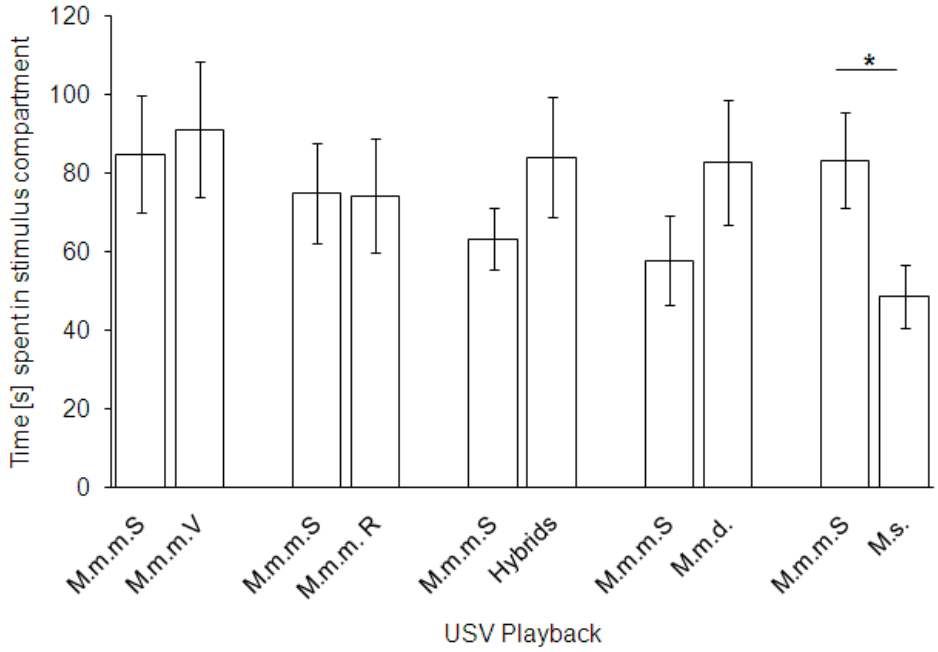

Figure 12: Time (mean ± SE) females (N = 20 except trial 2: M.m.m.S vs. M.m.m.R N = 19) spent in zones (Fence + Speaker zone + Middle zone) in proximity to different male USV playback. Single asterisks represent significance at a level of $p < 0.05$.

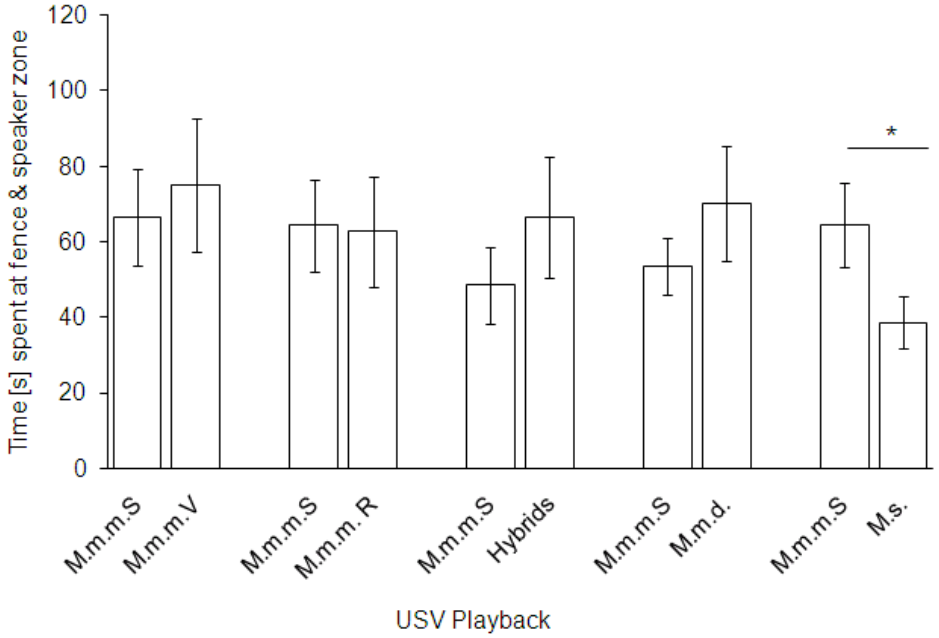

Figure 13: Time (mean ± SE) females (N = 20 except trial 2: M.m.m.S vs. M.m.m.R N = 19) spent in speaker zone and at fence in proximity to different male USV playback. Single asterisks represent significance at a level of $p < 0.05$.

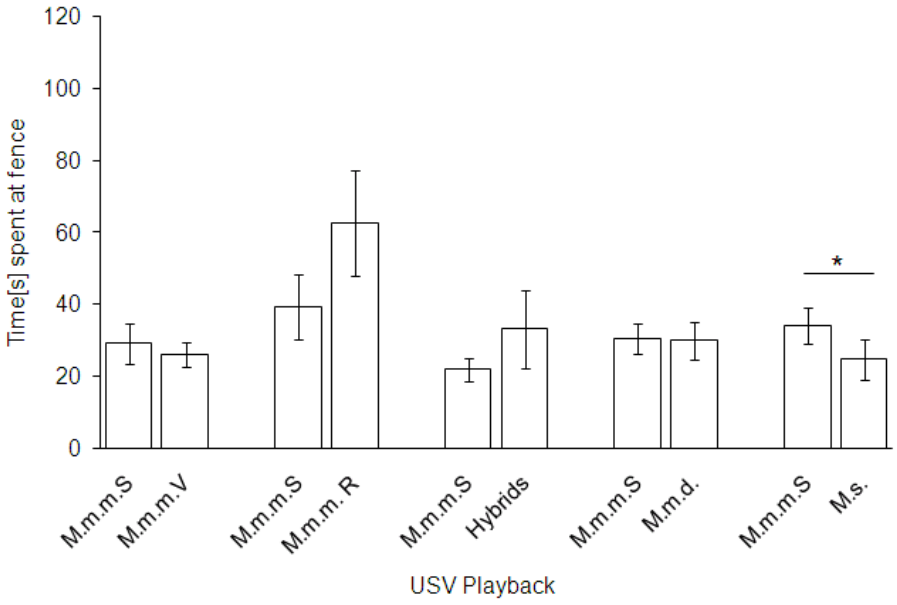

Figure 14: Time (mean± SE) females (N = 20 except trial 2: M.m.m.S vs. M.m.m.R N = 19) spent at the fence directly in front of the speaker in proximity to different male USV playback. Single asterisks represent significance at a level of $p < 0.05$.

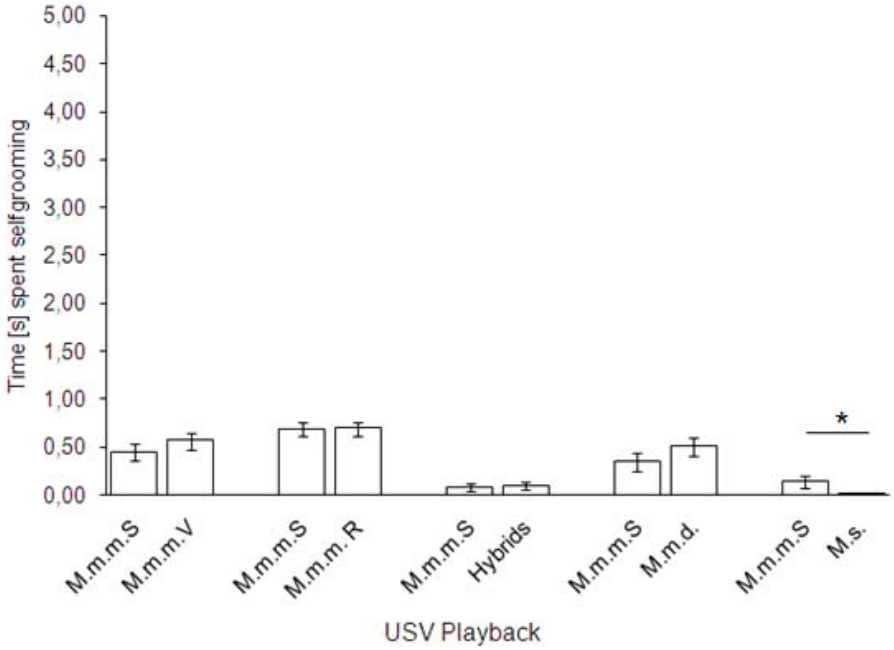

Figure 15: Time (mean ± SE) females (N = 20 except trial 2: M.m.m.S vs. M.m.m.R N = 19) spent self grooming in speaker zone in proximity to different male USV playback. Single asterisks represent significance at a level of $p < 0.05$.

Within trials with USV playback of their own population versus M.m.m. V females sniffed significantly longer at the urinary cue of their own population within both compartments (Mean (s) ± SE: 3.9 ± 5.6 vs. 2.1 ± 0.8; Wilcoxon signed rank test: N = 20, Z= - 2.012, p = 0.04) but sniffing behavior was independent of respective USV sound (Repeated measures ANOVA: F = 0.089, p = 0.768). Within all other playback preference test series female did not differ between enhancing urinary stimuli (for details see Appendix Tab. 15 -19).

The individually different numbers of complex syllables within the playbacks did not have an effect (M.m.m. S vs. M.m.m V: Linear mixed model: F = 0.075, p = 0.785; M.m.m.S vs. M.m.m.R: Linear mixed model: F = 0.487, p = 0.494; M.m.m.S vs. M.m.d.*:* Linear mixed model: F = 1,033, p = 0.322 ; M.m.m.S vs. Hybrids*:* Linear mixed model: F = 1.591, p = 0.222; M.m.m.S vs. M.s. *:* Linear mixed model: F = 3.695, p = 0.070) (data not shown).

4. Discussion

In this study, I investigated whether the courtship USVs of wild male mice is a species - specific vocalization by assessing distinct vocal features between *Mus* populations. Results demonstrated high variability in performance and USV features among different wild house mice populations and species. I surveyed features of syllables emitted by males to estimate spectrographic parameters and repertoire of males. I also tested the ability of female mice (M.m.m.S) to discriminate between male USVs of their own species (M.m.m.) and a subspecies (M.m.d.), a species (M.s.) and hybrid crosses (M.m.m. x M.m.d.) based on response behavior to male courtship USV playbacks by presenting simultaneous USV playbacks of a pool of the prior recorded males of several *Mus* populations. I measured retention times females stayed in proximity of respective USV playbacks. My experiments support the idea that male USVs are individually distinctive and used for species recognition. Additionally results showed that male courtship USVs attract females and might influence their mate choice behavior (Hoffmann 2008; Pomerantz, Nunez et al. 1983).

One of the most interesting results was the high variation in presence of vocalizing / non - vocalizing males between *Mus* species and among M.m.m. populations (Fig. 4). Males of different *Mus* populations were not vocalizing despite repeated recording attempts (50% of M.m.m.K; 60% of M.m.m.V; 56% of M.m.m.R; 33% of Hybrids; 80% of M.m.d). The ratio of non-vocalizing males differed significantly between *Mus* species (13% up to 79%) and was highest in M.m.d. (79% non - vocalizers) as well as between M.m.m. populations (12% up to 61%). High individual variation in USV emission has also been reported within inbred strains of laboratory mice but reported results did not provide rate of non - vocalizing mice (Nyby, Dizinno et al. 1976; Whitney, Stockton et al. 1979). Nyby (Nyby, Dizinno et al. 1976) reported a relatively low tendency of C57BL/6J male mice compared to another laboratory strain (DBA/2J). M.m.m. populations could be separated into ‘Outbred’ (heterozygote) and ‘Inbred’ (homozygote) based on naturally existing genetic variability. The proportion of non - callers of Inbred varied significantly from Outbred (Fig. 4). The greater proportion within Inbred might be due to lower genetic diversity. In this study

determination of heterozygote and homozygote mice is based on 6 microsatellite loci (Musolf et al., unpublished data) not currently tested another 9 loci to confirm genetic differences. Studies recently found females' preference for heterozygote males (Ilmonen, Stundner et al. 2009; Thom, Stockley et al. 2008). Ultrasonic vocalizations might be an adaptive trait shaped by sexual selection (Nyby 2009) with heterozygote genotype which could yield more dominant alleles for USVs than homozygote genotype (Brunelli, Vinocour et al. 1997). When vocalizing, males did not stop to emit USVs except Hybrids. Some males stopped calling after 2 - 3 recording trials (8 out 12 males). Previous studies assumed that mice, once they response to female urine with USVs, they never stop calling even when tested repeatedly (Nyby, Bigelow et al. 1983). If USVs serve as a courtship signal it is possible that males habituate to urine after several trials because no female appeared despite their efforts to attract one through USVs (Nyby, Dizinno et al. 1977; Pomerantz, Nunez et al. 1983). My results indicate an effect of genetic background on males' vocalization behavior. The FoxP2 gene is required as an important genetic locus subserving vocal communication in birds, human and mice (Groszer, Keays et al. 2008; Haesler, Wada et al. 2004; Teramitsu, Kudo et al. 2004). Shu et al. (Shu, Cho et al. 2005) showed that a disruption of the murine Foxp2 gene affects USV emission in pups. Recent work (Wang, Liang et al. 2008) showed effects of knockouts on dopamine neurons implicating acetylcholine signalling in the neural regulation of calling. M2 - and M5 muscarinic receptor knockout male mice significantly reduced calling whereas M4 - and Dopamine - D2 receptor knockouts showed no significant effect. However it is not clear whether these neurons or Foxp2 vary in wild house mice populations and how neural pathways influence vocalizing behavior of adult males (Nyby 2009; Roberts 1975). Unfortunately only a small number of males per populations and not all populations were repeatedly recorded as the main aim of this study was to get one USV recording per each male. Thus successful recorded males were not challenged further. Further recordings for determination of intra – individual variation and consistence in USV behavior should be conducted.

I found significant differences in males' latency to call and USV output rate when comparing species and populations. Latency varied highly among

species ranging from 0.5 to 1437.5 sec and differed significantly among M.m.m. populations (range from 1.32 to 1437.5 sec). In line with their higher proportion of non - vocalizers (Fig. 4 & Fig. 5), males of M.m.m.V (61% non - vocalizers) and M.m.m.R (52% non - vocalizers) as well as M.m.d. (79% non – vocalizers) emitted fewer USVs syllables with longer latencies (Fig.6 - 9). Same effect occurred within M.m.m.S and M.m.m.K. Besides a greater number of vocalizing males (M.m.m.S: 88% of vocalizing males; M.m.m.K.: 87% of vocalizing males) (Fig. 4) both M.m.m. populations had also shorter latency to call (Fig. 7) and a higher number of uttered syllables in 30 min recording (Fig. 9). Latency and call rate are influenced by genetics in rats (Sales 1979), laboratory adult mice and pups (Nyby and Whitney 1978; Roubertoux, Martin et al. 1996; Thornton, Hahn et al. 2005; Panksepp, Jochman et al. 2007) with dominant alleles coding for phenotypes with high vocalization rates and low latency (Brunelli, Vinocour et al. 1997). Differences in genetic diversity (heterozygote vs. homozygote) of M.m.m. populations might contribute to differences in USV performance, with higher USV emission rate and shorter latency to call of heterozygote mice. F1 hybrid mice appeared as more eager vocalizers than both of their parental populations. As shown in Figure 6 and Figure 8 both hybrid strains emitted more syllables with shorter latencies in 30 min recordings though not significant. Results conform to studies with F1 hybrids of laboratory house mice strains (Maggio and Whitney 1986; Hahn, Hewitt et al. 1987) and support their hypothesis of directional dominant inheritance of USV (Heterosis) (Maggio and Whitney 1986).

I found no relationship between latency / call rate and age. Though age has been reported to influence USV calling rate in birds (Gil and Gahr 2002; Radesäter, Jakobsson et al. 1987) and laboratory mice (D'Amato 1991; Warburton, Sales et al. 1989), based on changes in androgen level (Nyby, Wysocki et al. 1977). Male ultrasonic vocalizations (e.g. territorial urine marking) are considered to be influenced by social and sexual experience, like other reproductive behaviors (Lumley, Sipos et al. 1999; Nunez, Nyby et al. 1978; Ralls 1971). Several studies (Dizinno and Whitney 1977; Kerchner 2004) reported that castration and social submissive of laboratory male mice causes a decrease of vocalizations and scent marking whereas replacement or

implanting of testosterone maintain vocalizations and territorial marking (Nyby, Dizinno et al. 1976; Sipos and Nyby 1998). Social experience, another influencing factor, has already been shown to have no impact on wild mice calling rate in response to fresh female urine (Musolf et al. submitted).

The spectrographic analysis of recordings gives quantitative evidence for utterance of distinct syllable types which can be then discriminated among populations and species. In sum I identified seven different syllable types (Fig. 1). Accordingly repertoire estimation within and among populations was possible (Fig. 10). Syllable repertoire varied significantly among M.m.m. populations as well as between *Mus* species. Emission rate of all syllable types, except 'Complex 2', differed significantly between populations. Only one syllable type was commonly emitted by all individuals ('Frequency upsweep'; Fig. 10). Previous studies in laboratory mice mostly defined only two syllable types based on frequency values: low (~ 40 kHz) - and high frequency (~ 70 kHz) call (Gourbal, Barthelemy et al. 2004; White, Prasad et al. 1998). Portfors (Portfors 2007) determined different syllable types within ultrasonic vocalizations of laboratory male mice based on their spectrographic shape. Holy and Guo (Holy and Guo 2005) defined different syllable types based on pitch jumps within these syllables, which were arranged in a nonrandom temporal sequence. Detailed spectrographic analysis in wild mice USVs indicate more variability compared to laboratory mice UVSs (Kalcounis-Rueppell, Metheny et al. 2006; Miller and Engstrom 2007). Hence studies in wild house mice USVs might be more important for comparison of vocal communication in other animals as spectrographic analysis indicate higher variability of wild mice USVs.

Observed variation in repertoires may be due to genetic drift or perhaps if somehow reflects the ecological circumstances in which animals live (Owings and Morton 1998). Repertoire correlated with geographic variation as been demonstrated in courtship and territorial songs of songbirds (Nowicki and Searcy 2004), bats (Davidson and Wilkinson 2004) and among wild small rodents (e.g. *Baiomyinus* : Miller and Engstrom 2007). Geographical variation in song repertoire is attributed to genetic differences in populations as well as to learning ability of individuals (Baker 1982; Krebs and Davies 1993; Catchpole and Slater 1995). The song learning hypothesis suggests that individuals learn

species - specific vocal characteristics from conspecifics to convey group identity what can lead to local dialects among groups (Alcock 1989; Boughman and Moss 2003; Krebs and Davies 1993). Social groups, like mice, often consist of relatives, so vocalization similarities might be based on genetic similarity or are learned within a group, or both (Boughman and Moss 2003). Mice used in this study were reared in same room except M.s. (for details see section 2.1. Subject and housing). This could help to explain species differences I found. In case of M.m.m. and M.m.d., mice are separated in nature, but results show similarities and dissimilarities in USVs performances and features, thus rearing conditions might not be an influencing factor. However, we need studies that keep M.m.d. separated from M.m.m. as adults as well as in rearing to ascertain existence of species – specific vocalizations. If differences in USV's features are genetically affected it is unclear which factors maintain it and why populations differ in their vocalizing / non – vocalizing frequency. Adult ultrasound emission of mice emerges in males when mature (Nyby, Dizinno et al. 1976; Whitney, Coble et al. 1973) but sons might have learned calls of their fathers during early life like it is suggested in other species (e.g. birds: Zann 1990). Repertoire estimation reveals that the two hybrid strains share both repertoires of the parental population (M.m.m.V of Hybrid 2 and M.m.m.S of Hybrid 1) as well as of their maternal population (M.m.d.) (Fig. 10). Considering calls as sex - specific cues (sons copy their fathers, daughters copy their mothers) results assume inheritance of repertoire more than song learning (Boughman and Moss 2003). However, if song learning hypothesis applies to mice USVs has not been reported yet due to a lack of knowledge in functional significance of USVs and missing genetically studies in USV behavior. Cross - fostering or isolation (sons from their father) experiments are needed to understand learning acquisition in mice.

Discirminant function analysis of spectrographic patterns of USVs showed that syllables are individually distinctive (Tab. 2 & Fig. 11). This multivariate analysis, using frequency and temporal patterns of songs, has been used in studies in a wide range of species across many taxa to estimate individually stereotyped vocalizations, a prerequisite for individual vocal recognition (Gwilliam, Charrier et al. 2008; Nowicki and Searcy 2004; Sousa-

Lima, Paglia et al. 2002). To my knowledge, DFA has not been conducted with USVs of wild house mice populations before. Frequency and amplitude related parameters (peak and maximum frequency at start, peak amplitude and entropy at end) accounted for 83% of the variation of the common syllable type 'Frequency upsweep', with peak frequency as the most discriminating factor (Tab. 2). Individuals of M.s. were significantly separated from other species / populations showing higher values in frequency modulations and lower amplitude values (mean peak frequency: 69.8 kHz; mean maximum frequency: 72.6; mean amplitude: - 36.7; Appendix: Fig. 17 & 18; Tab. 14). As in other USV features, M.m.m. populations M.m.m.V and M.m.m.R showed different variation in frequency and amplitude values compared to M.m.m.S and M.m.m.K. In contrast to prior studies (Gourbal, Barthelemy et al. 2004; Panksepp, Jochman et al. 2007; Roubertoux, Martin et al. 1996) duration of calls did not contribute to population discrimination. This might be because analysis was conducted with only one syllable type in contrast to other studies (Gourbal, Barthelemy et al. 2004; Roubertoux, Martin et al. 1996).

I found significant variation in frequency, amplitude and entropy values (Appendix Tab. 10 – 14). Mean syllable frequency of 'Frequency upsweep' varied from 48 kHz to 82 kHz (Appendix Tab. 14) whereas studies in laboratory mice emitted calls around 40 kHz to 70 kHz (Gourbal, Barthelemy et al. 2004; White, Prasad et al. 1998). Courtship USVs are suspected to evolved by sensory exploitation of males to females' high – frequency sensitivity to pups ultrasonic calls (Hahn and Lavooy 2005; Smotherman, Bell et al. 1974). Ultrasonic calls of small rodents originally evolved for sound localization through degradation or attenuation (Bradbury and Vehrencamp 1998; Miller and Engstrom 2007; Smith 1979; Willot 2001). The variance between *Mus* species in frequency and amplitude might exist as a consequence of differing local environment (Krebs and Davies 1993; Geissler and Ehret 2001; Smith 1979). M.s. might produce vocalizations with spectral characteristics which are more effective in their habitat (Box 1). Additionally wild mice may have evolved signals with higher emission rate and higher frequency for maximum efficiency of sound transmission in open field and to discourage eavesdropping by potential predators (e.g. cats, owls: Bradbury and Vehrencamp 1998; Whitney,

Stockton et al. 1979). In contrast, the lack of predator pressure and habitat constraints might have lower the mean frequency of laboratory mice calls to reduce potential costs (Kalcounis-Rueppell, Metheny et al. 2006; Hoffmann 2008). In mammals (e.g. wolves: Tooze and Harrington 1990) and birds (Suthers and Zollinger 2002) the vocal individuality is influenced primarily by morphology structure of the vocal apparatus. But so far little is known about the vocal apparatus in mice.

Distinctive characteristics of USVs could be also influenced by variation in breeding systems (Smadja and Butlin 2009). Individual recognition can be advantageous in a polygamous breeding system such as within *Mus* species (Box 1). Signaling individuality and quality through acoustic cues can reduce conflict with neighbor territory males and increase reproductive success with potential mates. Species / populations must use signals which encode individual distinctiveness (Bradbury and Vehrencamp 1998; Owings and Morton 1998). It is enhanced when differences between populations are large and variation within individuals is minimal (Boughman and Moss 2003). Sexual selection can support and accelerate diversification in acoustic signals within and among populations (Smadja and Butlin 2009) and can explain resulted variation (Nowicki and Searcy 2004; Ryan and Rand 1993). Further individual signals are regarded as a means to maintain reproductive isolation between species (Smadja and Butlin 2009). M.m.m. and M.m.d. show continuus interbreeding along a hybrid zone in Europe (Ganem, Ginane et al. 2005; Smadja and Ganem 2002). It has been found in odor experiments that individuals of both subspecies show higher mating preference for conspecifics than in individuals away from hybrid zone, indicating that reproductive character displacement occurs. Mainly females of M.m.m. show exaggerated assortative mating within border of contact zone. I found no evidence but it needs to test mice from hybrid zone.

Spectral patterns of vocalizations can contain subtle but stable elements that could match with taxonomic boundaries (Miller and Engstrom 2007). M.m.d. differed in frequency of vocalizing / non – vocalizing males from M.m.m.S and M.m.m.K (Fig.4), but both subspecies showed also similarities in USV features (e.g. call rate , latency, spectrographic features; for details see Appendix Fig. 17 – 20). Whereas variation in USVs characteristics as well as in spectrographic

features of emitted syllables clearly distinguishes the two species, M.m.m. and M.s. Spectral characteristics (e.g. syllable structure and fundamental frequency) as well as the number of uttered syllables contribute to species identity as they are seen to be influenced by genetic constraints (McCracken and Sheldon 1997; Ryan and Rand 1993; Zimmermann, Vorobieva et al. 2000; Geissmann and Nijman 2006) and may correlate with genetic distances among taxonomic units (Packert, Martens et al. 2003). Phylogenic separation of the *Mus musculus* complex species and M.s. is genetically well supported (Guénet and Bonhomme 2003) whereas the taxonomical status of M.m.m. and M.m.d. is still debatted (Heth, Todrank et al. 2001; Tucker, Sandstedt et al. 2005) (Box 1). Therefore separation based on distinct features of USV might reveal taxonomically informative at the species level confirming phylogenic separation. Studying vocalizations as pyhlogenic relevant signal requires knowledge of genetics, develepomental and ecologic factors. These barriers complicate systematic studies of vocalization in free living songbirds and mammals (McCracken and Sheldon 1997; Miller and Engstrom 2007; Packert, Martens et al. 2003). Mice vocalizations could serve as an important trait to investigate evolution of song due to a short reproduction period of mice and the opportunity for testing and manipulating genetic influences on vocalizations (Wasserman, Palumbo et al. 2000). Using male USVs as a taxonomic trait may provide further conclusions into the existing phylogenic debate.

Besides spectrographic analysis playback experiments are used to determine vocal identity and recognition by conspecifics (e.g. birds: Reid, Arcese et al. 2006; ungulates: Reby and McComb 2003). Females retention time as well as self grooming behavior in proximity to male olfactory stimulus (e.g. urine scent mark) is seen to reflect sexual interest (Ferkin and Leonard 2005) so I measured both behaviors as a indicators for mating preference. Playback experiments showed that recorded male USV playbacks attract female significantly more than playback without USV (Fig. 2). These findings confirm previous studies in laboratory house mice that found that females are more attracted to vocalizing than devocalized males (Nyby, Whitney et al. 1978; Pomerantz, Nunez et al. 1983; Whitney, Coble et al. 1973). Additionally females did not differ between pooled male vocalization compared to vocalization of one

male (Fig. 2) indicating that my artificial created playback did not result in aversive behavior. The observed variation in male courtship USVs performance and characteristics suggests the existence of species – specific calls, but does not demonstrate individual recognition. The measured spectral patterns of male courtship USVs could be different from those used by females for individual vocal recognition (Searcy and Andersson 1986; Seyfarth and Cheney 2003). The risk of genetic problems ('inbreeding depression') and the ambition to maximize genetic compatibility within progeny ('outbreeding depression') may have evolved cues that have potential to inform mates about genetic compatibility (Penn and Potts 1999; Pusey 1987). The ability to identify potential mates males based on individually distinctive cues (e.g. calls) offers females the opportunity to mate with males who can afford genetic benefits (Gwilliam, Charrier et al. 2008; Vannoni and McElligott 2008), what occurs essentials regarding polygynous mating system mice (Box 1). In adult mice, it is known that signaling of individual identity occurs through odors and may be genetically controlled (Cheetham, Thom et al. 2007; Heth, Todrank et al. 2001; Penn and Fischer 2004). A study of Hoffmann (Hoffmann 2008) reported that wild female mice are able to discriminate between kin and non-kin male USVs suggesting male USVs may serve as a further mean for inbreeding avoidance acting as another secondary sexual trait. Preference for conspecifics only occurred when tested against different species (M.s.) (Fig. 12 – 15). This fits with the fact that though living occasionally in sympatry, mice of the *Mus musculus* complex and M.s. rarely produce offspring in nature and only sterile male offspring in laboratory (Guénet and Bonhomme 2003; Smadja and Ganem 2005). M.s. differed the most from other species in respect of spectrographic syllable. This leads to the assumption that information about male genotype might be perceived via spectrographic features of syllables.

Females showed a slight not significant preference for M.m.d. and Hybrids USVs which is contrary to odor preference tests, in which females showed significant preference for odor of conspecifics (Christophe and Baudoin 1998; Gouat, Patris et al. 1998; Smadja and Ganem 2007), but might provide an explanation of no strict species separation (e.g. Hybrid zones) and underline molecular data, regarding M.m.m. and M.m.d. as subspecies (Smadja and

Ganem 2002; Tucker, Sandstedt et al. 2005). Smadja and colleagues (Smadja and Ganem 2002; Smadja and Ganem 2007) evidenced that M.m.m. and M.m.d show distinct urinary signals in the border of the hybrid zone, allowing individuals to recognize consubspecifics and mating assortatively. However this assortative preference occurred only in the border of hybrid zone. Their results indicate asymmetrical character displacement of the receiver component of the 2 subspecies' mate recognition systems which might serve for further hybridization. Playback experiments with individuals from contact zone might show similar results in USVs and odor preference. Another explanation for the observed preference maybe the fact that presented USVs originated from the 4 vocalizers of M.m.d.. USVs are suggested to inform females about males' social status and quality (D'Amato 1991; Whitney, Stockton et al. 1979;). It might be that females might be more attracted by these males, because of greater quality of these recorded males than of males of M.m.m. population. Thus tests should be conducted with pooled USVs of more than 4 males of M.m.d.

Within playback experiments of M.m.m. populations females did not prefer male USVs of their own population (M.m.m.S) when tested against distinct population of the same species (M.m.m.V & M.m.m.R) (Fig. 12 – 15). Thus female may not suffer of a loss of reproductive benefits when preferring mates of different populations but same species. However it remains unclear if male courtship USVs have potential to inform females about males' genetics.

There was no effect of number of complex syllables on females' choice. Complex syllables appear irregularly in male vocalizations among all populations (Fig. 10) and might be more costly to produce as they contain more than one syllable. Hence complex syllables might contribute to extent the vocalizations complexity. There exist numerous studies in avian vocal behavior that documented the influence of song complexity on female mate choice thus inducing sexual selection (Searcy and Andersson 1986; Andersson and Iwasa 1996; Vallet, Beme et al. 1998). Regarding vocalization complexity as costly it might serve as an indicator for male phenotypic quality. However, the role of complex syllables in USVs in mice demand further investigations. As playbacks were standardized concerning number of syllable preference because of performance related traits could be excluded (Gil and Gahr 2002).

Females seemed to be more stressed in playback preference box what might resulted that they showed only small if any interest in urinary cues used as enhancing stimuli. Only in one of 5 test trials they significantly preferred urinary odor of their own population when tested against a distinct population (Playback experiments: M.m.m.S vs. M.m.m.V) but independent of respective playbacks. The fact that females are more stressed may also explain unexpected observed preference for male USVs playback of M.m.d. and Hybrids. Thus it needs to be clarified if female need more time to acclimate to the experimental apparatus to receive satisfying results in playback experiments.

In sum results indicate for the first time significant variability in USVs between wild *Mus* species and among *M. m. musculus* populations. Significant differences in USV performance dependent on the population of origin were found. Populations varied significantly in proportion of vocalizing males. Mainly M.m.d. stand out with a great absence of vocalizing males compared to other *Mus* species / subspecies. Latency to call and the amount of syllables uttered differed however, not significantly. Hybrids (M.m.m. x M.m.d.) appeared as more prolific vocalizers compared to their parental populations confirming the dominant inheritance theory (Maggio and Whitney 1986; Thornton, Hahn et al. 2005). Detailed spectrographic analysis of first 100 emitted calls gave quantitative evidence of the presence of distinct syllable types. Utterance of syllable types varied significantly among populations and might reveal characteristics comparable to bird songs (Holy and Guo 2005). The grouping of spectrographic characteristics of most common emitted syllable type (‘Frequency upsweep’) in the discriminant function analysis suggests presence of species-specific vocalizations enabling species recognition by acoustic signals (Baker 1982; Gwilliam, Charrier et al. 2008; Holy and Guo 2005; Miller and Engstrom 2007; Sousa-Lima, Paglia et al. 2002). Mainly individuals of M.s. were distinguished from other *Mus* populations whereas variance of vocal patterns between M.m.m. and M.m.d. populations was not significant. Song repertoire and spectrographic variability within populations and species could be attributed to genetic variability, ecological and geographical differences (Boughman and Moss 2003; Nowicki and Searcy 2004). Observed inter –

individual variation among species in latency, call rate and spectrographic features reveal new approach to use male mice USVs as phylogenic signal and identify potential homologous characters within species (McCracken and Sheldon 1997; Miller and Engstrom 2007). Results of USV features support subspecies status for M.m.m. and M.m.d. (Tucker, Sandstedt et al. 2005) as well as species status for M.s. However the analysis of only one syllable type ('Frequency upsweep') and the existence of high intra – individual variance within M.m.m. populations concerning USV performance may be considered as a limitation on the strength of my evidence. Further investigations of the entire vocalizations with greater sample size (especially in M.m.d.) should be required before male courtship USVs could be defined as species – specific calls including vocal signature. Further investigations on hybrid USVs should be conducted to determine inheritance of USV features.

My findings support the USV courtship hypothesis that females are attracted by male USVs (Pomerantz, Nunez et al. 1983). M.m.m. females significantly discriminated between male USVs of different species (M.m.m.S vs. M.s.). Thus vocalizations could be seen as a secondary sexual signal with potential to provide information of male species identity (D'Amato 1991; Nyby, Dizinno et al. 1976) on which sexual selection acts (Searcy and Andersson 1986). Further playback experiments with different populations could confirm my findings and explore the role of USVs characteristics on mate choice in more detail. To estimate which information is provided through USVs differences in preferred / unpreferred males' quality should be determined. Individuality is also conveyed through spectrographic parameters (Jorgensen and French 1998; Searby, Jouventin et al. 2004) thus it would be interesting to test females' preference for different syllable types and frequency.

Previous studies have only focused on mouse USVs of laboratory strains, whereas I found wild mice exhibit higher diversity and complexity in USVs. Studies of wild populations and species of USV characteristics combined with genetic analyses might enlighten functional significance and evolution of this behavior.

Finally, for further acoustic research, the acoustical environment in which animal are kept should be considered as it can alter vocal characteristics

(Portfors 2007). A noisy environment might directly influence mice USVs (Latham and Mason 2004). Thus it has to be investigated if male USVs recorded in the wild could reveal more differentiable vocalizations among *M. musculus* populations.

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7. Curriculum vitae

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Biographical data

Born 1st of July 1983 in Salzburg, Austria
Languages: German (mother tongue), English (speak, read and write fluently), French (basic), Italian (basic)

Education

2002 – present	Study of Biology, Universität Wien MS Diploma, Univesität Wien Experiments: Konrad Lorenz Institute for Ethology (KLIVV), Vienna Supervisor : Prof. Dr. Dustin Penn "Variation in ultrasonic courtship vocalization in male wild house mice (Genus *Mus*) "
2001 – 2002:	Study of Veterinary, University of Veterinary Medicine,Vienna
1993 – 2001:	AHS Tamsweg, Salzburg, Austria

Other activities:

2006 - 2008:	Student assistant, Konrad Lorenz Institute for Ethology (KLIVV), Vienna, Austria Supervisor: Prof. Dr. Dustin Penn, Dr. Kerstin Musolf "Role of MHC in female mate choice in wild house mice *Mus musculus musculus*"
2007:	Internship, Bioacoustics , Universität Wien Supervisor: Dr. Helmut Kratochvil, University of Vienna "Acoustic communication in giraffe (*Giraffa camelopardalis)*"
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8. APPENDIX

Table 3: Type and number (mean ± SD) of distinct syllable types uttered in 100 syllables among *musculus* populations and *Mus* species.

	Frequency Upsweep	Frequency downswee p	Constant Modulated	U shaped	U shaped inverted	Complex 2 elements	Complex 3 elements
M.m.m.V (N=10)	41.6±5.1	13.2±3.7	15.6±3.1	18.5±3.0	0.3±0.2	10.0±5.2	0.8±0.6
M.m.m.R (N=10)	50.1±8.0	17.5±4.3	19.0±3.6	5.4±1.3	7.6±1.9	0.3±0.3	0.0
M.m.m.S (N=10)	73.7±3.2	3.9±1.0	8.8±1.4	6.9±2.3	2.5±0.7	4.2±1.5	0.0
M.m.m.K (N=10)	79.8±4.3	1.1±0.5	3.6±0.7	2.0±0.6	3.0±1.1	9.6±4.1	0.9±0.5
Hybrid 1 (N=5)	62.7±4.3	13.4±2.6	9.9±1.5	6.5±2.2	1.0±1.0	5.7±3.1	0.8±0.4
Hybrid 2 (N=7)	52.5±8.0	7.0±1.6	7.9±2.2	19.3±3.9	0.0	12.0±5.4	1.1±0.9
M.m.d. (N=4)	38.5±7.4	24.2±3.7	7.6±3.0	8.5±2.8	4.2±1.5	15.2±7.4	1.8±0.8
M.s. (N=10)	57.5±4.8	4.1±1.0	10.7±2.3	0.2±0.1	13.4±2.6	10.8±3.4	3.3±1.5

Table 4: Intra-population comparison of all counted syllables 'frequency upsweep' (N = 3740) uttered by the 66 different animals. Mann – Whitney - U tests were performed to illustrate the differences between emitted rates of syllables by males of *musculus* populations and *Mus* species. Significant p - values after FDR control are highlighted.

Frequency upsweep	**M.m.m.V**	**M.m.m.R**	**M.m.m.S**	**M.m.m.K**	**Hybrid 1**	**Hybrid 2**	**M.s.**
M.m.m.V		Z=-0.341 P=0.733	Z=-3.569 **p≤0.001**	Z=-3.483 **p≤0.001**	Z=-2.272 **p=0.023**	Z=-0.879 p=0.379	Z=-1.667 p= 0.105
M.m.m.R	Z=-0.341 P=0.733		Z=-2.837 **p=0.005**	Z=-3.216 **p≤0.001**	Z=-1.410 p=0.159	Z=-0.342 p=0.733	Z=-1.627 p=0.105
M.m.m.S	Z=-3.569 **p≤0.001**	Z=-2.837 **p=0.005**		Z=-1.439 p=0.150	Z=-2.024 p=0.043	Z=-3.027 **p=0.002**	Z=-2.195 p=0.029
M.m.m.K	Z=-3.483 **p≤0.001**	Z=-3.216 **p≤0.001**	Z=-1.439 p=0.150		Z=-2.272 p=0.023	Z=-2.931 **p=0.003**	Z=-2.801 **p=0.004**
Hybrid 1	Z=-2.272 p=0.023	Z=-1.410 p=0.159	Z=-2.024 p=0.043	Z=-2.272 p=0.023		Z=-1.543 p=0.123	Z=-0.491 p=0.624
Hybrid 2	Z=-0.879 p=0.379	Z=-0.342 p=0.733	Z=-3.027 **p=0.002**	Z=-2.931 **p=0.003**	Z=-1.543 p=0.123		Z=-0.782 p=0.434
M.m.d.	Z=-0.925 p=0.355	Z=-1.062 p=0.288	Z=-2.832 **p=0.005**	Z=-2.835 **p=0.005**	Z=-2.449 **p=0.014**	Z=-1.521 p=0.131	Z=-2.409 **p=0.016**
M.s.	Z=-1.667 p= 0.105	Z=-1.627 p=0.105	Z=-2.195 **p=0.029**	Z=-2.801 **p=0.004**	Z=-0.491 p=0.624	Z=-0.782 p=0.434	

Table 5: Intra-population comparison of all recorded 'frequency downsweep' (N = 572) uttered by 66 different animals. Mann-Whitney-U tests were performed to illustrate the differences between emitted rates of syllables by males of *musculus* population and *Mus* species. Significant p - values after FDR control are highlighted.

Frequency downsweep	M.m.m.V	M.m.m.R	M.m.m.S	M.m.m.K	Hybrid 1	Hybrid 2	M.s.
M.m.m.V		Z=-0.493 p=0.622	Z=-1.863 p=0.062	Z=-3.185 **p=0.001**	Z=-0.368 p=0.713	Z=-1.323 p=0.186	Z=-1.903 p= 0.058
M.m.m.R	Z=-0.493 P=0.622		Z=-2.843 **p=0.004**		Z=-0.184 p=0.854	Z=-1.858 p=0.063	Z=-2.736 **p=0.006**
M.m.m.S	Z=-1.863 p=0.062	Z=-2.843 **p=0.004**		Z=-2.579 **p=0.01**	Z=-2.827 **p=0.005**	Z=-1.426 p=0.154	Z=-0.077 p=0.939
M.m.m.K	Z=-3.185 **p=0.001**	Z=-3.605 **p≤0.001**	Z=-2.579 **p=0.01**		Z=-3.165 **p=0.002**	Z=-2.948 **p=0.003**	Z=-2.621 **p=0.009**
Hybrid 1	Z=-1.323 p=0.186	Z=-1.858 p=0.063	Z=-2.827 **p=0.005**	Z=-3.165 **p=0.002**		Z=-2.126 p=0.033	Z=-2.707 **p=0.007**
Hybrid 2	Z=-1.323 p=0.186	Z=-1.858 p=0.063	Z=-1.426 p=0.154	Z=-2.948 **p=0.003**	Z=-2.126 p=0.033		Z=-1.278 p=0.201
M.m.d.	Z=-1.346 p=0.178	Z=-1.277 p=0.202	Z=-2.838 **p=0.005**	Z=-2.944 **p=0.003**	Z=-1.722 p=0.085	Z=-2.658 **p=0.008**	Z=-2.841 **p=0.004**
M.s.	Z=-1.903 p= 0.058	Z=-2.736 **p=0.006**	Z=-0.077 p=0.939	Z=-2.621 **p=0.009**	Z=-2.707 **p=0.007**	Z=-1.278 p=0.201	

Table 6: Intra-population comparison of all recorded 'constant modulated' (N = 680) uttered by 66 different animals. Mann-Whitney-U tests were performed to illustrate the differences between emitted rates of syllables by males of *musculus* populations and *Mus* species. Significant p - values after FDR control are highlighted.

Constant modulated	M.m.m.V	M.m.m.R	M.m.m.S	M.m.m.K	Hybrid 1	Hybrid 2	M.s.
M.m.m.V		Z=-0.265 p=0.791	Z=-1.289 p=0.197	Z=-2.820 **p=0.005**	Z=-0.924 p=0.356	Z=-1.573 p=0.116	Z=-1.250 p= 0.211
M.m.m.R	Z=-0.493 p=0.622		Z=-2.843 **p=0.004**	Z=-2.820 **p=0.005**	Z=-1.103 p=0.270	Z=-2.105 p=0.035	Z=-1.440 p=0.150
M.m.m.S	Z=-1.289 p=0.197	Z=-1.744 p=0.081		Z=-2.964 **p=0.003**	Z=-0.619 p=0.536	Z=-1.278 p=0.201	Z=-0.456 p=0.648
M.m.m.K	Z=-2.820 **p=0.005**	Z=-3.191 **p=0.001**	Z=-2.964 **p=0.003**		Z=-2.719 **p=0.007**	Z=-1.585 p=0.113	Z=-2.739 **p=0.006**
Hybrid 1	Z=-0.368 p=0.713	Z=-0.184 p=0.854	Z=-0.619 p=0.536	Z=-2.719 **p=0.007**		Z=-1.143 p=0.253	Z=-0.061 p=0.951
Hybrid 2	Z=-1.323 p=0.186	Z=-1.858 p=0.063	Z=-1.278 p=0.201	Z=-1.585 p=0.113	Z=-1.143 p=0.253		Z=-1.028 p=0.304
M.m.d.	Z=-1.847 p=0.065	Z=-1.628 p=0.103	Z=-0.713 p=0.476	Z=-0.791 p=0.429	Z=-1.112 p=0.266	Z=-0.572 p=0.567	Z=-0.851 p=0.395
M.s.	Z=-1.250 p= 0.211	Z=-1.440 p=0.150	Z=-0.456 p=0.648	Z=-2.739 p=0.006	Z=-0.061 p=0.951	Z=-1.028 p=0.304	

Table 7: Intra - population comparison of all recorded 'u shaped' (N = 506) uttered by 66 different animals. Mann-Whitney-U tests were performed to illustrate the differences between emitted rates of syllables by males of *musculus* population and *Mus* species. Significant p - values after FDR control are highlighted.

U shaped	M.m.m.V	M.m.m.R	M.m.m.S	M.m.m.K	Hybrid 1	Hybrid 2	M.s.
M.m.m.V		Z=-3.080 **p=0.002**	Z=-2.585 **p=0.010**	Z=-3.268 **p=0.001**	Z=-2.399 **p=0.016**	Z=-0.392 p=0.695	Z=-3.839 **p≤0.001**
M.m.m.R	Z=-3.080 **p=0.002**		Z=-0.308 p=0.758	Z=-1.912 P=0.056	Z=-0.560 p=0.575	Z=-2.891 **p=0.004**	Z=-3.704 **p=0.006**
M.m.m.S	Z=-2.585 **p=0.010**	Z=-0.308 p=0.758		Z=-1.452 p=0.146	Z=-0.186 p=0.853	Z=-2.401 **p=0.016**	Z=-3.277 **p=0.001**
M.m.m.K	Z=-3.268 **p=0.001**	Z=-1.912 P=0.056	Z=-1.452 p=0.146		Z=-2.041 **p=0.041**	Z=-3.332 **p=0.001**	Z=-2.927 **p=0.003**
Hybrid 1	Z=-2.399 **p=0.016**	Z=-0.560 p=0.575	Z=-0.186 p=0.853	Z=-2.041 **p=0.041**		Z=-2.030 p=0.042	Z=-3.202 **p=0.001**
Hybrid 2	Z=-0.392 p=0.695	Z=-2.891 **p=0.004**	Z=-2.401 **p=0.016**	Z=-3.332 **p=0.001**	Z=-2.030 **p=0.042**		Z=-3.609 **p≤0.001**
M.m.d.	Z=-2.131 **p=0.033**	Z=-0.857 p=0.391	Z=-0.498 p=0.619	Z=-2.155 p=0.031	Z=-0247 p=0.805	Z=-1.701 p=0.089	Z=-3.268 **p=0.001**
M.s.	Z=-3.839 **p≤0.001**	Z=-3.704 **p=0.006**	Z=-3.277 **p=0.001**	Z=-2.927 **p=0.003**	Z=-3.202 **p=0.001**	Z=-3.609 **p≤0.001**	

Table 8: Intra - population comparison of all recorded 'u shaped inverted' (N = 279) syllables uttered by 66 different animals. Mann-Whitney-U tests were performed to illustrate the differences between emitted rates of syllables by males of *musculus* population and *Mus* species. Significant p - values after FDR control are highlighted.

U shaped inverted	M.m.m.V	M.m.m.R	M.m.m.S	M.m.m.K	Hybrid 1	Hybrid 2	M.s.
M.m.m.V		Z=-3.762 **p≤0.001**	Z=-2.354 **p=0.019**	Z=-2.353 **p=0.019**	Z=-0.158 p=0.874		Z=-3.869 **p≤ 0.001**
M.m.m.R	Z=-3.762 **p≤0.001**		Z=-1.977 p=0.048	Z=-1.976 p=0.048	Z=-2.414 **p=0.016**		Z=-2.045 p=0.041
M.m.m.S	Z=-2.354 **p=0.019**	Z=-1.977 p=0.048		Z=-0.077 p=0.939	Z=-1.427 p=0.154		Z=-3.526 **p≤ 0.001**
M.m.m.K	Z=-2.353 **p=0.019**	Z=-1.976 p=0.048	Z=-0.077 p=0.939		Z=-1.422 p=0.155		Z=-3.224 **p=0.001**
Hybrid 1	Z=-0.158 p=0.874	Z=-2.414 **p=0.016**	Z=-1.427 p=0.154	Z=-1.422 p=0.155			Z=-2.969 **p=0.003**
Hybrid 2							
M.m.d.	Z=-2.833 **p=0.005**	Z=-1.140 p=0.254	Z=-2.832 **p=0.005**	Z=-0.647 p=0.518	Z=-1.799 p=0.072		Z=-2.551 **p=0.011**
M.s.	Z=-3.869 **p≤0.001**	Z=-2.045 p=0.041	Z=-3.526 **p≤0.001**	Z=-3.224 **p=0.001**	Z=-2.969 **p=0.003**		

Table 9: Inter-population comparison of all recorded 'Complex 3' (N = 67) syllables uttered by 66 different animals. Mann – Whitney - U tests were performed to illustrate the differences between emitted rates of syllables by males of *musculus* population and *Mus* species.

Complex 3	M.m.m.V	M.m.m.R	M.m.m.S	M.m.m.K	Hybrid 1	Hybrid 2	M.s.
M.m.m.V				Z=-0.398 p=0.691	Z=-1.022 p=0.307	Z=-0.848 p=0.397	Z=-1.989 p=0.047
M.m.m.R							
M.m.m.S							
M.m.m.K	Z=-0.398 p=0.691				Z=-0.694 p=0.488	Z=-0.514 p=0.607	Z=-1.741 p=0.082
Hybrid 1	Z=-1.022 p=0.307			Z=-0.694 p=0.488		Z=-0.175 p=0.861	Z=-1.071 p=0.284
Hybrid 2	Z=-0.848 p=0.397			Z=-0.514 p=0.607	Z=-0.175 p=0.861		Z=-1.117 p=0.264
M.m.d.	Z=-1.403 p=0.161			Z=-1.178 p=0.239	Z=-0.891 p=0.373	Z=-0.797 p=0.426	Z=-0.360 p=0.719
M.s.	Z=-1.989 p=0.047			Z=-1.741 p=0.082	Z=-1.071 p=0.284	Z=-1.117 p=0.264	

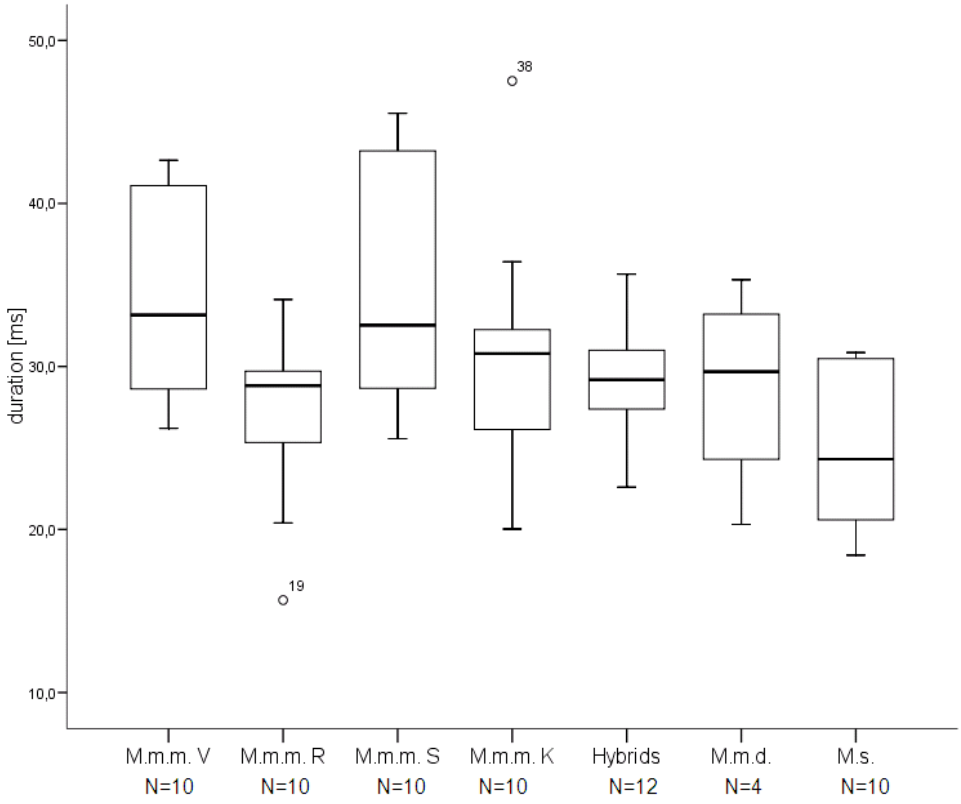

Figure 16: Duration (s) (Mean ± SD) of syllable type 'frequency upsweep' among *musculus* populations and between *Mus* species. No significant variance appeared.

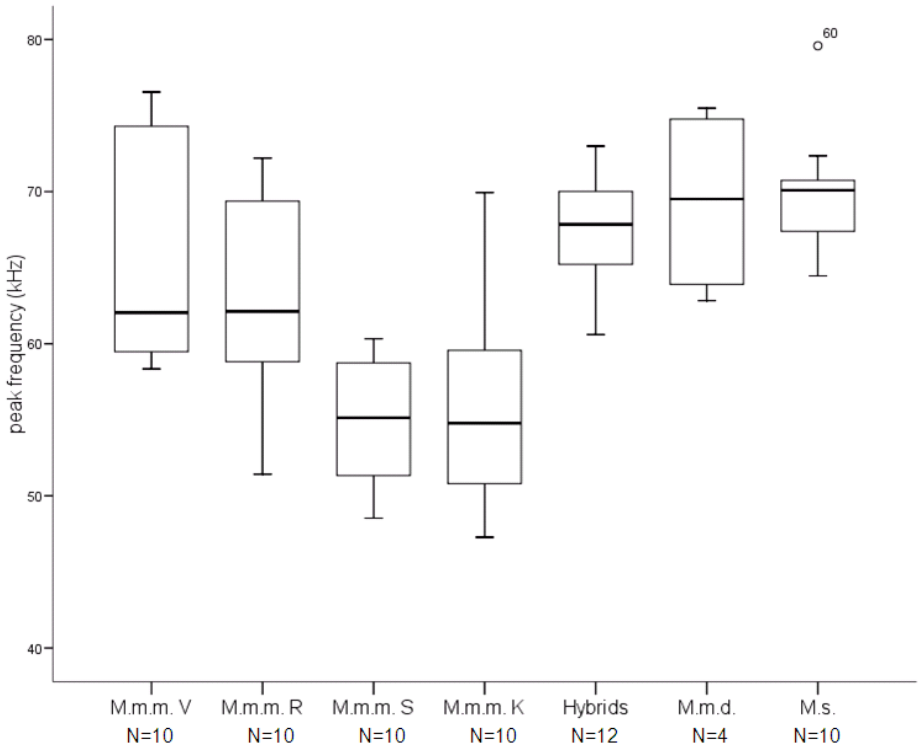

Figure 17: Peak frequency (kHz) (Mean ± SD) at start of 'frequency upsweep' among musculus populations and between *Mus* species.

Table 10: Intra - population comparison of spectrographic parameter 'peak frequency' using a MANOVA to illustrate differences among *musculus* populations and between *Mus* species. Significant p - values are highlighted.

	M.m.m.V	**M.m.m.R**	**M.m.m.S**	**M.m.m.K**	**Hybrids**	**M.m.d.**	**M.s.**
M.m.m.V		p=1.000	**p=0.002**	**p=0.009**	p=1.000	p=1.000	p=1.000
M.m.m.R	p=1.000		p=0.058	p=0.227	p=1.000	p=1.000	p= 0.183
M.m.m.S	**p=0.002**	p=0.058		p=1.000	**p≤0.001**	**p=0.001**	**p≤0.001**
M.m.m.K	**p=0.009**	p=0.227	p=1.000		**p≤0.001**	p=0.005	**p≤0.001**
Hybrids	p=1.000	p=1.000	**p≤0.001**	**p≤0.001**		p=1.000	p=1.000
M.m.d.	p=1.000	p=1.000	**p=0.001**	**p=0.005**	p=1.000		p=1.000
M.s.	p=1.000	p= 0.183	**p≤0.001**	**p≤0.001**	p=1.000	p=1.000	

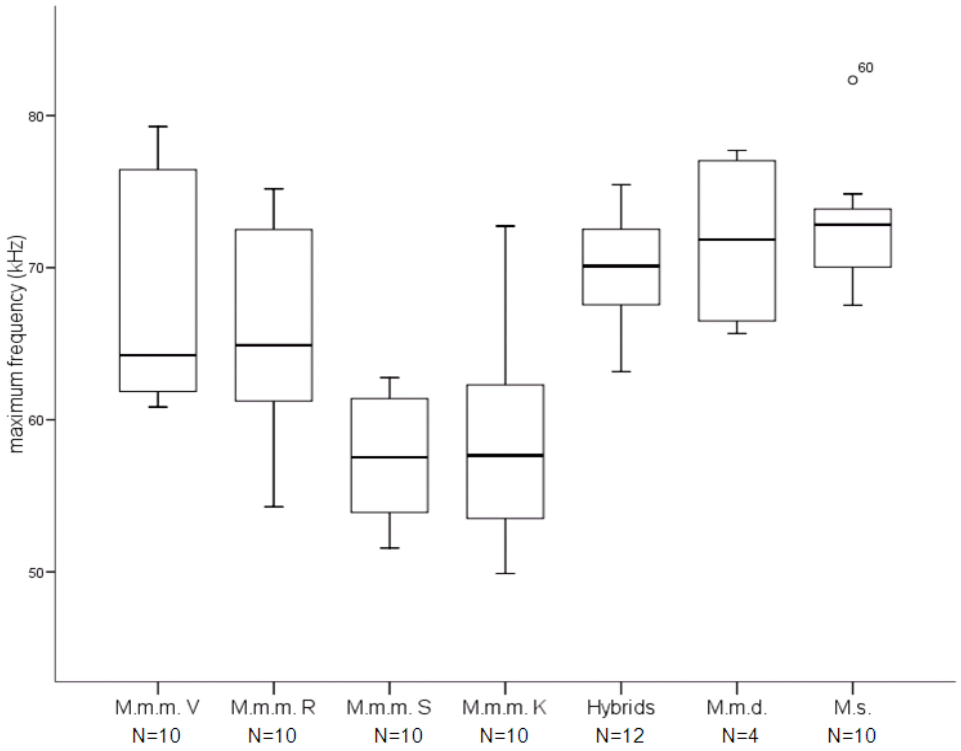

Figure 18: Maximum frequency (kHz) (Mean ± SD) at end of 'frequency upsweep' among *musculus* populations and between *Mus* species.

Table 11: Intra- population comparison of spectrographic parameter 'maximum frequency' using a MANOVA to illustrate differences among *musculus* populations and between *Mus* species. Significant p - values are highlighted.

	M.m.m.V	**M.m.m.R**	**M.m.m.S**	**M.m.m.K**	**Hybrids**	**M.m.d.**	**M.s.**
M.m.m.V		p=1.000	**p =0.002**	**p =0.014**	p=1.000	p=1.000	p=1.000
M.m.m.R	p=1.000		p= 0.041	p= 0.194	p=1.000	p=1.000	p=1.000
M.m.m.S	**p =0.002**	**p= 0.041**		p=1.000	**p≤0.001**	**p =0.002**	**p≤0.001**
M.m.m.K	**p =0.014**	p= 0.194	p=1.000		**p= 0.001**	**p= 0.006**	**p≤0.001**
Hybrids	p=1.000	p=1.000	**p≤0.001**	**p= 0.001**		p=1.000	p=1.000
M.m.d.	p=1.000		**p =0.002**	p=1.000	p=1.000		p=1.000
M.s.	p=1.000	p=1.000	**p≤0.001**	**p≤0.001**	p=1.000	p=1.000	

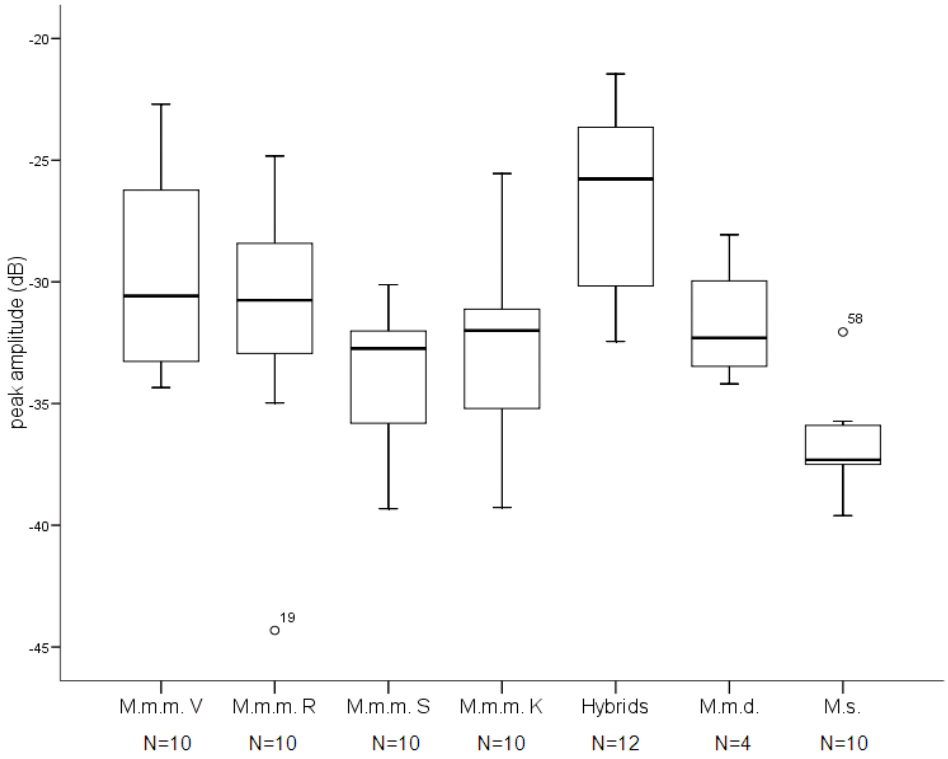

Figure 19: Peak amplitude (dB) (Mean ± SD) at end of 'frequency upsweep' among *musculus* populations and between *Mus* species.

Table 12: Intra - population comparison of spectrographic parameter 'Peak Amplitude' using a MANOVA to illustrate differences among *musculus* populations and between *Mus* species. Significant p - values are highlighted.

	M.m.m.V	**M.m.m.R**	**M.m.m.S**	**M.m.m.K**	**Hybrids**	**M.m.d.**	**M.s.**
M.m.m.V		p=1.000	p= 0.562	p=1.000	p=1.000	p=1.000	**p= 0.002**
M.m.m.R	p=1.000		p=1.000	p=1.000	p= 0.053	p=1.000	p= 0.074
M.m.m.S	p= 0.562	p=1.000		p=1.000	**p= 0.001**	p=1.000	p=1.000
Mm.m.K	p=1.000	p=1.000	p=1.000		**p= 0.012**	p=1.000	p= 0.261
Hybrids	p=1.000	p= 0.053	**p= 0.001**	**p= 0.012**		p= 0.435	**p≤ 0.001**
M.m.d.	p=1.000	p=1.000	p=1.000	p=1.000	p= 0.435		p= 0.571
M.s.	**p= 0.002**	p= 0.074	p=1.000	p= 0.261	**p≤ 0.001**	p= 0.571	

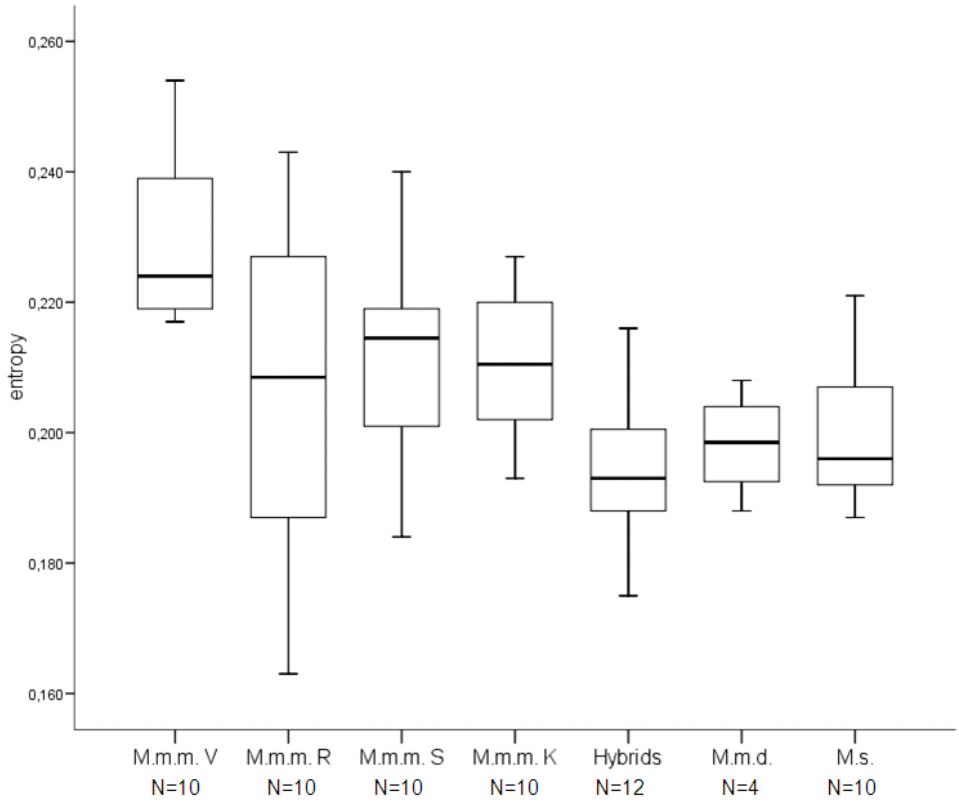

Figure 20: Entropy (Mean ± SD) at end of 'frequency upsweep' among *musculus* populations and between *Mus* species.

Table 13: Inter- population comparison of spectrographic parameter 'Entropy' using a MANOVA to illustrate differences among *musculus* populations and between *Mus* species. Significant p - values are highlighted.

	M.m.m.V	**M.m.m.R**	**M.m.m.S**	**M.m.m.K**	**Hybrids**	**M.m.d.**	**M.s.**
M.m.m.V		**p= 0.037**	p= 0.195	p= 0.146	**p≤ 0.001**	**p= 0.017**	**p= 0.001**
M.m.m.R	**p= 0.037**		p= 1.000	p= 1.000	p= 0.806	p= 1.000	p= 1.000
M.m.m.S	p= 0.195	p= 1.000		p= 1.000	p= 0.173	p= 1.000	p= 1.000
M.m.m.K	p= 0.146	p= 1.000	p= 1.000		p= 0.232	p= 1.000	p= 1.000
Hybrids	**p≤ 0.001**	p= 0.806	p= 0.173	p= 0.232		p= 1.000	p= 1.000
M.m.d.	**p= 0.017**	p= 1.000	p= 1.000	p= 1.000	p= 1.000		p= 1.000
M.s.	**p= 0.001**	p= 1.000	p= 1.000	p= 1.000	p= 1.000	p= 1.000	

Table 14: Mean (± SD) and range of 4 syllable parameters of most common emitted syllable type 'frequency upsweep' (N = 3740) used in MANOVA and univariate F test between males of different *musculus* populations and *Mus* species (Wilks'$\Lambda_{24;196.57}$ = 0.07974. p ≤ 0.001).

Variable	M.m.m.V (N=10) Range Mean (± SD)	M.m.m.R (N=10) Range Mean (± SD)	M.m.m.S (N=10) Range Mean (± SD)	M.m.m.K (N=10) Range Mean (± SD)	Hybrids (N=12) Range Mean (± SD)	M.m.d. (N=4) Range Mean (± SD)	M.s. (N=10) Range Mean (± SD)	$F_{6.59}$	p level
Peak frequency (start)	58.3 – 76.5 65.6 (±7.3)	51.4 – 72.2 62.8 (±7.2)	48.5 – 60.3 54.8 (±4.2)	47.3 – 69.9 56.0 (±6.8)	62.8 – 73.0 67.4 (±3.4)	62.8 – 75.5 69.3 (±6.4)	64.5 – 79.6 69.8 (±4.3)	10.49	<0.001
Maximum frequency (start)	60.8 – 79.3 67.9 (±7.4)	54.3 – 75.2 65.6 (±7.3)	51.6 – 62.8 57.3 (±4.1)	49.9 – 72.7 58.7 (±6.9)	63.2 – 75.5 69.9 (±3.4)	65.7 – 77.7 71.7 (±6.1)	67.5 – 82.3 72.6 (±4.2)	10.50	<0.001
Peak Amplitude (end)	-34.3 – 22.7) -29.79 (±4.1)	-44.3 – 24.8) -31.64 (±5.3)	-39.3 – 30.2 -33.61 (±2.8)	-39.3 – 25.5) -32.42 (±3.9)	-32.5 – 21.5) -26.57 (±3.7)	-34.5 – 21.5) -31.72 (±2.6)	-39.6 – 32.1 -36.74 (±2.0)	7.67	<0.001
Entropy (end)	0.217 – 0.254 0.2292 (±0.01)	0.163 – 0.243 0.2075 (±0.02)	0.184 – 0.240 0.2114 (±0.02)	0.193 – 0.227 0.2107 (±0.01)	0.175 – 0.243 0.1941 (±0.01)	0188 – 0.208 0.1983 (±0.01)	0.187 – 0.221 0.1997 (±0.01)	6.15	<0.005

Table 15: Raw data of playback experiment (Trial: male USV of M.m.m. vs. M.m.m.V). Retention times of females in three proximity zones and times females spent for specific behaviours in the speaker zone of the two stimuli compartments.

Trial	Animal	Time in middle zone (M.m.m.S)	Time in speaker zone (M.m.m.S)	Time at fence (M.m.m.S)	Time spent selfgrooming (M.m.m.S)	Time spent sniffing (M.m.m.S)	Time in middle zone (Strange)	Time in speaker zone (Strange)	Time at fence (Strange)	Time spent selfgrooming (Strange)	Time spent sniffing (Strange)
M.m.m.V	1	22.655	30.595	34.397	0.000	3.233	12.388	14.372	22.045	36.417	2.360
M.m.m.V	2	2.723	9.741	42.213	0.000	0.000	6.343	9.436	*43.219*	0.000	1.554
M.m.m.V	3	9.453	17.212	4.668	21.880	5.887	11.521	258.511	0.000	0.000	1.315
M.m.m.V	4	8.667	20.485	61.617	82.102	1.578	5.500	40.635	45.835	86.470	0.000
M.m.m.V	5	17.135	206.104	24.123	230.227	12.380	9.228	7.815	20.881	28.696	3.905
M.m.m.V	6	5.269	12.998	24.836	0.000	5.448	0.000	61.047	*46.413*	107.460	0.000
M.m.m.V	7	0.000	0.000	0.000	0.000	2.411	5.357	261.119	42.161	303.280	0.495
M.m.m.V	8	6.959	32.043	28.246	0.000	0.782	12.607	111.485	34.436	145.921	0.000
M.m.m.V	9	20.487	30.033	42.351	0.000	2.147	13.083	65.170	40.267	105.437	1.553
M.m.m.V	10	9.815	133.181	9.908	143.089	23.729	4.753	39.571	6.342	45.913	7.344
M.m.m.V	11	96.543	59.080	64.821	123.901	1.198	9.892	18.367	21.931	40.298	0.000
M.m.m.V	12	17.773	23.277	102.017	125.294	1.143	6.199	0.000	31.890	0.000	0.000
M.m.m.V	13	26.315	7.837	14.865	22.702	0.770	10.429	11.152	10.081	21.233	0.000
M.m.m.V	14	27.889	84.467	35.300	119.767	5.928	40.509	20.165	34.327	54.492	14.888
M.m.m.V	15	17.415	27.503	27.091	54.594	1.378	11.274	18.902	23.466	42.368	3.222
M.m.m.V	16	29.014	14.330	36.937	0.000	6.337	24.936	15.269	*40.254*	0.000	2.899
M.m.m.V	17	14.392	24.318	15.493	39.811	2.046	26.788	17.274	26.477	43.751	1.503
M.m.m.V	18	0.000	2.131	13.191	15.322	1.068	9.869	7.465	18.991	26.456	0.000
M.m.m.V	19	11.326	6.449	0.000	6.449	0.000	0.000	0.000	0.000	0.000	0.000
M.m.m.V	20	17.851	5.682	4.208	0.000	0.000	78.506	21.600	15.638	0.000	1.627
Mean		18.084	37.373	29.314	49.257	3.873	14.959	49.968	26.233	54.410	2.133

Table 16: Raw data of playback experiment (Trial: male USV of M.m.m.S vs. M.m.m.R). Retention times of females in three proximity zones and times females spent for specific behaviours in the speaker zone of the two stimulus compartments.

Trial	Animal	Time in middle zone (M.m.m.S)	Time in speaker zone (M.m.m.S)	Time at fence (M.m.m.S)	Time spent selfgrooming (M.m.m.S)	Time spent sniffing (M.m.m.S)	Time in middle zone (Strange)	Time in speaker zone (Strange)	Time at fence (Strange)	Time spent selfgrooming (Strange)	Time spent sniffing (Strange)
M.m.m.R	1	16,067	16,252	37,035	53,287	4,651	4,622	12,821	39,092	51,913	2,068
M.m.m.R	2	5.308	25.028	48.978	74.006	0.223	4.604	25.938	31.773	57.711	5.050
M.m.m.R	3	33.445	61.068	34.398	95.466	6.518	1.912	6.657	21.849	28.506	6.716
M.m.m.R	4	3.593	59.437	174.685	234.122	6.716	2.476	8.158	*22.358*	30.516	12.370
M.m.m.R	5	2.273	3.038	19.980	23.018	4.139	18.004	26.554	*34.145*	60.699	0.398
M.m.m.R	6	5.995	3.804	25.978	29.782	3.614	13.979	14.954	29.518	44.472	1.396
M.m.m.R	7	0.000	0.000	*0.000*	0.000	0.000	0.000	0.000	0.000	0.000	0.000
M.m.m.R	8	20.168	24.820	51.785	76.605	4.854	1.637	17.115	*6.296*	23.411	5.206
M.m.m.R	9	32.140	30.503	34.506	65.009	3.774	21.672	23.033	*31.084*	54.117	4.082
M.m.m.R	10	10.596	18.965	81.484	100.449	5.216	27.522	74.850	*34.084*	108.934	5.276
M.m.m.R	11	5.972	55.439	16.422	71.861	2.276	4.842	83.726	20.502	104.228	12.394
M.m.m.R	12	7.775	18.479	68.605	87.084	1.507	11.055	9.913	28.290	38.203	0.951
M.m.m.R	13	4.613	2.823	0.000	2.823	1.594	21.907	22.268	25.741	48.009	4.756
M.m.m.R	14	3.459	80.040	17.085	97.125	1.123	6.924	19.027	47.255	66.282	5.182
M.m.m.R	15	6.924	30.605	58.719	89.324	11.212	3.459	80.040	*17.085*	97.125	2.862
M.m.m.R	16	25.562	32.182	20.436	52.618	5.439	49.544	14.636	*14.707*	29.343	6.348
M.m.m.R	17	10.299	3.358	17.510	20.868	0.000	12.104	21.488	14.595	36.083	0.000
M.m.m.R	18	4.001	9.267	41.374	50.641	1.733	2.549	1.198	9.427	10.625	0.489
M.m.m.R	19	0.120	0.000	0.000	0.000	0.000	0.000	279.105	*19.263*	298.368	4.535
M.m.m.R	20	0.000	0.000	0.000	0.000	0.000	10.490	1.904	0.000	1.904	0.000
Mean		9.592	24.150	37.471	61.621	3.155	11.299	38.451	21.472	59.923	4.106

17: Raw data of playback experiment (Trial: male USV of M.m.m.S vs. Hybrids). Retention times of females in three proximity zones and times females spent for specific behaviours in the speaker zone of the two stimulus compartments.

Trial	Animal	Time in middle zone (M.m.m.S)	Time in speaker zone (M.m.m.S)	Time at fence (M.m.m.S)	Time spent selfgrooming (M.m.m.S)	Time spent sniffing (M.m.m.S)	Time in middle zone (Strange)	Time in speaker zone (Strange)	Time at fence (Strange)	Time spent selfgrooming (Strange)	Time spent sniffing (Strange)
Hybrids	1	9.334	1.232	8.681	0.000	0.000	7.183	4.462	5.099	0.000	0.000
Hybrids	2	7.136	9.278	26.562	0.000	1.401	10.680	8.109	47.275	0.000	0.679
Hybrids	3	24.355	5.694	40.689	0.000	3.354	19.065	101.401	37.580	15.077	12.412
Hybrids	4	9.097	32.356	46.861	0.000	0.000	0.000	9.600	9.568	0.000	0.000
Hybrids	5	11.429	32.907	36.909	14.058	4.099	12.631	116.749	*24.509*	78.856	2.902
Hybrids	6	1.628	7.099	*12.257*	0.000	0.000	0.000	256.779	6.274	32.739	0.308
Hybrids	7	6.850	10.535	53.211	0.000	3.470	10.735	14.731	72.550	0.000	0.874
Hybrids	8	4.405	1.665	4.537	0.000	0.955	16.410	12.158	11.691	0.000	1.114
Hybrids	9	14.077	35.674	*47.169*	0.000	0.000	15.891	3.103	0.000	0.000	7.407
Hybrids	10	7.811	76.148	30.793	48.293	0.000	6.148	86.862	*56.628*	75.071	7.881
Hybrids	11	0.000	17.403	*14.675*	0.000	0.000	82.068	4.042	63.045	0.000	0.000
Hybrids	12	0.000	90.664	9.445	86.041	0.955	12.176	43.431	29.074	12.508	0.661
Hybrids	13	28.535	8.356	24.731	0.000	0.955	4.146	12.786	37.227	0.000	0.969
Hybrids	14	5.666	34.944	58.737	17.874	4.320	13.956	49.271	45.069	11.935	5.614
Hybrids	15	20.104	38.922	66.018	2.575	4.099	8.583	17.028	*25.992*	0.000	4.306
Hybrids	16	7.108	17.184	47.067	0.000	0.781	6.701	85.527	48.258	53.716	0.000
Hybrids	17	8.966	6.703	*12.095*	0.000	1.563	5.619	13.160	22.709	0.000	1.190
Hybrids	18	10.516	16.940	*45.113*	0.000	1.659	3.744	5.958	56.352	0.000	2.126
Hybrids	19	4.853	3.550	4.005	0.000	0.000	0.000	0.000	*0.000*	0.000	0.000
Hybrids	20	10.459	11.519	22.430	0.000	0.000	0.000	0.000	0.000	0.000	1.695
Mean		9.616	22.939	30.599	8.442	1.381	11.787	42.258	29.945	13.995	2.507

Table 18: Raw data of playback experiment (Trial: male USV of M.m.m.S vs. M.m.d.). Retention times of females in three proximity zones and times females spent for specific behaviours in the speaker zone of the two stimulus compartments.

Trial	Animal	Time in middle zone (M.m.m.S)	Time in speaker zone (M.m.m.S)	Time at fence (M.m.m.S)	Time spent selfgrooming (M.m.m.S)	Time spent sniffing (M.m.m.S)	Time in middle zone (Strange)	Time in speaker zone (Strange)	Time at fence (Strange)	Time spent selfgrooming (Strange)	Time spent sniffing (Strange)
M.m.d.	1	27.471	28.961	35.583	0.000	52.115	39.317	16.532	*41.838*	0.000	0.000
M.m.d.	2	12.755	7.122	20.155	0.000	2.400	5.194	13.863	*34.344*	0.000	34.344
M.m.d.	3	18.590	26.400	21.032	47.432	1.690	23.256	38.634	*55.106*	93.740	102.538
M.m.d.	4	4.830	2.499	10.221	0.000	0.000	9.240	24.021	224.672	248.693	0.000
M.m.d.	5	0.000	10.125	10.552	20.677	0.000	0.000	242.643	8.819	251.462	0.000
M.m.d.	6	0.000	0.000	0.000	0.000	0.000	0.000	7.329	17.166		0.000
M.m.d.	7	4.148	0.000	*0.000*	0.000	0.000	0.000	0.989	6.785	7.774	0.000
M.m.d.	8	11.643	5.633	4.981	0.000	6.270	38.102	76.566	24.620	101.186	68.719
M.m.d.	9	15.509	5.925	35.552	0.000	1.770	16.582	33.167	41.421	74.588	5.916
M.m.d.	10	12.351	164.025	27.919	191.944	0.890	0.000	17.779	5.810	23.589	9.880
M.m.d.	11	0.000	0.000	0.000	0.000	0.000	32.207	2.474	0.000	2.474	0.000
M.m.d.	12	11.776	36.362	18.867	55.229	0.910	9.541	21.442	12.134	33.576	0.720
M.m.d.	13	13.494	25.893	24.669	0.000	3.558	9.585	6.519	9.269	15.788	7.746
M.m.d.	14	43.066	87.571	24.560	112.131	28.010	15.558	45.806	8.995	54.801	13.560
M.m.d.	15	16.130	5.414	41.244	0.000	1.210	26.773	68.372	51.332	119.704	5.810
M.m.d.	16	14.597	37.032	38.524	75.556	0.820	4.472	16.849	32.634	0.000	3.010
M.m.d.	17	2.592	19.396	36.020	55.416	4.090	15.869	16.088	16.147	32.235	0.800
M.m.d.	18	3.009	40.621	48.859	89.480	0.000	19.181	14.050	67.747	0.000	0.000
M.m.d.	19	3.563	15.450	26.516	0.000	3.010	0.000	0.000	0.000	0.000	0.000
M.m.d.	20	6.102	12.177	13.236	25.413	0.000	30.561	5.204	5.333	10.537	0.000
Mean		11.081	26.530	21.925	33.664	5.337	14.772	33.416	33.209	59.453	12.652

Table 19: Raw data of playback experiment (Trial: male USV of M.m.m.S vs. M.s.). Retention times of females in three proximity zones and times females spent for specific behaviours in the speaker zone of the two stimulus compartments.

Trial	Animal	Time in middle zone (M.m.m.S)	Time in speaker zone (M.m.m.S)	Time at fence (M.m.m.S)	Time spent selfgrooming (M.m.m.S)	Time spent sniffing (M.m.m.S)	Time in middle zone (Strange)	Time in speaker zone (Strange)	Time at fence (Strange)	Time spent selfgrooming (Strange)	Time spent sniffing (Strange)
M.s.	1	15.098	13.779	44.653	39.952	3.482	38.544	19.466	32.414	0.000	1.672
M.s.	2	8.288	9.097	30.263	0.206	2.677	6.404	29.864	*24.482*	0.000	0.000
M.s.	3	3.237	9.894	6.027	0.000	11.345	0.000	5.982	9.185	0.000	16.368
M.s.	4	3.293	201.435	8.000	0.000	3.069	4.599	15.798	2.817	0.000	3.115
M.s.	5	17.578	47.623	59.561	0.000	3.469	12.246	20.089	26.552	0.000	0.000
M.s.	6	0.000	36.058	39.852	0.000	0.000	0.000	12.660	20.488	0.000	6.630
M.s.	7	108.256	40.983	30.530	0.000	1.551	1.879	4.243	7.530	7.749	0.000
M.s.	8	47.185	19.745	24.053	0.000	2.413	0.966	3.497	6.172	0.000	2.204
M.s.	9	12.441	27.413	51.872	0.000	2.369	18.431	20.565	43.990	0.000	0.000
M.s.	10	11.885	23.139	88.295	0.000	4.607	14.476	39.062	61.662	0.000	7.987
M.s.	11	21.254	55.317	55.083	0.000	3.950	13.708	9.829	17.451	0.000	3.232
M.s.	12	3.184	12.506	58.265	79.155	1.453	6.215	8.230	*78.441*	0.000	5.208
M.s.	13	1.778	32.197	31.377	2.205	6.229	0.935	9.019	23.703	0.000	0.000
M.s.	14	22.535	53.026	43.402	5.140	1.240	39.287	15.193	23.449	0.000	3.244
M.s.	15	4.387	54.507	*28.431*	0.000	0.000	17.141	18.311	7.392	0.000	2.195
M.s.	16	15.175	15.000	17.377	0.000	3.997	8.928	10.107	14.918	0.000	6.628
M.s.	17	4.376	7.136	20.298	32.287	0.000	1.804	2.829	0.000	0.000	1.434
M.s.	18	7.409	7.425	41.281	0.000	0.000	8.964	17.828	*90.620*	0.000	1.413
M.s.	19	0.000	0.000	0.000	23.682	2.338	0.000	11.220	0.000	6.447	0.000
M.s.	20	6.563	1.092	3.127	121.636	8.970	3.133	3.698	4.698	0.000	9.766
Mean		**15.696**	**33.369**	**34.087**	**15.213**	**3.158**	**9.883**	**13.875**	**24.798**	**0.789**	**3.555**

Zusammenfassung

Männliche Hausmäuse (*Mus musculus*) geben in der Anwesenheit von Weibchen oder Weibchen-Urin Laute im Ultraschallbereich (>20 kHz) von sich. Diese Laute zeigen einige Merkmale vergleichbar mit Vogelgesang wie zum Beispiel bestimmte Silbentypen, die in Phrasen auftreten können. Diese Ultraschalllaute (USV = **U**ltra**S**onic **V**ocalization) werden als Signal des männlichen Balzverhalten angesehen. Sie dienen dazu, Weibchen anzulocken und ihre Bereitschaft zur Verpaarung zu erhöhen. Ähnlich wie Vogelgesang könnten USVs Information über die Herkunft sowie Qualität eines Männchens beinhalten.

Die Mehrheit der bisherigen Studien über USVs wurde an Labormäusen durchgeführt. Nur wenig ist über USVs und ihre verhaltensökologische Bedeutung in wilden Hausmaus Arten bekannt. In meiner Studie habe ich deswegen die Laute von verschiedenen wilden Hausmaus-Populationen als auch Arten untersucht und versucht, Unterschiede in USVs festzustellen. Zusätzlich habe ich Wahlversuche mit Hausmaus-Weibchen durchgeführt, um zu testen, ob diese in der Lage sind, USVs verschiedener Populationen und Arten zu diskriminieren.

Mit Weibchen-Urin stimulierte Männchen der Unterarten *Mus musculus musculus*, *Mus musculus domesticus*, zwei Hybridlinien aus Kreuzungen beider Arten (*musculus* x *domesticus*) und der Art *Mus spicilegus* (alle F1 Nachkommen wild gefangener Individuen) wurden aufgenommen und ihre Laute analysiert. Dabei zeigte sich eine signifikant unterschiedliche Bereitschaft der Männchen zu singen, welche Populations - abhängig war mit dem schlechtesten Resultat von nur 21% Sängern in der *M m. domesticus* Population. Weitere Analysen erfolgten mit den Aufnahmen von singenden Männchen. Pro Population wurde die Stichprobenanzahl auf 10 Individuen gesetzt, die bis auf für die *M. m. domesticus* Population (hier N = 4) aufgrund der oben erwähnten schlechten Singbereitschaft erreicht wurde. Eine spektrographische Analyse der Gesänge ermöglichte eine Bestimmung von 7 verschiedenen Silbentypen sowie deren unterschiedliche Äußerungsanzahl in den diversen Mauspopulationen. Der Gebrauch unterschiedlicher Silbentypen (Repertoire) unterschied sich signifikant innerhalb aller Populationen und Arten. Eine kanonische Diskriminanzanalyse von 4 spektrographischen Parametern des am meisten verwendeten Silbentyps (‚Frequency

upsweep‘) zeigte, dass Populationen auch anhand dieser Parameter trennbar sind. Aufgrund der resultierenden Unterschiede zwischen den Arten kann man annehmen, dass auch in USVs von Hausmäusen artspezifische Merkmale aufweisen was eine Arterkennung annhand von akustischen Signalen ermöglicht. Des weiteren könnten USVs als phylogenetisches Merkmal in Taxonomie Studien verwendet werden, wie es bereits in einigen Singvögelarten angewendet wurde.

Mit Hilfe von Playbackversuchen wurden Weibchen einer Population (*M. m. musculus*) auf ihre Fähigkeit, zwischen USVs von verschiedenen Populationen und Arten zu unterscheiden, getestet. Vorausgehend wurden Weibchen auf ihre Reaktion auf männliche USVs getestet. Weibchen verbrachten mehr Zeit in der Nähe von Playback mit USVs verglichen zu Playback ohne USVs (Hintergrundgeräusch). Des weiteren zeigten sie keine unterschiedliche Präferenz zwischen Playbacks von gepoolten USVs und USVs eines Männchens (alle USVs von M.m.m.S). Individuelle Playbacks bestanden aus einem Pool aus den zuvor aufgenommenen männlichen USVs der verschiedenen Hausmaus-Populationen und Arten. Ich präsentierte den Weibchen verschiedene dichotomische Kombinationen, wobei sie immer zwischen den Lauten ihrer eigenen Populationen und einer fremden Population, Unterart oder Art wählen konnten. Eine signifikante Bevorzugung für die Laute von Artgenossen trat nur bei Versuchen mit einer fremden Art (*Mus spicilegus*) auf, während bei allen anderen Kombinationen keine signifikante Diskriminierung gefunden wurde. Die Ergebnisse deuten darauf hin, das Weibchen von männlichen Ultraschalllauten angelockt werden und sie diese zur Unterscheidung von Männchen unterschiedlicher Arten verwenden.

Meine Arbeit ist die erste vergleichende Studie über Ultraschall - Lautgebungen in wilden Hausmäusen und erweitert damit das bisherige Wissen von hauptsächlich Laborstudien der Ultraschall - Lautgebung bei Kleinsäugern. Meine Ergebnisse zeigen, dass USVs artspezifisch sind, die sich durch verschiedene Gesangsparameter unterscheiden lassen. Weibchen können diese möglicherweise zur Diskriminierung nutzen. Somit bietet diese Studie den Ausgangspunkt für weitere Forschungen bezüglich der Funktion und Information welche USVs beinhalten können (z. B. Qualität, Identität eines Männchens).