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Courtship vocalizations of wild house mice show highly dynamic changes and correlate with male copulatory success

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Courtship vocalizations can influence mate choice and reproductive success and thus evolve through sexual selection. House mice, *Mus musculus*, are intensively studied; however, little is known about their vocalizations emitted during courtship sequences and whether and/or how they influence male copulatory success. To address these questions, we recorded the behaviour and vocalizations of pairs of wild house mice across distinct phases of courtship and mating. Over 53 000 vocalizations were detected and classified, and of these ca. 90% were ultrasonic (USV) and 10% were broadband (BBV) vocalizations, presumably emitted by males and females, respectively. Mice altered their vocal rate, composition and repertoire at each stage of courtship and mating. They increased the emissions of all simple USVs while reducing other calls upon contact with a potential mate. Then, once males began mounting and engaging in other sexual interactions, the pairs emitted more complex calls, especially harmonic USVs and BBVs with spectral nonlinearities. Vocalizations were closely associated with male mating behaviour and peaked in rates and complexity just before males approached the female to mount. USV bouts began earlier and contained more complex syllables when mounting attempts ended in copulation. As courtship progressed, the timing of USV and BBV emissions became tightly synchronized, as with duetting of songbirds. We observed several differences in the vocal repertoire and spectral features of calls between mice that successfully copulated with ejaculation and those that did not. USV emission was positively correlated with male sexual behaviours, especially among copulating mice, suggesting that the effect of USV emission on male mating success depends on their sexual behaviour, and vice versa. Our results show that the courtship vocalizations of wild house mice are more complex and dynamic than those previously reported and provide the first evidence for vocalizations that correlate with and predict male copulatory success.

Courtship vocalizations have been studied in many taxa and a wide variety of species, including insects (Sivinski et al., 1984; Souza et al., 2004), amphibians (Gerhardt & Schwartz, 2001), fish (Amorim et al., 2015), birds (Kroodsma & Byers, 1991) and mammals (Behr & von Helversen, 2004; McComb, 1991). The courtship songs of birds are particularly well known for their beauty and complexity, and males and females of some species perform duets, 'slotting their notes in and around each other's with such precision that the two songs can sound like one' (Yong, 2022, pp. 223–234). Courtship vocalizations have been shown to influence male mating success in many species, and to evolve through sexual selection (Andersson, 1994; Catchpole, 1987; Searcy & Andersson, 1986). Male vocalizations and female auditory perception are suspected to coevolve, as males tap into and exploit existing sensory biases in female auditory systems, and vice versa (Ryan, 2018). Our study focused on the courtship vocalizations of wild house mice, *Mus musculus musculus*, to determine how they change over different stages of courtship and mating, and to test whether their calls influence male copulatory success.

House mice are the most intensively studied species in the biomedical sciences; however, their vocalizations have not been studied for very long, because they are mostly ultrasonic, outside of the auditory range of human hearing (>20 kHz; Asaba, Hattori, et al., 2014; Musolf & Penn, 2012; Sales & Pye, 1974; Zippelius & Schleidt, 1956). Mice produce ultrasonic vocalizations (USVs), particularly in opposite-sex interactions, and especially during sexual behaviour when males sniff females and during mating (Nyby, 1983; Sales, 1972). Both sexes emit USVs during male–female interactions (Neunuebel et al., 2015), but USVs are mainly (84%–96%) emitted by males (Heckman et al., 2017; Sterling et al., 2023; Warren et al., 2018). USVs can be classified into 10 to 15 different types of 'calls' or 'syllables' that vary in complexity

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(laboratory mice: Grimsley et al., 2011; Scattoni et al., 2008; wild house mice: Hoffmann et al., 2012a; Zala et al., 2020). The courtship USVs of male mice are usually uttered in bouts composed of one or more syllable types and in a nonrandom order, and thus they have characteristics of birdsong (Holy & Guo, 2005). Unlike songbirds, mice do not require vocal learning (Mahrt et al., 2013), although females appear to acquire preferences for male USVs through sexual imprinting (Asaba, Okabe, et al., 2014). Several genetic backgrounds, single mutations and deletions have been found to influence the USVs of laboratory mice, including genes associated with some human diseases. The USVs of laboratory mouse models are therefore increasingly used to study neurodevelopmental and psychiatric disorders (Fischer & Hammerschmidt, 2011; Scattoni et al., 2009). Mouse USVs have also been investigated to better understand the neural mechanisms that control vocal emission (Gao et al., 2019) and auditory perception (Tasaka et al., 2018) in mammals.

However, despite the growing number of studies on USVs, little is known about the vocalizations emitted by mice during courtship and mating or their dynamics. Previous studies have found that laboratory mice begin sexual interactions by emitting mostly simple USVs, and then, just before and during male mounting, they increase the emission of complex USVs with harmonic elements (Finton et al., 2017; Hanson & Hurley, 2012; Matsumoto & Okanoya, 2016). Because opposite-sex interactions were only examined during brief periods (5–20 min) and only until mounting began, further studies are needed to analyse vocalizations emitted during the entire courtship and mating sequence (before, during and after ejaculation). Two early studies assessed USVs emitted by laboratory mice before and during ejaculation. Nyby (1983) found that mice vocalize at constant rates during courtship until male ejaculation, and then abruptly cease until the next copulatory series. White et al. (1998) reported shifts in the numbers of USVs emitted immediately before and during copulation, but they found no changes in their spectrotemporal features as courtship progressed until the male ejaculated. These two early studies were limited to USVs detected at 40 and 70 kHz (and none were classified). All previous studies used mice that were repeatedly sexually stimulated or females were manipulated with oestrogen to induce oestrus, and most used mice that had been prescreened so that only individuals that displayed (males) or allowed (females) mounting behaviour were investigated. Thus, it is unclear whether the results will generalize to wild house mice or even to laboratory mice showing normal variation in sexual behaviour.

It is also unclear whether the vocalizations emitted by mice during courtship influence male copulatory success, although several results are consistent with this hypothesis. First, male mice increase USV emission and alter the types of USVs and their frequency (Hz) upon encountering an adult female or their scent (Marconi et al., 2020; Musolf et al., 2010; Zala et al., 2017a), indicating that sexually mature females are males' intended target receivers. Second, females approach the playbacks of male USVs (Beck et al., 2023; Hammerschmidt et al., 2009; Musolf et al., 2010; Pomerantz et al., 1983). Moreover, they show preferential attraction towards complex over simple USVs (Chabout et al., 2015) and the USVs of certain males, depending on their species (Musolf et al., 2015) and individual familiarity or kinship (Asaba, Okabe, et al., 2014; Musolf et al., 2010). Third, females spend more time near and more frequently visit males that are vocally intact compared with devocalized males (Pomerantz et al., 1983) and show stronger preferences for high vocalizing males (Tschida et al., 2019). Male devocalization does not affect the male mounting rate or female lordosis (Asaba et al., 2017), although muted males achieve fewer intromissions than sham-operated males during opposite-sex interactions (Nomoto et al., 2018). Fourth, USVs provide much information about males that females may utilize to assess potential mating partners (species recognition: Musolf et al., 2015; individual identity: Hoffmann et al., 2012b; Marconi et al., 2020; health: Lopes & König, 2016; social status: D'Amato, 1991; Wang et al., 2011). Fifth, courtship USVs emitted during the initial male–female interaction correlate positively with a male's subsequent reproductive success (Asaba et al., 2017; Kanno & Kikusui, 2018; Nicolakis et al., 2020). However, most of these findings were from inbred strains of laboratory mice, which differ in courtship and mating behaviour from wild house mice (Estep et al., 1975), and no studies have analysed male mating behaviour until ejaculation. Therefore, studies are needed to test whether USVs influence successful copulation, especially in wild house mice.

House mice also emit sonic vocalizations, partly audible to human ears, which may also influence mating behaviour, and there are two types of these calls. First, mice sometimes emit broadband vocalizations (BBVs) or 'squeaks', composed of multiple harmonics, either simple (or 'pure') ones or showing many complex spectral nonlinearities (Finton et al., 2017; Grimsley et al., 2011; Lupanova & Egorova, 2015). Mouse BBVs appear to be primarily emitted by females during opposite-sex interactions (Grimsley et al., 2013; Lupanova & Egorova, 2015), and it has been suggested that these calls function to repel unwanted approaches by courting males, as their emission is tightly coupled with defensive behaviour, at least during the early stages of courtship (Barthelemy et al., 2004; Finton et al., 2017; Sugimoto et al., 2011). However, BBVs are also emitted during male sexual mountings without female rejection (Sales, 1972), and therefore, their signalling functions are unclear and may depend on social context, as found with other rodent vocalizations (Hurley & Kalcounis-Rueppell, 2018). BBVs may also signal information about a caller's individual identity (Finton et al., 2017) and affective state. Second, house mice emit mid-frequency vocalizations (MFVs), which are similar in shape to USVs, but mostly or entirely <20 kHz (laboratory mice: Grimsley et al., 2016; and labelled 'low-frequency vocalizations' in wild house mice: Zala et al., 2020). To our knowledge, no studies have investigated the emission of BBVs or MFVs over the entire courtship sequence or tested whether they influence successful copulation in mice. USVs and BBVs are emitted during mounting, presumably by the male and female, respectively (Finton et al., 2017; Grimsley et al., 2013). The close timing of these two types of calls raises the interesting possibility that the vocal exchange between the sexes influences copulatory behaviour, similar to the courtship duets of some songbirds (Hall, 2009).

We recorded the USVs and sonic vocalizations of opposite-sex pairs of wild house mice (F1 of wild-caught house mice) over ca. 40 h (mainly during the dark phases of the light cycle) and addressed the following aims. First, we classified and analysed USVs, MFVs and BBVs in these recordings to determine whether and how they change over time and during distinctive stages of courtship and mating. We also employed fine-scale analyses during the late stages of courtship to assess the precise timing of the different vocalizations emitted just seconds before and during males' attempts to mount and copulate with a female. Second, to determine whether any particular vocalizations predict and correlate with male copulatory success, we compared the vocalizations of males that copulated with ejaculation with those of males that failed to successfully copulate during the 40 h observation period. We examined particular candidate USVs and their acoustic features, which have been suggested to influence female attraction to males in previous studies (e.g. number of complex calls, Chabout et al., 2015; and especially harmonic syllable types, Hanson & Hurley, 2012; Matsumoto & Okanoya, 2016; length and frequency (Hz) of USVs, Barthelemy et al., 2004; Matsumoto & Okanoya, 2016; Nicolakis et al., 2020) and conducted additional

exploratory analyses of other types of calls. Furthermore, we investigated whether BBVs are associated with females' efforts to repel males' mating advances (Finton et al., 2017), and whether BBVs are emitted in synchrony with USVs during mating.

METHODS

Subjects and Housing

We studied 83 F1-offspring of wild-caught house mice, originally trapped in nine locations in Vienna and surroundings, Austria (mean and SD distance between the locations: 15 ± 10 km). The wild-caught mice (P generation) were bred by crossing individuals from different locations to avoid inbreeding. The offspring were weaned at 21 days of age and were kept in mixed-sex groups with their siblings until the age of 35 days. From week 5, females were housed in groups of up to three mice, and males were housed alone until the start of the study. All mice were virgins at the time of the study. Owing to constraints in P breeding success, we included two age classes: 17 males and 35 females with a mean ± SD age of 9 ± 1 months and 25 males and six females with a mean ± SD age of 17 ± 1 months. Mice were kept in standard type IIL cages (37 × 21 cm and 14 cm high; Tecniplast, Germany) with water and food ad libitum. Each cage contained woodchips as bedding material (ABEDD, Austria) and environmental enrichment, which included a polycarbonate nestbox (Tecniplast, Germany), cotton nesting material (Ehret, Austria) and a cardboard tube. At every cage change, a seed mixture (8 g) was added to the cage as dietary enrichment. Standard conditions in the colony rooms were a 12:12 h light:dark cycle with dark light (red) on at 1500 h and mean ± SD room temperature of 21 ± 1 °C.

Study Design and Procedures

We generated 42 opposite-sex pairs and assigned each mouse to a partner by systematically outcrossing the families. One individual female was recorded twice, as we had to separate her from the first male and paired her with another male after 18 days. We recorded each pair of mice over ca. 40 h and primarily during the dark (red-light) phases of the light cycle to monitor their mating behaviour and vocalizations. The recordings began at the onset of the dark period at 1500 h and ended 2 days later at 0700 h during the light period. Pairs were recorded in two separate cages, which were designed to analyse distinct phases of courtship (Fig. 1). First, we utilized a method of gradually introducing the sexes, which previously revealed significant changes in the number and types of vocalizations emitted by mice during the initial phases of courtship interactions, and the number and mean length of these USVs predicted pairs' latencies to the first litter (Nicolakis et al., 2020). We placed the mice into a standard type III cage ('cage 1': 43 × 27 cm and 15 cm high, UNO, Netherlands) with a transparent perforated Plexiglas divider to separate the mice, as described in the study by Zala et al. (2017a). Their vocalizations were recorded and analysed during the 10 min before (Fig. 1a: 'precontact' or 1-PC phase) and 10 min after (Fig. 1b: 'first contact' or 2-FC phase) the partition was removed to allow direct, physical interactions between the mice.

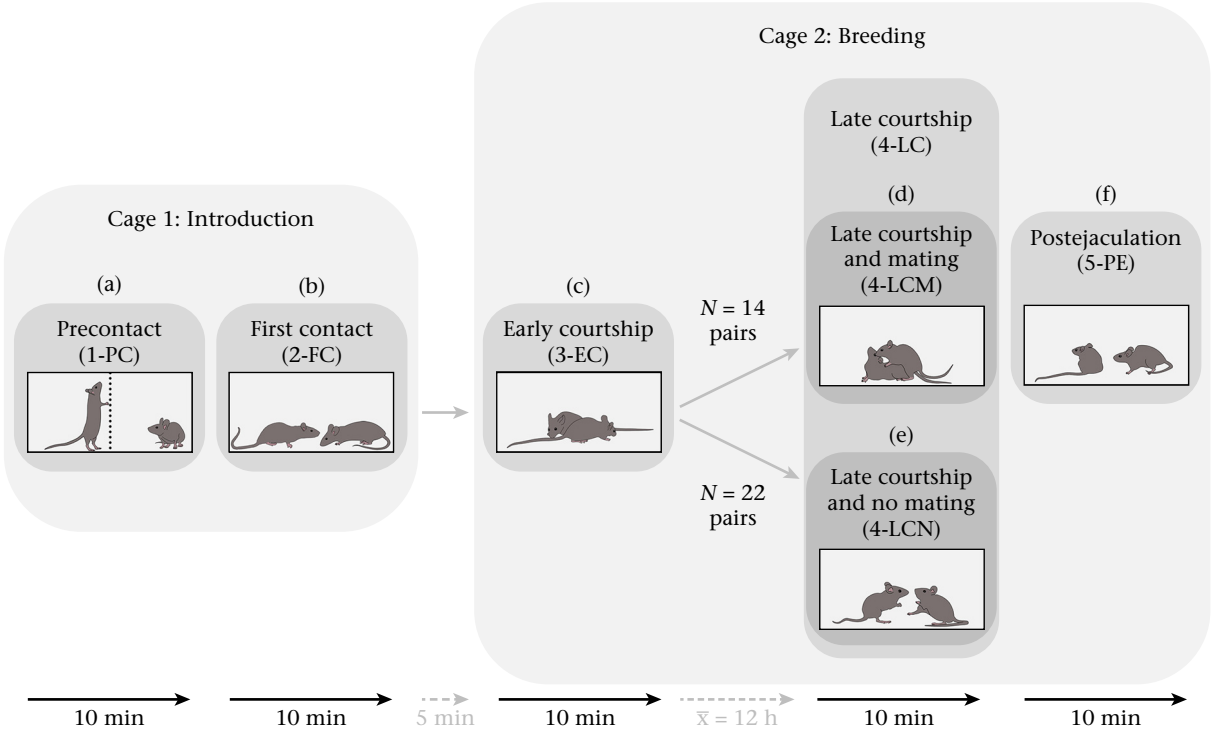

Figure 1. Procedures for recording and analysing vocalizations in pairs of mice during different stages of courtship and mating. (a) Each pair ($N = 42$) was placed into a specially designed cage to gradually introduce the mice and were first recorded for 10 min while separated by a perforated, transparent divider, which prevented direct contact ('precontact' or 1-PC phase). (b) After the divider was removed, the mice were recorded for 10 min during their first direct interactions ('first contact' or 2-FC phase). (c) Most mice ($N = 36$ pairs) were then immediately transferred to a breeding cage where after 5 min of acclimatization, they were recorded for another 10 min ('early courtship' or 3-EC phase). Recordings continued over the next 2 nights and videos were examined to distinguish between pairs that copulated successfully with ejaculation and those that did not. (d) We analysed recordings of pairs that successfully copulated ($N = 14$) during the 10 min before the first ejaculation ('late courtship and mating' or 4-LCM phase). (e) To analyse vocalizations of pairs that did not copulate with ejaculation ($N = 22$), we selected 10 min of recordings when males showed high rates of sexual behaviour during the first night ('late courtship and no mating' or 4-LCN phase). (f) Vocalizations of the mice that successfully copulated were also analysed for 10 min following the first ejaculation ('postejaculation' or 5-PE phase). In total, 40 and 50 min of vocalizations were analysed per pair (noncopulating and copulating pairs, respectively). Black arrows indicate the order in which the phases were recorded and their duration and dashed grey lines indicate the intervals between the phases (the interval between the 3-EC and 4-LC stage was ca. 3–36 h, mean 12 h).

We refer to these first two recordings as the introduction phase, which can also be considered the beginning of early courtship. Second, after 20 min of recordings inside cage 1, we placed the pair into the male's home cage ('cage 2': standard type IIL cage, as described above) for breeding. The cage was transferred to another room where recordings were immediately conducted. Owing to space limitations, only 36 of 42 pairs recorded inside cage 1 were recorded afterwards. In cage 2, we recorded the mice over the next 2 days. On the first night, recordings started between 1530 and 1730 h depending on the end of the recordings in cage 1 (six pairs per day were tested consecutively) and ended at 0700 h of the following day. The pairs were left undisturbed inside their cages and on the second night were recorded again from 1500 to 0700 h. We later evaluated recordings of each pair over 10 min during the early stage of courtship, which started after 5 min of acclimatization inside cage 2 (Fig. 1c: 'early courtship' or 3-EC phase). To select the time frames to analyse the mice during the late stages of courtship, we selected segments that depended on the males' courtship behaviour. We first manually scanned all videos to detect copulations, and for the $N = 14$ pairs that copulated successfully with ejaculation, vocalizations were evaluated over the 10 min preceding the end of the first ejaculation (Fig. 1d: 'late courtship and mating' or 4-LCM phase; interval between 3-EC and 4-LCM 16 $\pm$ 12 h, median 10 h) and the 10 min following ejaculation (Fig. 1f: 'postejaculation' or 5-PE phase). To analyse the vocalizations of the remaining $N = 22$ pairs that did not successfully mate (no ejaculation was observed during the two nights), we selected the 10 min segment containing the highest rate of male sexual behaviour (see Analyses of Behavioural Contexts) during the first night (Fig. 1e: 'late courtship and no mating' or 4-LCN phase; interval between 3-EC and 4-LCN 10 $\pm$ 2 h, median 10 h). We compared the calls of 'copulating' males versus these 'noncopulating' males during their most active sexual behaviour (rather than selecting an arbitrary period, which would likely result in comparing sexual versus nonsexual behaviour), although this comparison may be overly conservative (see Discussion).

To determine the female oestrous stage, vaginal smears were obtained at 1100 h preceding the first recordings; however, we did not track the oestrous stages afterwards to avoid potential handling effects. Cytology was examined using a light microscope (200$\times$ magnification, 20$\times$ objective and 10$\times$ ocular), and oestrous stage was evaluated by the presence and proportion of nucleated epithelial cells, cornified epithelial cells and leukocytes (Byers et al., 2012). Females in the pro-oestrous ($N = 1$) and oestrous ($N = 13$) stages were merged as 'oestrous' and females in the metoestrous ($N = 9$) and dioestrous ($N = 16$) stages were classified as 'nonoestrous'. For three females, the stage could not be unequivocally determined due to low quantities of cells, and 'nonoestrous' was assumed.

Audio and Video Recordings

For all audio recordings, ultrasound microphones (USG Electret Ultrasound Microphone; Avisoft Bioacoustics/Knowles FG) were placed 10 cm above the cages and connected to an analogue-digital converter (UltraSound Gate 116–200 and UltraSound Gate 416Hb; Avisoft Bioacoustics, Germany). Audio recordings were acquired using the RECORDER USGH-software (Avisoft-RECORDER Version 4.2) with a 300 kHz sampling rate and 16-bit format. Recordings in cage 1 were run continuously, whereas in cage 2, the 'whistle tracking' setting in the Avisoft recorder was selected (minimum duration 5 ms, range: 20–250 kHz). Videos were generated using IP cameras (DCS-3710, D-Link Systems) and the software iSpy (https://www.iSpy Connect.com).

Vocalization Analyses

We processed the acquired WAV files with the software S_TOOLS-STx (Version 5.0.6, 10071, Acoustics Research Institute, Vienna, Austria) and used the Automatic Mouse Ultrasound Detector (A-MUD 3.2) to detect USVs (Zala et al., 2017b, 2020). The following spectrographic parameters were extracted from each USV: length (duration in ms), mean amplitude (dB), frequency (mean, minimum and maximum in kHz) and frequency bandwidth (maximum–minimum frequency in kHz). Intersyllable intervals (ISI), i.e. the silence between USVs within bouts (the distance between any two USVs with <300 ms) and interbout intervals (IBI), i.e. the silence between bouts of USVs (distances between two USVs with >300 ms), were calculated following Marconi et al. (2020). We removed all false positive segments from the files. USVs were manually classified into 11 syllable types (adapted from Grimsley et al., 2011; Hanson & Hurley, 2012; Musolf et al., 2015; Scattoni et al., 2008; Zala et al., 2020; Fig. 2a): (1) short ('s'): vocalizations <10 ms in length; (2) flat ('f'): vocalizations without changes in frequency >5 kHz; (3) down ('d'): vocalizations with a decrease in frequency >5 kHz; (4) up: vocalizations with an increase in frequency >5 kHz; (5) u-shaped ('u'): vocalizations with a decrease and then an increase in frequency, each >5 kHz; (6) u-shaped inverted ('ui'): vocalizations with an increase followed by a decrease in frequency, each >5 kHz; (7) complex ('c'): vocalizations with three or more directional changes in frequency, each >5 kHz; (8) complex 2 ('c2'): vocalizations with a jump in frequency without time separation; (9) complex 3 ('c3'): vocalizations with two or more jumps in frequency without time separation; (10) harmonic ('h'): vocalizations with at least one harmonic component and with or without frequency jumps (this type of call thus merges all types of USVs that have harmonics); (11) unstructured ('un'): vocalizations that do not meet any aforementioned criteria (labelled 'unclassified' or 'unclassifiable' in Marconi et al., 2020; Nicolakis et al., 2020; Zala et al., 2020). Unstructured syllables appear either entirely within the ultrasonic frequency range >20 kHz or have audible components <20 kHz (Fig. 2b). To investigate a simpler classification, the 11 syllable types were pooled into four major classes as follows: short ('s'), simple (combining 'f', 'd', 'up', 'u' and 'ui'), complex (combining 'c', 'c2', 'c3' and 'h') and unstructured ('un') calls (categories adapted from Nicolakis et al., 2020; Fig. 2a). Additionally, across the 11 syllable types, we quantified USVs that were mainly or entirely in the ultra-high frequency range >91 kHz (Hoffmann et al., 2012b; Zala et al., 2020; Fig. 2b) and the appearances of sidebands, i.e. bands adjacent to the fundamental elements (described as 'subharmonics' in Grimsely et al., 2011 and 'harmonics' in Scattoni et al., 2008; Fig. 2b).

We also analysed sonic vocalizations (<20 kHz; Fig. 2c), including MFVs, which are often similar in shape to USVs but mostly <20 kHz (adapted from Grimsley et al., 2016), and BBVs (Finton et al., 2017; also labelled 'low-frequency harmonic vocalizations'; 'low-frequency vocalizations' or 'squeaks' in Portfors, 2007; Wang et al., 2008; Zala et al., 2020). BBVs show stacks of harmonic elements with fundamental frequencies <10 kHz and harmonic bands often reaching into the ultrasonic range (Grimsley et al., 2011, 2013; Lupanova & Egorova, 2015). We distinguished two classes, pure versus nonlinear BBVs (Finton et al., 2017). Pure BBVs are defined by their pure harmonic components and typically minor modulations in frequencies (Finton et al., 2017; Lupanova & Egorova, 2015), whereas nonlinear BBVs exhibit additional structures in their spectral features, such as subharmonics (sequences of additional harmonics at integer fractions of the fundamental elements) or segments of noise-like structures (termed 'chaos' or 'deterministic chaos'; Wilden et al., 1998). It is not known whether these two types of BBVs provide different signalling functions,

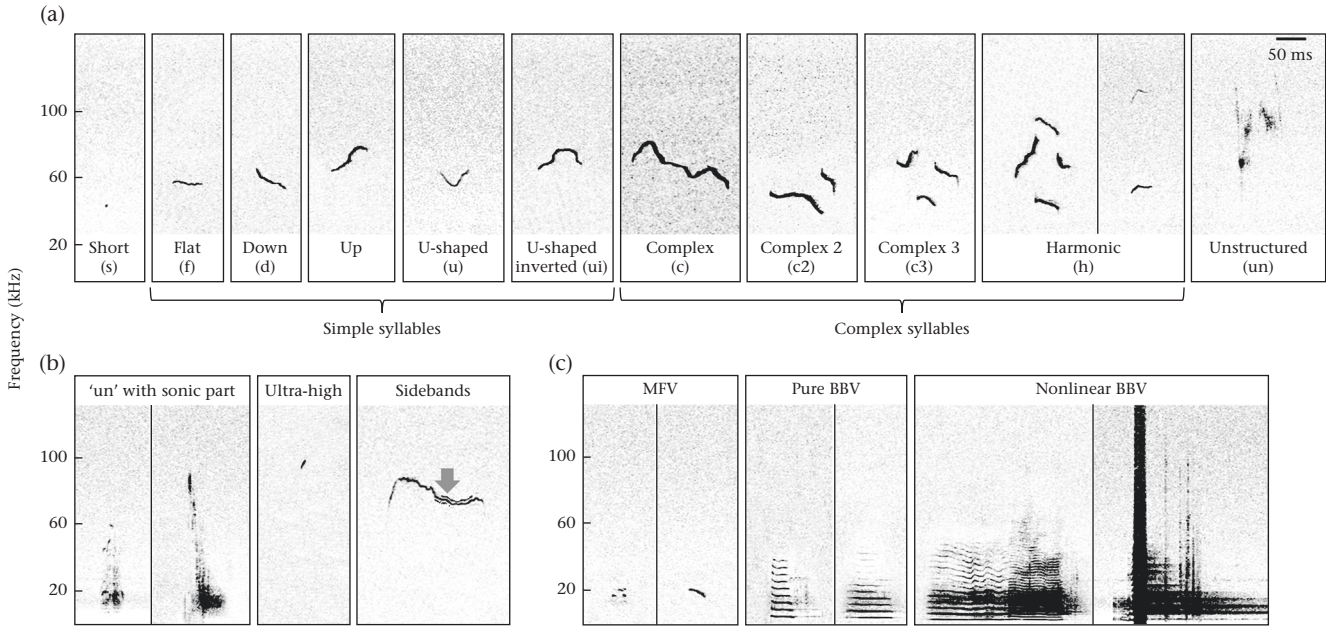

Figure 2. Examples of spectrograms of the different vocalization types. (a) Ultrasonic vocalizations (USVs) were classified into 11 syllable types that belong to four major classes (short, simple, complex and unstructured syllables). (b) Two unstructured USV syllables (which can extend into the sonic range <20 kHz), one ultra-high USV (mainly or completely >91 kHz) and a USV with sidebands. (c) Sonic vocalizations (<20 kHz) include mid-frequency vocalizations and broadband vocalizations, which were classified into pure and nonlinear classes, based on the absence or presence of spectral nonlinearities.

although nonlinearities potentially convey information about female identity and oestrous state (Finton et al., 2017).

Analyses of Behavioural Contexts

Male sexual behaviour was assessed during the 'late courtship' stage (Fig. 1d and e: 4-LCM and 4-LCN phases). The time and number of occurrences of six male sexual behaviours were evaluated and summed as 'mating attempts' for analyses: (1) 'approach': male approaches female's posterior end from a distance of more than one mouse length and in a directed manner; (2) 'chase': male chases the female from a short distance and female flees from the male; (3) 'mount attempt': male approaches and lifts forelegs onto female's posterior end without successfully mounting; (4) 'head mount attempt': male attempts to mount female's head, as in (3); (5) 'mount': male mounts the female from the posterior end but without pelvic thrusts (usually lasting <1 s); (6) 'head mount': male mounts female's head with or without pelvic thrusts. In addition to mating attempts, we monitored (7) 'copulation': a male mounted a female from the posterior end with visible pelvic thrusts, with or without ejaculation. Male house mice and other rodents exhibit a series of copulations without ejaculation (intromissions) as part of their courtship before ejaculation. Ejaculation was evaluated when males tipped over on their side and remained immobile. Except for three males, all males that achieved copulations also ejaculated within 40 h of observation. We also assessed two female behaviours: (1) female approaching the male from a distance of more than one mouse length that resulted in bodily contact and (2) female defensive behaviours, such as kicking the male or adopting an upright posture towards the male with their mouth open.

None of the males mounted (and only three males attempted to mount) the female during their first direct interactions (Fig. 1b and c: 2-FC and 3-EC phases), which was expected because courtship in wild mice typically spans over many hours or even days. These early phases are equivalent to the 'precopulatory' or 'intromission-latency' periods previously described for rodent courtship phases (Dewsbury, 1988; Pomerantz & Clemens, 1981). Males that successfully mated during the study ($N = 14$) had variable numbers of intromissions within the 10 min before the male first ejaculated (Fig. 1d: 4-LCM phase, which corresponds to the 'copulatory' or 'ejaculation-latency' period in Dewsbury, 1988; Pomerantz & Clemens, 1981). We expected males to have a refractory period, i.e. the period after ejaculation and before mice begin copulating again ('postejaculatory' period or interval in Dewsbury, 1988; Pomerantz & Clemens, 1981). We did not observe any sexual behaviour in 12 of the 14 pairs during the 10 min after ejaculation in our study (Fig. 1f: 5-PE phase).

We matched audio and video recordings within 1 s to analyse vocalizations occurring in association with specific male courtship behaviours and focused on the comparison of (failed) male mating attempts versus copulations. Changes in USV syllable type composition and their spectrotemporal features in relation to these behaviours were examined, separating between vocalizations that were emitted 'prior' to and 'during' the mating attempt or copulation. USVs detected within sequences (i.e. <1 s between two USVs) that started before and continued until the onset of the behaviour were labelled as calls 'prior' to the behaviour. USVs emitted from the start of the behaviour until dismount (for copulations and mountings) or until 2 s after the start of the event (for all mating attempts except mountings) were considered calls 'during' the mating attempt or copulation. USVs emitted during the remaining time of the 10 min recording while the pair was not interacting or they were performing nonsexual behaviours, were considered as 'nonsexual'. Furthermore, we examined the timing of USVs and BBVs occurring in the 5 s before, during and 5 s after each behavioural event.

Statistical Analyses

All statistical tests were conducted using RStudio (RStudio version 4.2.1; R Core Team, 2022). Relationships between courtship phases, behavioural contexts and vocalizations were investigated with repeated measures analysis using general (LMM) or

generalized linear mixed-effects models (GLMM), conducted using the packages 'lme4' (version 1.1.30) and 'glmmTMB' (version 1.1.6). Pair identities were included in all models to compute random intercepts. Subjects were of two distinct age classes; therefore, we included male and female age (older/younger) as fixed factors in the models to control for them. Significant effects of male and female age are reported in the Appendix; however, due to the imbalance of pairs with older versus younger females ($N = 6$ and $N = 35$, respectively), any effects of female age should be interpreted with caution. For the analysis of the first two recording phases (1-PC and 2-FC), the female oestrous state (oestrous/nonoestrous) was included as a fixed factor, but not in the analyses of later courtship phases because the observation period spanned over a total of two days. When analysing the differences between copulating and noncopulating pairs during courtship phases, we first explored the interaction of group (copulating/noncopulating pairs) and phase. Results are presented when the group * phase interaction is significant. We also ran a reduced model that lacked interaction but contained main effects. For analyses of vocalizations associated with specific behavioural contexts at the 'late courtship' stage (4-LC, including 4-LCM and 4-LCN phases), group (copulating/noncopulating pairs) was included as an additional fixed factor in all models.

Because the distributions of all vocalization count data were highly right-skewed, models were constructed using a negative binomial family. For analyses of rates of USVs and BBVs emitted over five 1 s time bins preceding mating attempts versus copulations, we used data from each observed behavioural event and entered the interaction of behaviour and time bin as fixed factors and pair identity and individual observation as random factors in the GLMM. Changes in the USV repertoire size (number of different syllable types used within a 10 min recording; from 0 to 11 types) over courtship phases were tested using LMM when assumptions of normality of residuals and homoscedasticity of the data were met; otherwise, a GLMM with Poisson distribution was conducted. The models were tested for overdispersion with the 'dispersion_glmer' function (package 'blmeco', version 1.4). To analyse changes in USV spectrotemporal parameters between courtship phases or behavioural contexts, we extracted data from each USV and used random intercepts and by pair random slopes models with the gamma or occasionally the inverse Gaussian distribution family. We visualized the appropriate model distributions using the 'fitdist' function (package 'fitdistrplus', version 1.1.8). Proportional changes in BBV/USV overlap between courtship phases and groups were tested using a GLMM and the beta family argument. Because copulating pairs were found to mate in the first ($N = 10$) or second night ($N = 4$), we also explored whether courtship duration correlated with vocalizations emitted during the 4-LCM phase. Thus, all models were initially conducted with night (first/second night) as a fixed factor. None of the tested response variables were significantly affected; thus, the factor was excluded from further analyses. We calculated the significance of the fixed factors using the 'Anova' function from the package 'car' (version 3.1-0). When applicable, post hoc Tukey's tests were performed using the package 'multcomp' (version 1.4.20) or 'emmeans' (version 1.8.1.1). Visualization of some results (e.g. total number of USVs and syllable repertoire) suggested that copulating versus noncopulating pairs differed in the first stages of courtship; therefore, we conducted post hoc tests for these results.

To test for differences in pairs' USV composition (numbers emitted of each of the 11 syllable types) between courtship phases or behavioural contexts, we used a multivariate approach, analysis of similarities (ANOSIM), using the package 'vegan' (version 2.6.4). The simper function was applied to examine the contribution of syllable types to patterns of dissimilarity by pairwise comparison of phases or behavioural contexts. We tested for differences in the dispersion among the compared variables as they might lead to confounding results. We used nonmetric multidimensional scaling (NMDS) plots based on Bray–Curtis dissimilarity calculations for visualization of the results. To test for differences in the vocal composition of copulating versus noncopulating pairs during courtship phases, we used permutational multivariate analysis of variance (PERMANOVA) to analyse the interaction between the group and courtship phases.

Spearman rank correlation tests were used to assess the relationship among vocalization types, USV syllable types and USV numbers and syllable diversity. Fold changes for visualization of dynamics in syllable type usage among phases were calculated by dividing the numbers of each syllable type from the first and the subsequent phase for each pair.

We used the 'Song Overlap Null model Generator' (Masco et al., 2016) to test whether the occurrences of temporal overlaps between USVs and BBVs at the 4-LC stage were greater than chance expectations. We ran 1000 randomizations (using the 'KeepGaps' method that maintains the duration of vocalizations and their intervals during the randomization procedure to account for constraints on their timing) for each pair to generate a null distribution and compared the predicted amount of overlap expected due to chance to the observed number of overlaps.

Results are considered statistically significant at $\alpha = 0.05$. We provide mean and SD for descriptive statistics, whereas we provide estimates and SEs for results from our models unless stated otherwise.

Ethical Note

This study was discussed and approved by the institutional Ethics and Animal Welfare Committee (ETK-03/03/2017) in accordance with the Good Scientific Practice guidelines and national legislation. The recordings were conducted during a regular breeding cycle for colony maintenance, eliminating the need to use animals solely for the experimental recordings and avoiding unnecessary pregnancies. Handling of mice was avoided, and transfers to a new cage were performed by guiding mice into a transfer bottle. The experimental protocol had been used and refined in our laboratory numerous times, thus allowing us to work with minimal stress on the animals. During their cohousing period, breeding pairs were closely monitored for fighting or signs of fighting. We separated two pairs due to small bite marks on their tails, and these individuals were checked and did not require medical treatment. All mice were returned to the colony after breeding.

RESULTS

Overall, we detected a total of 53 018 vocalizations, comprising 46 370 USVs (87%), 1539 MFVs (3%) and 5109 BBVs (10%). We classified USVs into 11 syllable types and BBVs into pure and nonlinear types. The mice vocalized during all phases of courtship and mating (Fig. 1), except for one pair that ceased to vocalize after ejaculation, and they mostly emitted simple classes of USVs overall (77% of all USVs). The mice emitted a median of 159 USVs (273 ± 312) per 10 min segment, but there was enormous variation in USV emission among pairs (range 0–2045 USVs per 10 min). The number of USVs per recording (USV emission rate) correlated logarithmically with the number of syllable types (USV repertoire; $r_S = 0.829$, $P < 0.001$; Fig. A1a). USV numbers per recording correlated with the number of BBVs ($r_S = 0.374$, $P < 0.001$), but not with the number of MFVs. The numbers of MFVs and BBVs were negatively correlated ($r_S = -0.250$, $P < 0.001$).

The numbers of most (9/11) USV syllable types were significantly intercorrelated, and their correlations showed a

hierarchically structured pattern (Fig. 3a): (1) simple USVs were correlated with other types, particularly with other simple types (the only exception was the 'ui' syllable, which clustered with complex types); (2) complex USVs were correlated with other USVs, especially with other complex types; (3) 's' calls correlated with most other USV types, but only weakly; and (4) 'un' calls only correlated with the emission of 's' syllables. We therefore used multivariate tests for most analyses, although we also used univariate tests on the changes of the four major USV classes (simple, complex, 's' and 'un') over time and courtship phases.

The composition of USV syllable types emitted by the mice changed significantly across the different phases of courtship and male sexual behaviour (behavioural context; Fig. A1b). Using fold changes to visualize vocal dynamics showed that simple USV types, and particularly 'up' calls, increased dramatically once the mice came into first direct contact (2-FC phase; Fig. 3b). Then, several hours later in courtship, when males increased the rate of mounting and other sexual behaviours (4-LC phase), they shifted to increasing the numbers of complex types of USVs, and especially 'h' calls (Fig. 3c). The majority (56%) of 'h' USVs had at least one frequency jump, and most (89%) of these had two jumps. They were found to be emitted predominantly in the seconds preceding male copulatory behaviour. These results were statistically significant, and we describe the changes in vocal emission in more detail in the following sections.

Vocal Dynamics During the Introduction

The mice vocalized at low rates while they were still separated by the divider during 'precontact' (Fig. 1a: 1-PC phase), and then, during the 'first contact' stage and their first direct interactions (Fig. 1b: 2-FC phase), they significantly increased the number and types of USVs (USV numbers: GLMM: $\chi^2_1 = 5.27$, $P = 0.022$; USV repertoire: LMM: $\chi^2_1 = 7.08$, $P = 0.008$; Fig. 4a and b). Multivariate analysis of the USV composition showed significant differences between the two stages (ANOSIM: $R = 0.168$, $N = 42$, $P = 0.001$; Fig. 4c). During 2-FC, the mice increased the emission of all five simple call types ('f', 'd', 'up', 'u' and 'ui') and two of the four complex types ('c' and 'h'), whereas they reduced the number of 'un' USVs (Fig. 4d). The 'un' USVs, which typically have a very broad frequency bandwidth and can extend into the audible frequency range, also changed their shape and frequency, so that a lower proportion of 'un' calls reached <20 kHz during this stage (LMM: $\chi^2_1 = 14.90$, $P < 0.001$).

We found significant changes in USV spectrotemporal features during this introduction phase. During 1-PC, the mice emitted many USVs in the ultra-high frequency range (>91 kHz), the majority (69%) of which were short (<10 ms), and then during 2-FC, they reduced their numbers (1-PC: 20 ± 42 ultra-high USVs per pair (9 ± 16% of USVs); 2-FC: 2 ± 5 (1 ± 3%); GLMM: $\chi^2_1 = 34.24$, $P < 0.001$; see also 'Vocalizations of Copulating Versus Noncopulating Pairs' with Fig. 9f and Fig. A1b for these results and other stages). At the 2-FC stage, the mean length (duration) of USV syllables significantly increased (1-PC: 28 ± 18 ms; 2-FC: 37 ± 19 ms; GLMM: $\chi^2_1 = 21.98$, $P < 0.001$) and their frequency decreased (mean frequency: 1-PC: 71 ± 16 kHz; 2-FC: 68 ± 9 kHz; GLMM: mean frequency: $\chi^2_1 = 13.13$, $P < 0.001$; minimum frequency: $\chi^2_1 = 12.88$, $P < 0.001$; maximum frequency: $\chi^2_1 = 8.88$, $P = 0.003$).

We also observed significant changes in the sonic calls emitted by the mice. During 1-PC, the number of MFVs was high and correlated with the number of USVs ($r_S = 0.466$, $P = 0.002$), and then, at 2-FC, their emission declined (GLMM: $\chi^2_1 = 175.2$, $P < 0.001$; Fig. 5a). In contrast, BBVs, like USVs, increased in number during 2-FC (GLMM: $\chi^2_1 = 92.08$, $P < 0.001$; Fig. 5b). Before contact, most BBVs showed pure harmonic structures, but most BBVs contained nonlinearities when the sexes came into direct contact (1-PC: 59 ± 39% pure harmonic BBVs/total BBVs per pair; 2-FC: 38 ± 26%).

Vaginal smears were collected 4–6 h before the first recording and one-third (14/42) of the females were found to be in the oestrous stage. We found no evidence that the female oestrous state

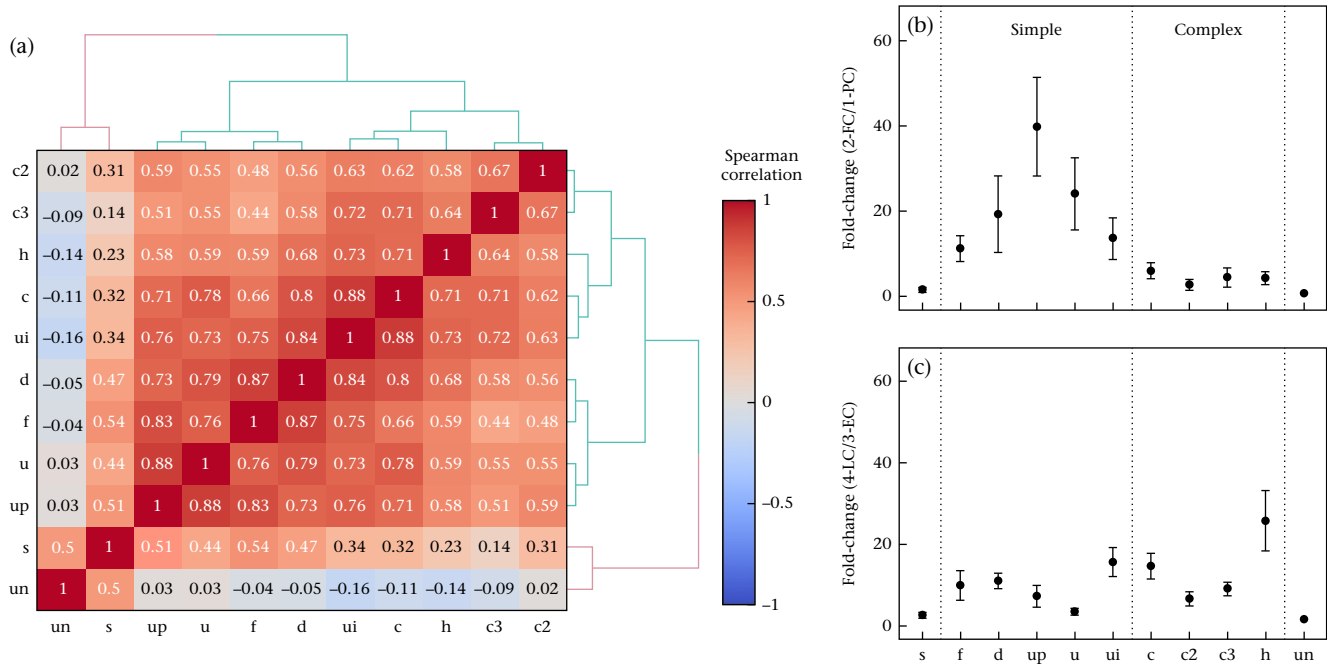

Figure 3. (a) Correlation matrix comparing the relationships in the numbers of 11 ultrasonic vocalization (USV) syllable types ('s', 'f', 'd', 'up', 'u', 'ui', 'c', 'c2', 'c3', 'h' and 'un') measured per recording, showing Spearman rank correlation coefficients and a dendrogram of their correlation-based distances. (b) Fold changes in the numbers of USV syllable types emitted during the pairs' introduction ('first contact'/'precontact' stage) in cage 1 ($N = 42$ pairs) and then during (c) the following two stages ('late courtship'/ 'early courtship' stage) in cage 2 ($N = 36$ pairs). Means ± SEM are shown.

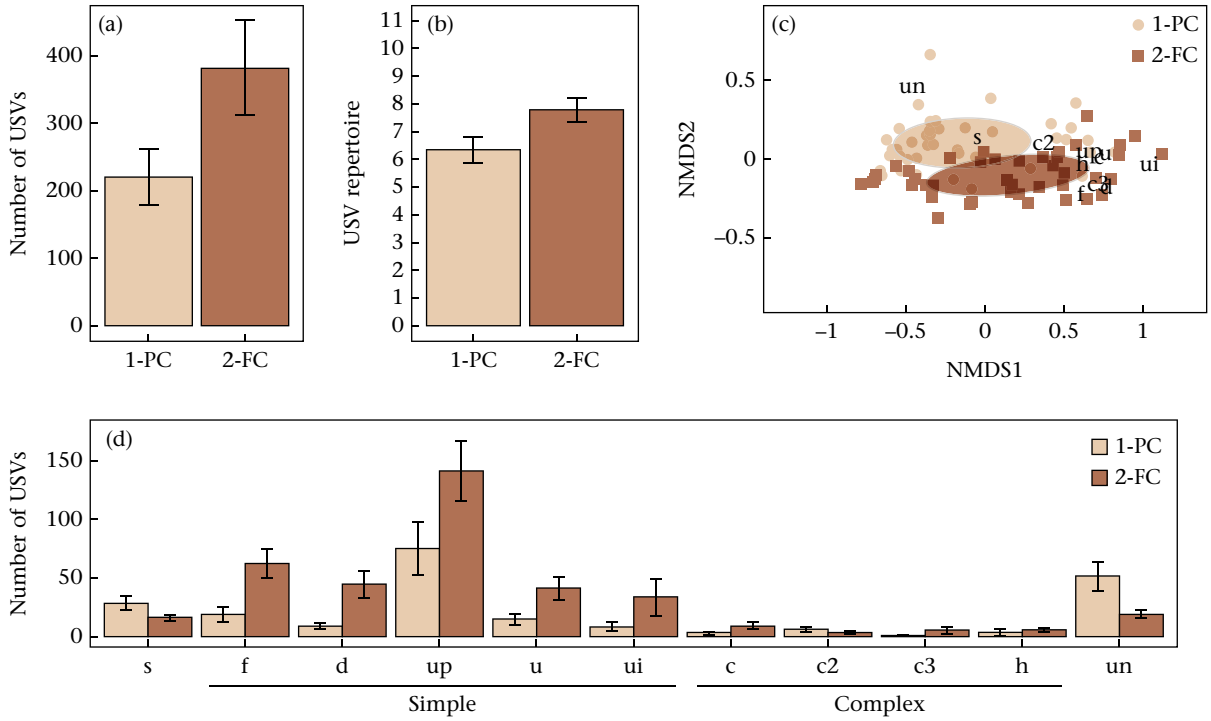

Figure 4. Ultrasonic vocalizations (USVs) emitted during pairs' introduction in cage 1, showing (a) USV numbers and (b) repertoire (diversity of USV syllable types) and comparing 'precontact' (1-PC) versus 'first contact' (2-FC) stages. (c) Cluster analysis comparing the composition of USV syllable types emitted during the two different stages (NMDS plot of USV composition; ellipses represent SD about the centroid for each stage; stress value = 0.08). (d) Numbers of each USV syllable type emitted during the two stages (Fig. 3b displays these results as fold changes). Bar charts show means ± SEM for each of the two 10 min recordings (N = 42 pairs).

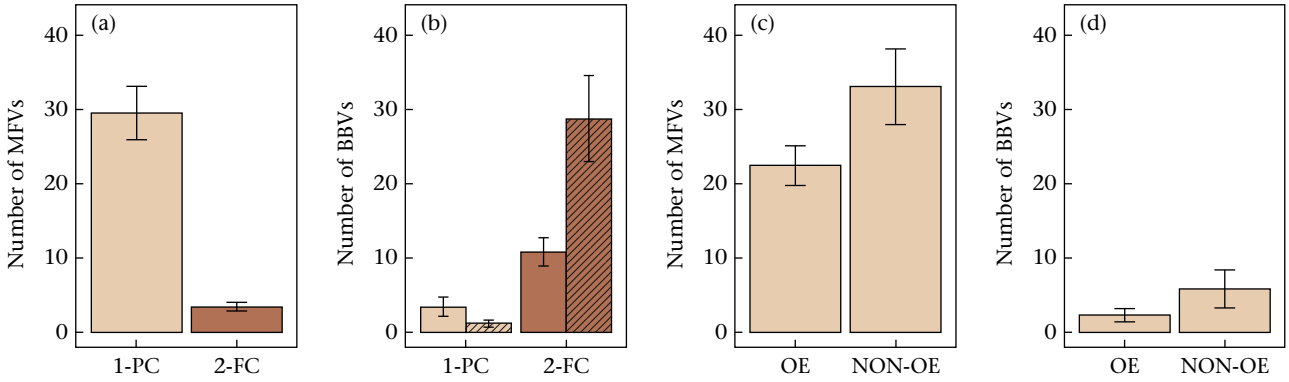

Figure 5. Sonic calls emitted during the introduction of the sexes in cage 1, showing the emission of (a) mid-frequency vocalizations (MFVs) and (b) broadband vocalizations (BBVs), comparing 'precontact' (1-PC) versus 'first contact' (2-FC) stages. The BBVs include the comparison of emitted pure harmonic (solid bars) and nonlinear (striped bars) types. Number of (c) MFVs and (d) BBVs emitted during 1-PC in females in sexual oestrous ('OE'; N = 14) versus nonoestrous ('NON-OE'; N = 28) stages. Means ± SEM are shown.

influenced the number, diversity or spectral features of USVs emitted in cage 1. However, the female oestrous stage affected the number of MFVs over the two phases, and they were emitted more among pairs with nonoestrous females (GLMM: $\chi^2_1 = 6.61$, $P = 0.01$; Fig. 5c; 2-FC not shown). Female oestrous state was also associated with lower numbers of BBVs during 1-PC (GLM: $\chi^2_1 = 4.07$, $P = 0.044$; Fig. 5d), but not during 2-FC.

Vocal Dynamics Before, During and After Copulation, and Ejaculation

We analysed video recordings of the mice over the next 2 nights (cage 2) and found that 14/36 (39%) of pairs successfully mated (i.e. copulations with intromissions and at least one ejaculation). We compared the vocalizations of the 14 pairs during three main stages of male sexual behaviour in courtship (10 min recording each), which included: (1) 'early courtship', the period after introduction but before any mountings occurred (Fig. 1c: 3-EC); (2) 'late courtship and mating', the period just before and during male ejaculation (Fig. 1d: 4-LCM); and (3) 'postejaculation', the period directly following male ejaculation (Fig. 1f: 5-PE). As expected, the mice showed similar USV emission rates and vocal repertoires over the 20 min of their first direct interactions (2-FC in cage 1 and 3-EC in cage 2), except that they continued to reduce emissions of 's' and 'un' syllables and sonic MFVs. Males exhibited female anogenital sniffing during these early stages but did not engage in any mounting behaviour until several hours later. Females' initial oestrous stage at this time had no effect on whether the mice eventually copulated or not. During the first night and within 16 h after assessing the oestrous state, 6/12 of the females in oestrous state

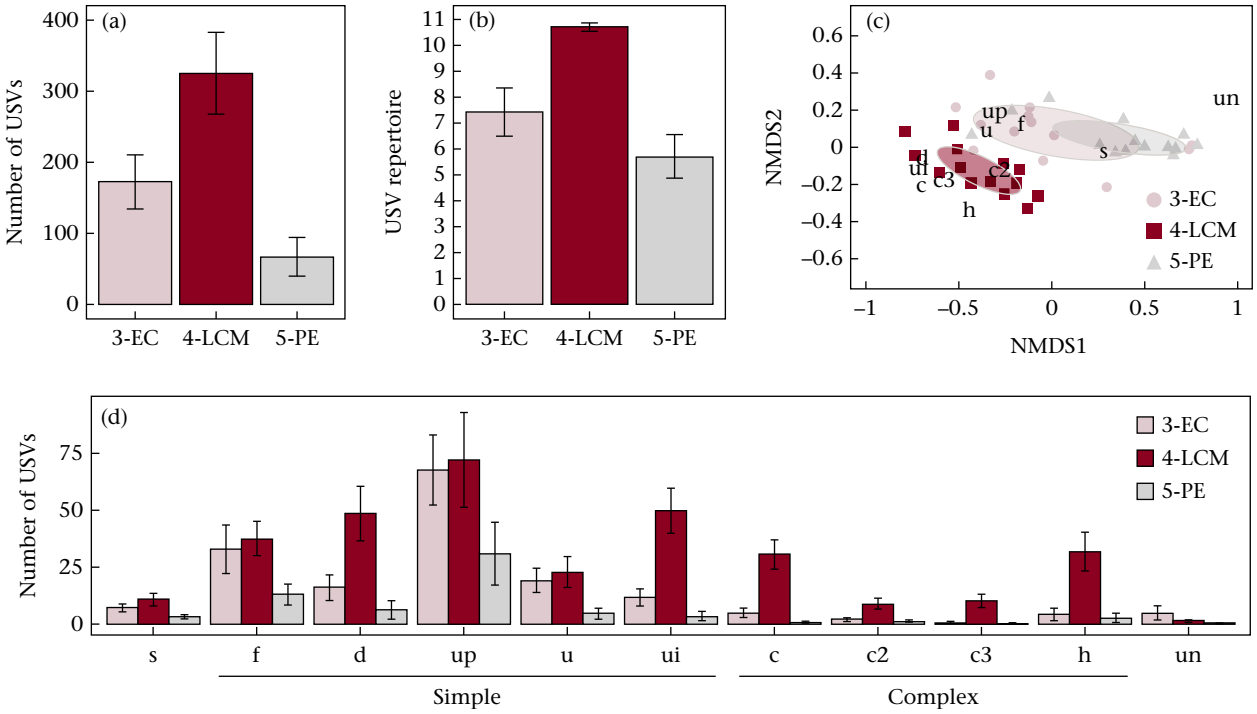

Figure 6. Ultrasonic vocalizations (USVs) emitted during three stages of male courtship and mating behaviour, 'early courtship' (3-EC), 'late courtship and mating' (4-LCM) and 'postejaculation' (5-PE). Changes in (a) USV numbers and (b) repertoire (0–11 syllable types). (c) USV composition (NMDS plot; stress value = 0.09) and (d) numbers of 11 USV syllable types across the three courtship stages. Bar charts show means ± SEM for each of the three 10 min recordings (N = 14 pairs).

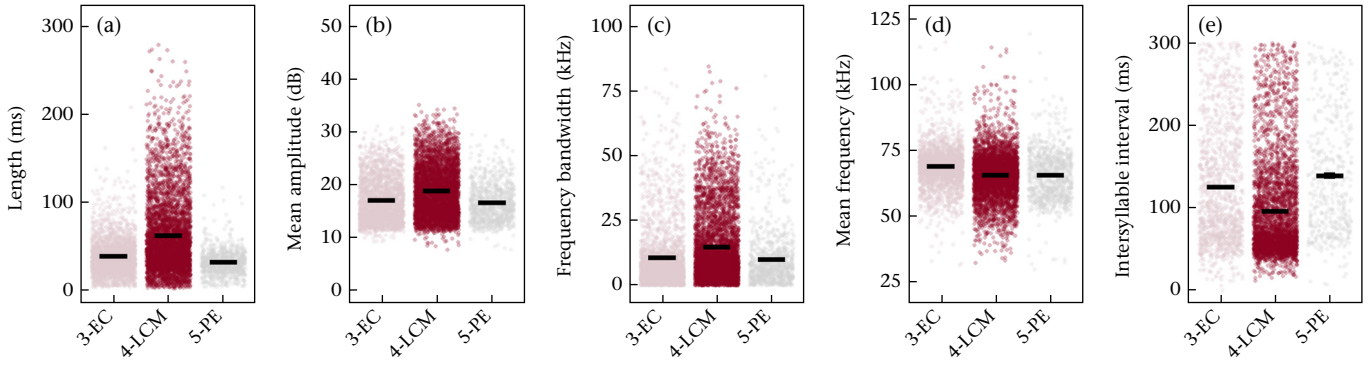

Figure 7. Spectrotemporal features of ultrasonic vocalizations (USVs) emitted from 14 pairs during 'early courtship' (3-EC), 'late courtship and mating' (4-LCM) and 'postejaculation' (5-PE), showing (a) syllable length (duration), (b) mean amplitude, (c) frequency bandwidth, (d) mean frequency and (e) intersyllable intervals (intervals <300 ms). Points represent raw data, and horizontal bars represent means ± SEM; because SEM are small the error bar is not visible.

and 4/24 of the nonoestrous females mated. Four other females initially in nonoestrous stages mated during the second night.

We found that the number and diversity of USVs emitted by the mice showed significant changes over time and across the three stages (USV numbers: GLMM: $\chi^2_2 = 25.51$, $P < 0.001$; USV repertoire: LMM: $\chi^2_2 = 30.54$, $P < 0.001$; Fig. 6a and b). Compared with 3-EC, the mice had increased USV numbers during 4-LCM just before and during male ejaculation (post hoc: $P = 0.008$), and then USV numbers dropped again following male ejaculation (5-PE; post hoc: $P < 0.001$). USV emission ceased for several minutes following ejaculation; however, most mice soon resumed vocalizing and USV numbers returned to 3-EC levels. None of the pairs emitted their full syllable repertoire during 3-EC or 5-PE, whereas the majority (11/14 pairs) produced all 11 syllable types during 4-LCM (post hoc: 4-LCM versus other stages: $P \leq 0.001$). Similar syllable type diversity was observed in the 3-EC and 5-PE stages. The composition of USV syllable types differed significantly over the three stages of courtship (ANOSIM: $R = 0.341$, $N = 14$, $P = 0.001$; Fig. 6c and d). Specifically, all complex syllable types ('c', 'c2', 'c3' and 'h') showed higher abundances during 4-LCM than during the 3-EC and 5-PE stages. Additionally, 'd' and 'ui' were more common before (4-LCM) than after ejaculation (5-PE). The USVs 's', 'un' and some simple calls ('f', 'up' and 'u') appeared more often at the 3-EC stage than during 5-PE.

Most USV spectrotemporal features showed significant changes over the three stages, particularly during the mating phase (4-LCM; GLMM: length: $\chi^2_2 = 94.87$, $P < 0.001$; mean amplitude: $\chi^2_2 = 27.85$, $P < 0.001$; frequency bandwidth: $\chi^2_2 = 56.47$, $P < 0.001$; mean frequency: $\chi^2_2 = 10.52$, $P = 0.005$; minimum frequency: $\chi^2_2 = 6.67$, $P = 0.036$; ISI: $\chi^2_2 = 23.17$, $P < 0.001$; Fig. 7a–e). During 4-LCM, USVs increased in length (duration), mean amplitude and frequency bandwidth and were emitted at

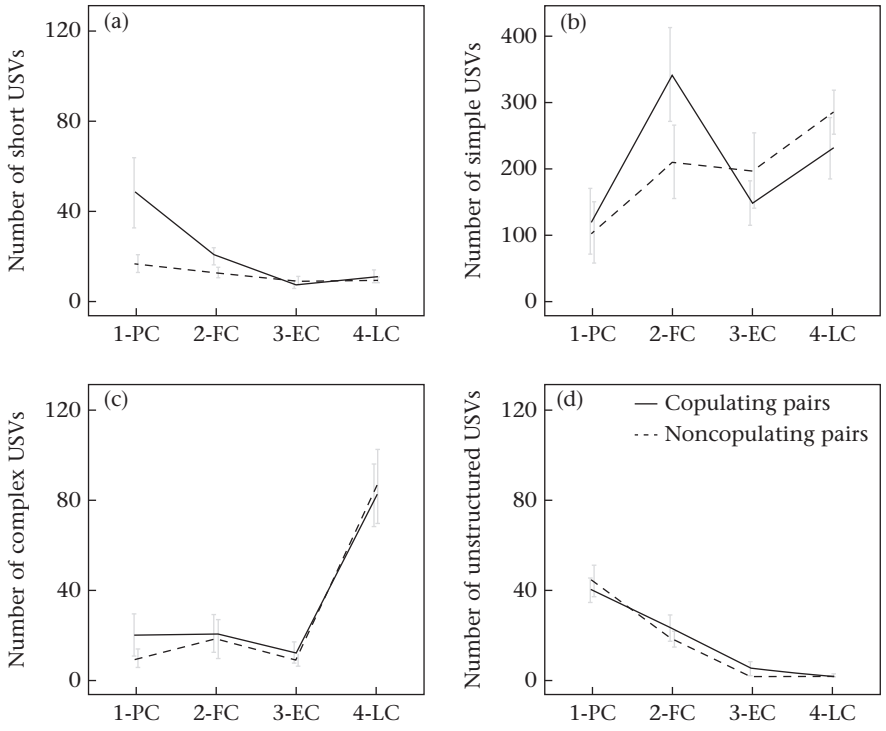

Figure 8. Ultrasonic vocalization (USV) emission of copulating (solid line, $N = 14$) versus noncopulating mice (dotted line, $N = 22$) over time and four courtship phases (1-PC to 4-LC), showing numbers of (a) short ('s'), (b) simple, (c) complex and (d) unstructured ('un') USVs. Note that these graphs differ in scale because simple USVs were emitted at higher rates than other syllable classes. Means ± SEM are shown for each of the four 10 min recordings.

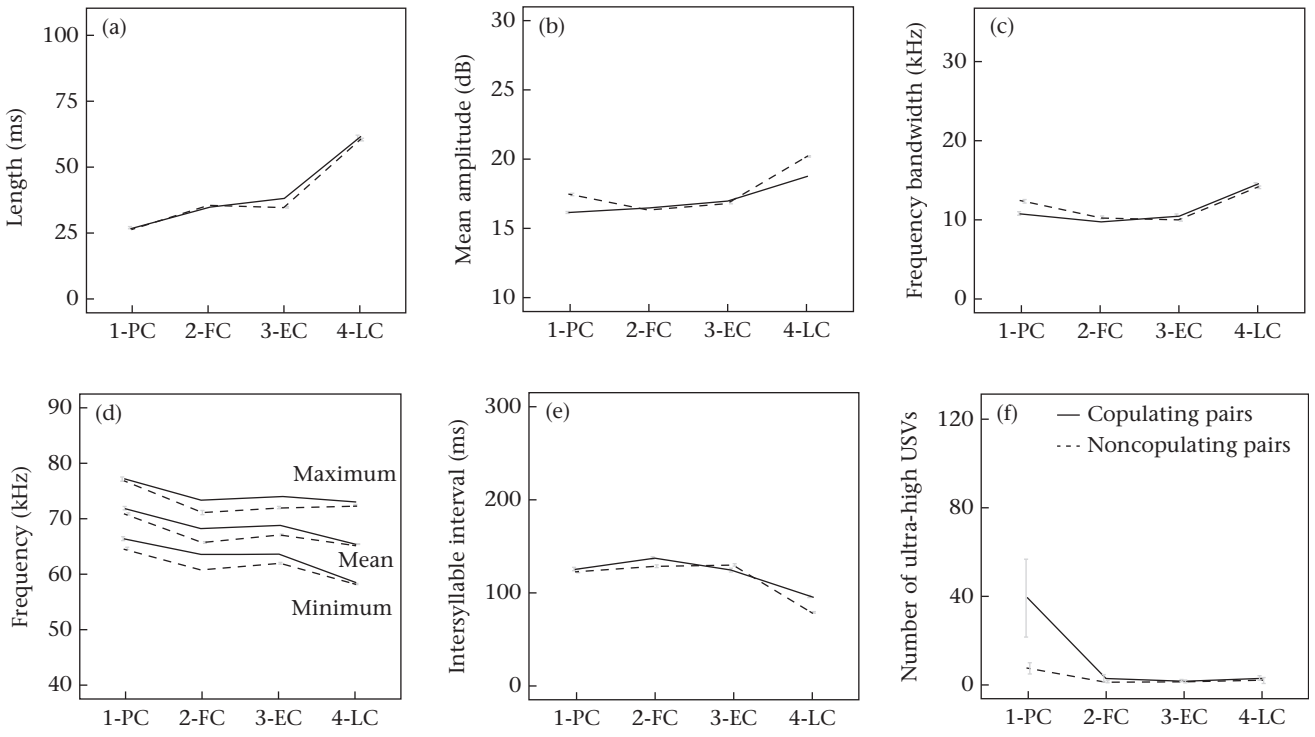

Figure 9. Ultrasonic vocalization (USV) spectrotemporal features of copulating (solid line, $N = 14$) versus noncopulating pairs (dotted line, $N = 22$) over time and four stages of courtship (1-PC to 4-LC), showing (a) USV length (duration), (b) mean amplitude, (c) frequency bandwidth, (d) frequency and (e) intersyllable intervals (intervals <300 ms). (f) Number of USVs at ultra-high frequencies (>91 kHz). Means ± SEM are shown for each of four 10 min recordings.

lower mean and minimum frequencies compared with 3-EC (post hoc: length/amplitude/bandwidth: $P < 0.001$; mean frequency: $P = 0.005$; minimum frequency: $P = 0.031$). After ejaculation (5-PE), these changes in USV spectrotemporal features were reversed (post hoc: 4-LCM versus 5-PE: length/amplitude/bandwidth: $P < 0.001$; minimum frequency: $P = 0.043$), and again, their calls showed similar features as those observed during 3-EC, except for the mean frequency, which stayed at lower levels (post hoc: 3-EC versus 5-PE: $P = 0.037$). ISIs were significantly shorter in 4-LCM than in 3-EC and 5-PE (post hoc: all $P < 0.001$) and did not differ between 3-EC and 5-PE. There was no significant effect of courtship phase on maximum call frequency (Hz; GLMM: $\chi^2_2 = 3.86$, $P = 0.145$). Mice emitted only a few ultra-high USVs over the three stages (see below for 3-EC and 4-LCM stages), but their rates declined following ejaculation (GLMM: $\chi^2_2 = 7.69$, $P = 0.021$; post hoc: 4-LCM versus 5-PE: $P = 0.016$).

We found significant changes in all sonic vocalizations during these three stages of courtship (GLMM: MFV: $\chi^2_2 = 8.66$, $P = 0.013$; nonlinear BBV: $\chi^2_2 = 68.79$, $P < 0.001$; pure BBV: $\chi^2_2 = 52.99$, $P < 0.001$). The numbers of MFVs were relatively low for all stages (3-EC: 2 ± 4 MFVs per pair; 4-LCM: 3 ± 3; 5-PE: 1 ± 1), whereas there was a significant increase in nonlinear BBVs during 4-LCM (nonlinear BBV: 3-EC: 19 ± 18; 4-LCM: 42 ± 29; 5-PE: 2 ± 4; pure BBV: 3-EC: 10 ± 8; 4-LCM: 19 ± 14; 5-PE: 1 ± 3; post hoc: nonlinear BBV: 3-EC versus 4-LCM: $P = 0.007$). The numbers of MFVs and nonlinear and pure BBVs dropped after ejaculation, with lower emission of all BBVs in the 5-PE phase than during 3-EC (post hoc: MFV: $P = 0.01$; BBV: all $P < 0.001$).

Vocalizations of Copulating and Noncopulating Pairs

To determine whether any vocalizations were associated with or predicted male mating success, we compared the vocalizations of pairs that successfully copulated until ejaculation ($N = 14$) versus those that failed to mate with ejaculation ($N = 22$) within the 2-day observation period. We examined the vocalizations of these two groups over time and courtship phases, including all stages that preceded male mating behaviour (Fig. 1a–c: 1-PC to 3-EC phases) and the 4-LC stage (Fig. 1d and e: 4-LCM and 4-LCN phases), when both groups engaged in high rates of male sexual behaviour.

Mice generally increased their USV emission and syllable type repertoire over time and courtship stages, and these changes included copulating and noncopulating pairs (Table A1, Fig. A2a and b). Visualization of the results suggests that pairs that successfully copulated emitted more USVs and had a larger USV repertoire over the first two courtship stages, but we detected no significant group differences in the multiple comparisons of the phases. All pairs of mice showed an initial increase in simple USVs upon first contact of the sexes and then higher emissions of the complex syllable class at the late courtship stages, whereas emissions of 's' and 'un' calls decreased over time (Fig. 8a–d, Table A1). Copulating mice consistently emitted a higher diversity of syllable types and produced more USVs of the complex syllable class than the other pairs, but these differences were not statistically significant (GLMM: USV repertoire: $\chi^2_1 = 3.78$, $P = 0.052$; complex USV: $\chi^2_1 = 3.56$, $P = 0.059$). We found a significant interaction between the group of mice and the courtship phase on the emission of 's' calls (GLMM: $\chi^2_3 = 10.85$, $P = 0.013$), and 's' syllables were produced more often by copulating than noncopulating mice at the first courtship stage (1-PC: post hoc: $P = 0.007$). Our multivariate analyses detected no differences in the USV composition between copulating and noncopulating pairs over the courtship phases, but we found a main effect of group and phase (PERMANOVA, group: $F_{1,136} = 1.86$, $P < 0.001$; phase: $F_{3,136} = 20.53$, $P < 0.001$; group*phase: $F_{3,136} = 1.04$, $P = 0.319$). Generally, the mice emitted more 'd' and 'ui' and all four complex types during the late (4-LC) stages compared with the preceding stages. 'S' calls showed greater abundance in copulating pairs than in noncopulating pairs. USVs containing sidebands showed similar emission patterns as those observed for complex calls (Table A1, Fig. A2c), although at very low numbers of USVs (2 ± 8 USVs with sidebands/10 min).

In addition to changes in syllable type composition, all mice showed increased length (duration), amplitude and frequency bandwidth, and they also lowered the frequency of their USVs over time and courtship stages (Fig. 9a–d, Table A2). We found a significant interaction between group and courtship phase on mean and minimum USV frequencies (GLMM: mean frequency: $\chi^2_3 = 9.91$, $P = 0.019$; minimum frequency: $\chi^2_3 = 9.21$, $P = 0.027$) and an effect of group on maximum frequency (GLMM: $\chi^2_1 = 5.04$, $P = 0.025$). Post hoc pairwise comparisons showed that in the first 10 min recording (1-PC), copulating pairs emitted USVs at higher mean and minimum frequencies than noncopulating pairs (mean frequency: $P = 0.029$; minimum frequency: $P = 0.044$), and they generally emitted USVs at higher maximum frequencies. Consistent with this finding, copulating pairs also emitted more ultra-high USVs (GLMM: $\chi^2_1 = 6.89$, $P = 0.009$; Fig. 9f, Table A1). In both groups, the intervals between USVs within a bout (ISI) decreased over time (Fig. 9e), whereas the interbout interval (IBI) remained unchanged (Table A2); however, copulating pairs generally emitted calls with longer ISIs than noncopulating ones (GLMM: $\chi^2_1 = 9.20$, $P = 0.002$).

Examination of the spectral changes among the 11 syllable types indicated that most, but not all, types followed these general temporal patterns (Fig. A3). Notably, 'up' calls, the most common syllable type overall (35% of all USVs), showed only minor changes in duration and frequency bandwidth over time and courtship phases. Complex 'h' calls showed the highest mean amplitude and lowest mean and minimum frequencies among all syllable types at the 4-LC stage.

The emission of MFVs declined sharply in all pairs after the 1-PC stage and remained low (Table A1, Fig. A2d). Both groups of mice emitted more BBVs when the pair interacted directly and further increased the numbers of nonlinear BBVs during 4-LC (Fig. 10, Table A1). Overall, we found that the majority of BBVs had spectral nonlinearities and that pure harmonic BBVs were emitted more often by copulating than by noncopulating pairs (GLMM: $\chi^2_1 = 3.89$, $P = 0.049$).

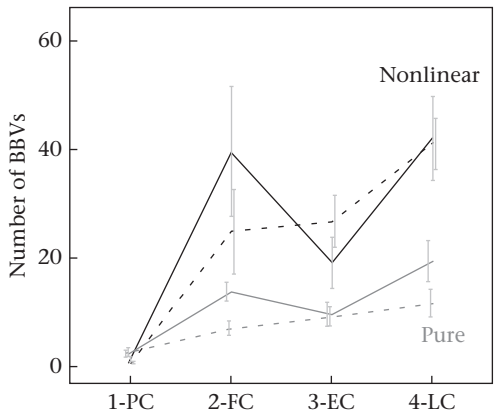

Figure 10. Broadband vocalizations (BBVs) emitted by copulating (solid lines, $N = 14$) versus noncopulating mice (dotted lines, $N = 22$) over time and four courtship phases (1-PC to 4-LC). We separated BBVs into purely harmonic (grey lines) and nonlinear (black lines) classes. Means ± SEM are shown for each of four 10 min recordings.

Vocal Dynamics During Sexual Behaviour

We conducted more detailed analyses of male sexual behaviour and vocalizations of all pairs at the 4-LC stage (Fig. 1d and e: 4-LCM and 4-LCN). There were many failed mating attempts among copulating males that successfully ejaculated (54 ± 29% of the attempts to mate failed in this group) and the noncopulating mice that never ejaculated. We used finer scale, second-by-second temporal analyses to compare the vocalizations associated with copulatory behaviour (intromissions with and without ejaculation) versus unsuccessful mating attempts (without copulation).

We found an extremely close temporal association between male behaviour and vocalizations. USV numbers positively correlated with rates of male sexual behaviour (GLM: $F_{1,33} = 13.29$, $P < 0.001$), and the correlation slope was stronger among the mice that mated (copulating versus noncopulating pairs: $t = -3.09$, $P = 0.004$; Fig. 11a). The majority of USVs were associated with copulations among those pairs, and mice simultaneously emitted USVs during all observed copulations. Both groups also emitted USVs during nearly all (98%) unsuccessful mating attempts. Mice increased vocalization rates directly before the males' attempts to mount, and we detected USVs in the seconds before all copulations as well as 95% of failed mating attempts.

We further examined USV syllable composition in the context of male sexual behaviour by comparing USVs emitted in the seconds before and during copulation, a failed mating attempt, and in a nonsexual context (i.e. in the absence of any sexual behaviour). We found significant differences in the USV types emitted in the different contexts (ANOSIM: $R = 0.066$, $N = 36$, $P = 0.001$; Fig. 11b and c). Mice emitted significantly more complex 'h' calls just preceding copulations compared with failed mating attempts (prior to and during). During copulatory mounts, USVs with one frequency jump ('c2') and 's' syllables were more common when compared with other contexts of sexual behaviour. The complex 'c' and 'h' and simple 'ui' calls appeared more often in the seconds before unsuccessful mating attempts compared with after their initiation. 'Un' calls and all simple USVs (except for 'ui') were more likely to be emitted during nonsexual periods than in contexts of sexual behaviour (successful or unsuccessful).

We tested whether USV spectrographic characteristics were associated with male mating behaviours and found that they differed significantly between behavioural contexts (GLMM: length: $\chi^2_4 = 126.7$, $P < 0.001$; mean amplitude: $\chi^2_4 = 62.44$, $P < 0.001$; frequency bandwidth: $\chi^2_4 = 120.76$, $P < 0.001$; mean frequency: $\chi^2_4 = 19.19$, $P < 0.001$; minimum frequency: $\chi^2_4 = 48.95$, $P < 0.001$; maximum frequency: $\chi^2_4 = 35.54$, $P < 0.001$; ISI: $\chi^2_4 = 235.35$, $P < 0.001$; Fig. 12a–d, Table A3, Fig. A5). The mice emitted USVs that increased in length (duration), mean amplitude, frequency bandwidth and maximum frequency right before they exhibited sexual behaviour (for unsuccessful mating attempts and copulations compared with nonsexual periods; post hoc: all $P < 0.001$). Upon the onset of copulatory mounts, USV length, mean amplitude and frequency bandwidth decreased again (post hoc: all $P < 0.001$; see also Fig. 12e for the changes in USV length in the

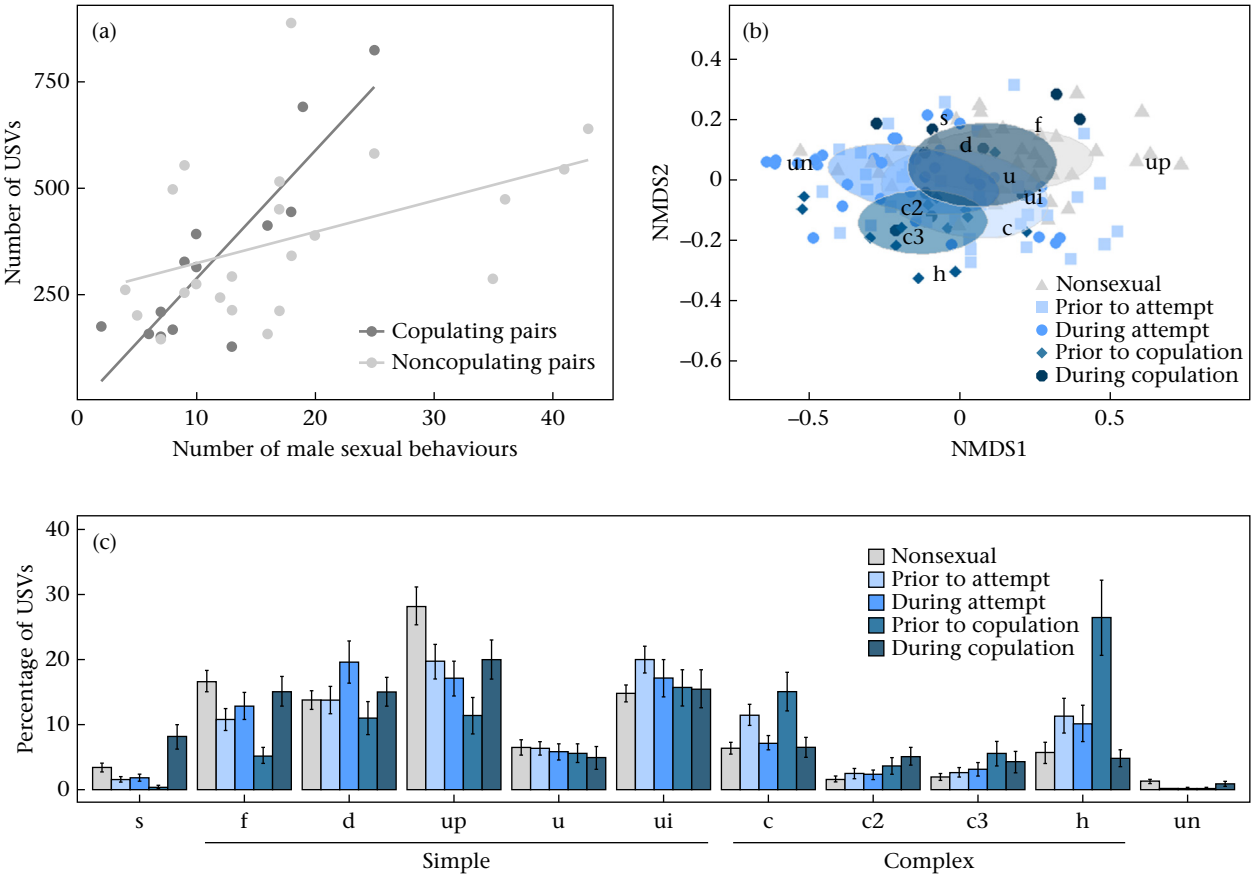

Figure 11. Male sexual behaviours and ultrasonic vocalizations (USVs) emitted at the 4-LC stage ($N = 36$ pairs). (a) Relationship between the rate of male sexual behaviours, including mating attempts and copulations, and USV emission in copulating ($N = 14$) and noncopulating pairs ($N = 22$). Syllable types emitted during different contexts of male sexual behaviour are presented as (b) NMDS plot of the composition of USV syllable types (stress value = 0.15) and (c) bar plot of the percentages (means ± SEM) of the 11 syllable types. Mating attempts and copulations were separated into periods directly before and after their initiation and compared with nonsexual periods. Proportions account for different rates of mating attempts and copulations. The numbers and rates/s for each syllable type are shown in Fig. A4.

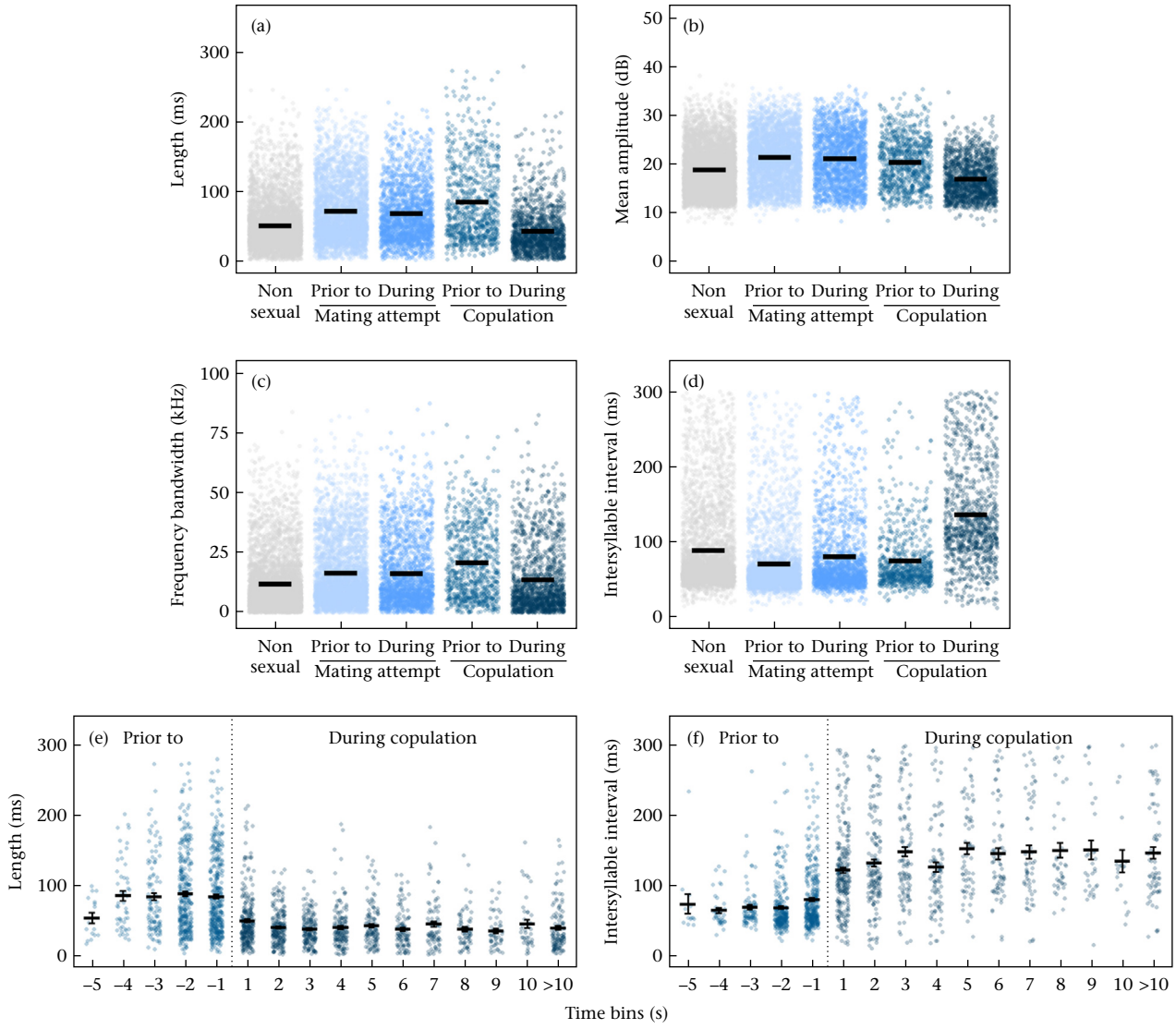

Figure 12. Spectrotemporal features of ultrasonic vocalizations (USVs) associated with male sexual behaviour, showing (a) USV length (duration), (b) mean amplitude, (c) frequency bandwidth and (d) intersyllable intervals (ISI). Male mating attempts and copulations were separated into the periods just prior to and during the behaviour and compared to nonsexual time periods (10 min recordings in 36 pairs). Dynamics of (e) USV length and (f) ISI in 1 s time bins over the 5 s before copulations and during copulatory mounts. Points represent raw data, and horizontal bars represent means ± SEM. For small SEM the error bars are not visible.

seconds prior to and during copulations). Minimum USV frequency also dropped just before copulations, and once a male successfully mounted, syllables increased in their minimum and mean frequency (post hoc: all $P \leq 0.006$). USVs emitted in the seconds preceding copulations (versus those preceding failed mating attempts) showed longer durations and frequency bandwidths as well as a lower minimum frequency (post hoc: length/bandwidth: $P < 0.001$; minimum frequency: $P = 0.039$). During mating attempts, USVs decreased in length after their initiation compared with the seconds right before the event (post hoc: $P = 0.016$), without changes in other spectral features. Shorter ISIs were found in the seconds preceding a mating attempt (compared with nonsexual time periods) and intervals increased again with the start of this behaviour (post hoc: all $P < 0.001$). ISIs were particularly long during copulatory mounts compared with all other measured contexts (post hoc: all $P < 0.001$; see Fig. 12f for the changes in ISI directly before and during copulations). Overall, noncopulating pairs emitted USVs at significantly higher amplitudes than copulating pairs (GLMM: $\chi^2_1 = 7.72$, $P = 0.005$).

Mice emitted few MFVs at the 4-LC stage (Fig. A2d), but when they occurred, they were usually emitted during male sexual behaviour (63 ± 45% of MFVs per pair emitted in the 5 s around and during the behaviour). Emission of BBVs was even more closely associated with male (unsuccessful and successful) mating behaviour (74 ± 19% of BBVs per pair). There were no differences in the emission patterns of the two BBV classes in connection with male sexual behaviour, and we subsequently pooled their numbers for analyses. BBV numbers correlated positively with numbers of male sexual behaviours, although this trend was not statistically significant (GLM: $F_{1,33} = 3.78$, $P = 0.06$). As with USVs, whenever copulations were observed, they were accompanied by many BBVs; however, there were no differences in the overall number of BBVs between copulating and noncopulating pairs at this courtship stage (Fig. 10). BBV emission was not correlated with occurrences of defensive behaviours by the female (GLM: $F_{1,33} = 2.05$, $P = 0.161$), contrary to what we expected, and we found a positive correlation between the number of times that females approached the male and BBV emission (GLM: $F_{1,33} = 6.79$, $P = 0.014$).

We further investigated whether the precise 'timing' of vocal emission predicted copulatory success by examining the 5 s period preceding copulations versus failed attempts to mount the female. We found significant differences in the distribution of USVs emitted shortly (five 1 s time bins) before the behaviour (GLMM: $\chi^2_4 = 13.68$, $P = 0.008$), which showed that when mice began calling at higher rates earlier (at −2 s; post hoc: $P = 0.005$), this was more likely to result in copulation (Fig. 13a). Additionally, we found that the duration of vocalization sequences (bouts) that started before and continued until initiation of the behaviour was longer for copulations compared with failed mating attempts (1.9 ± 1.1 s between vocalization start and onset of copulation; 1.4 ± 1 s interval for mating attempts; LMM: $\chi^2_1 = 4.53$, $P = 0.033$). BBVs emitted in the 5 s leading to copulations versus unsuccessful attempts also differed in their distributions over the five 1 s time bins (GLMM: $\chi^2_4 = 52.45$, $P < 0.001$). More BBVs occurred at earlier time points before copulations (at −4 and −3 s), whereas we observed more BBVs 1 s before a failed mating attempt (post hoc: all $P \leq 0.026$; Fig. 13b).

During copulatory mounts, the mice continued to call: USV rates decreased over time, whereas BBVs first decreased and then increased towards the termination of mating (Fig. 13c). USV sequences always ended before or at the time of dismount. After a short pause, mice resumed vocalizing (36 ± 39 s interval between dismount and first USV following dismount; not including ejaculations that are followed by a longer break in USV emission; see Vocal Dynamics Before, During and After Copulation and Ejaculation).

We found no temporal overlaps of more than one USV in the spectrograms, but we found many overlaps between USVs and BBVs. The proportion of BBVs overlapping with USVs was significantly higher at the 4-LC stage than the preceding stage in cage 2 (3-EC: 10 ± 16% of BBVs overlapping with USVs per pair; 4-LC: 60 ± 21%; GLMM: $\chi^2_1 = 118.64$, $P < 0.001$) and no differences in the proportion of overlaps between copulating versus noncopulating pairs were found ($\chi^2_1 = 2.16$, $P = 0.142$). The large numbers of USVs and BBVs during male sexual behaviour may have increased the probability of temporal overlaps only by chance. However, using the 'Song Overlap Null Model Generator' tool (Masco et al., 2016), we observed that the number of BBVs that overlapped with USVs was greater than chance expectations in 35 of 36 pairs. The proportion of BBV/USV overlaps also differed significantly between the behavioural contexts of their emission (LMM: $\chi^2_4 = 74.35$, $P < 0.001$; Fig. 14a, Table A4). More specifically, as expected, higher proportional overlaps were observed within 5 s before an (unsuccessful or successful) attempt to mount, as USVs and BBVs increased in rates compared to nonsexual time periods (post hoc: all $P \leq 0.005$). However, we found that fewer BBVs overlapped with USVs during copulatory mounts, although pairs continued to vocalize (post hoc: all $P < 0.001$). Fig. 14b shows the distribution of timing in the initiation of all USVs within 5 s of each BBV. The highest probability in the relative timing of USVs was just prior (145 ms) to the start of BBV emission. For temporal overlaps between the two vocalization types, significantly more USVs preceded BBVs in time than vice versa (Wilcoxon signed-ranks test: $V = 562$, $N = 36$, $P < 0.001$).

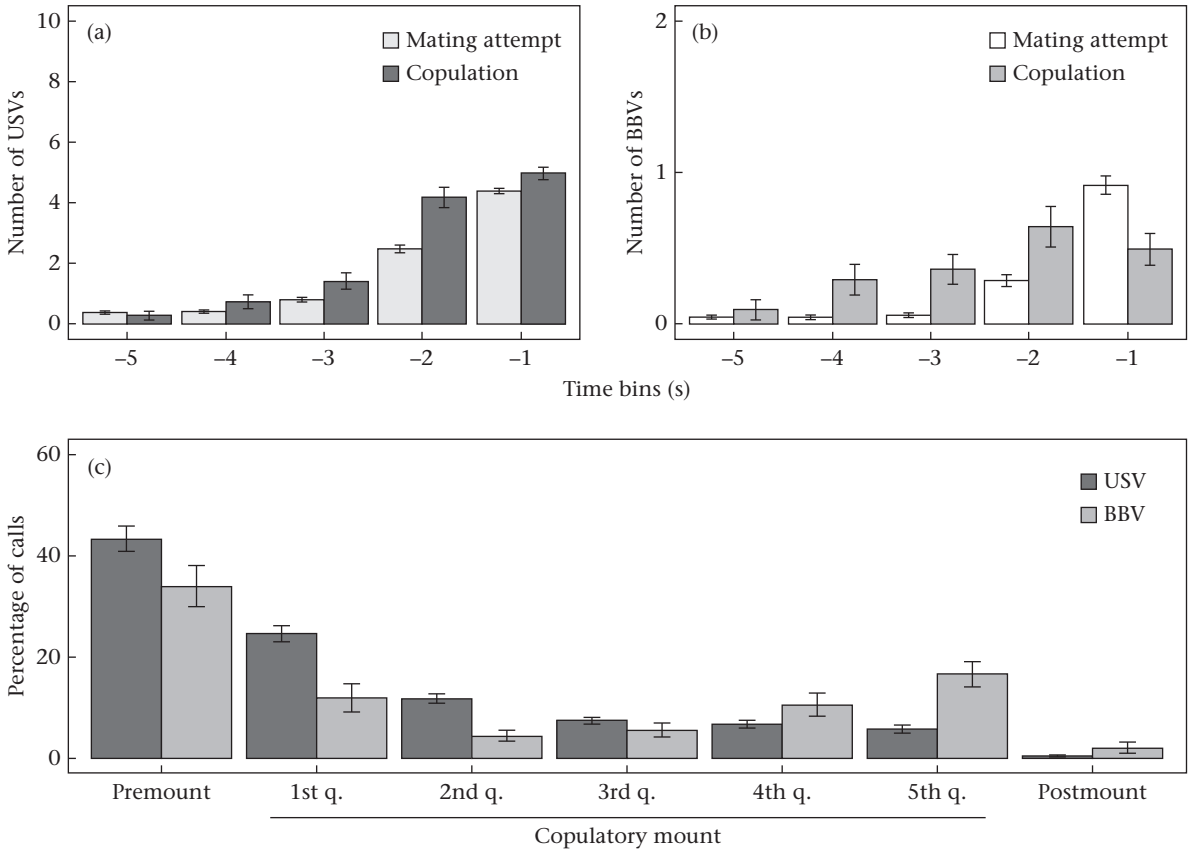

Figure 13. Vocalizations emitted in the seconds before male sexual behaviour, showing numbers of (a) ultrasonic (USVs) and (b) broadband (BBVs) vocalizations over five 1 s time bins relative to the start of a copulation versus failed mating attempt ($N = 36$ pairs). (c) Emission of USVs and BBVs associated with copulations ($N = 14$ pairs), shown as the percentage of calls emitted during the 5 s period before female mounting (premount), during copulation (copulatory mount) and in the 5 s following copulatory dismount (postmount). Vocalizations during the copulatory mount were calculated by dividing the total duration of each mount into five equal parts (quintiles (=q.); mean duration of mounts was 16 ± 8 s). Means ± SEM are shown.

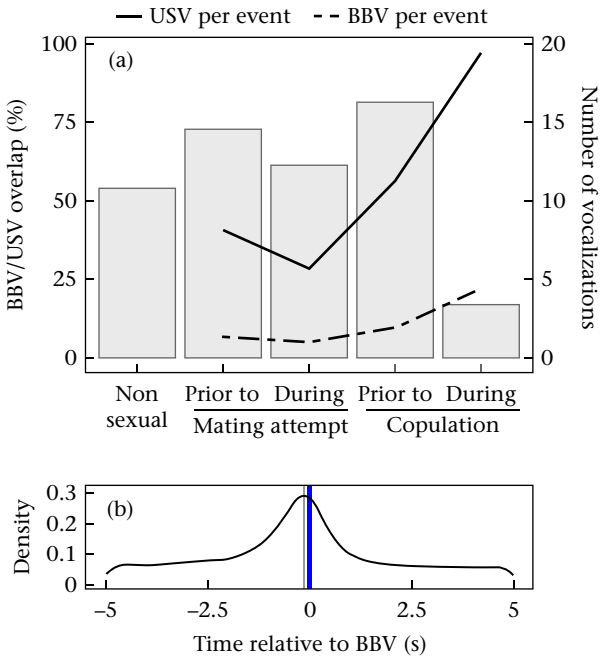

Figure 14. Synchrony of ultrasonic (USVs) and broadband (BBVs) vocalizations emitted during sexual and nonsexual behaviours, showing (a) percentage of BBV/USV overlap per behavioural context: during nonsexual periods and in the 5 s before and during mating attempts and copulations. Bars show the mean percentage of BBVs overlapping with USVs, and lines show the mean number of USVs (solid line) and BBVs (dotted line) measured in the respective context during a total of 476 mating attempts and 75 copulations in 36 pairs. (b) Density plot of time intervals between USV versus BBV emission (calculated for all USVs emitted within 5 s to each BBV). The blue vertical line indicates the beginning of BBVs, and the grey vertical line indicates the probability peak of the relative timing of USVs.

DISCUSSION

We recorded each pair of mice over approximately 40 h and analysed their vocalizations and behaviour during different stages of courtship and mating for 40 to 50 min in total. Our main findings include the following. First, the courtship vocalizations of the mice were surprisingly complex compared with previous studies, as they showed more dynamic changes and also changes in dynamics (changes in the changes themselves; Figs. 3–6, Fig. A1b). The pairs produced distinctive vocal repertoires and compositions during each phase of courtship, and their calls became increasingly complex over time, especially once males began female mounting. Second, late in courtship, the emission of USVs and BBVs was closely timed with male sexual behaviour, peaking in rates and vocal complexity just before males attempted to mount the female (Figs. 11–13). Moreover, in the seconds before a male's approach to mount, the emission of USVs and BBVs became closely synchronized (Fig. 14), suggesting that males and females duet, as in some songbirds. Third, ca. 40% of males successfully copulated during the study (with at least one ejaculation), and we found several differences between the vocalizations of copulating and noncopulating pairs, including some that predicted subsequent copulatory success (Figs. 8–10, 11a). The number of male sexual behaviours within recordings was positively correlated with USV emission, and this relationship was stronger for copulating mice than for noncopulating mice. This finding suggests that the influence of male sexual behaviour on copulatory success depends on USV emission, and vice versa. Fourth, we found that the vocalizations emitted just seconds before a male's successful attempt to copulate differed in timing and in certain acoustic features from those that preceded unsuccessful mounting attempts (Figs. 12 and 13). Finally, after ejaculation, the mice briefly paused and then resumed vocalizing but were emitting mostly simple calls, as during the early stages of courtship.

Our results provide the first analyses of types of courtship vocalizations emitted by house mice during the main stages of courtship and mating (including before, during and after copulation and ejaculation). They also provide the first evidence of courtship vocalizations in house mice that correlate with and predict male copulatory success (ejaculation), consistent with previous reports that USVs influence male reproductive success (Nicolakis et al., 2020). Although our results provide correlational evidence for these candidate vocalizations, further experimental tests are required to make inferences about causation. However, such experiments will be challenging because our results also suggest that courtship in house mice involves a dynamic vocal interplay between the sexes (bidirectional and multistep 'copulatory dialogues'; Rodríguez et al., 2012), which complicates experimental approaches. We address our findings in greater detail in the subsequent sections.

Overall Vocalizations

Of the ca. 53 000 vocalizations, 87% were USVs, which were classified into 11 syllable types. We confirmed that mice emitted higher levels of syllable type diversity when they vocalized at high rates, at least until they reached the complete repertoire (Fig. A1a; Nicolakis et al., 2020). We found that the numbers of most (9/11) syllable types were intercorrelated, and we identified a hierarchical pattern in their correlations (Fig. 3a). These results indicate differences in the usage of the four major classes of USVs (simple, complex, 's' and 'un'), and therefore, their emissions may have different functions. These results also seem to suggest that the 11 USVs could be merged into four syllable types (Abbasi et al., 2022). However, by limiting the analyses to these major classes, we would have failed to detect significant differences within these classes because the intercorrelations changed with the stage of courtship and in the context of male sexual behaviour. Moreover, because only certain USVs within the major classes were associated with male copulatory success, they may differ in their signalling functions. The mice also emitted BBVs and MFVs (10% and 3% of calls, respectively), which were negatively correlated, and similar to USVs, the emission of sonic calls depended on the stage of courtship and sexual behaviour. Owing to these intercorrelations, we used univariate and multivariate analyses.

We did not identify the vocalizer within pairs; however, this limitation should not affect our conclusions, as they do not necessarily depend on which sex produced the vocalizations. Previous studies on male laboratory mice found that males emit the vast majority of USVs during opposite-sex interactions (Heckman et al., 2017; Neunuebel et al., 2015; Sangiamo et al., 2020; Sterling et al., 2023; Wang et al., 2008; Warren et al., 2018; Whitney et al., 1973). In line with these results, we found that overlaps between two USVs in a spectrogram were rare and most occurred only during the first recording stage (1-PC). All these examples included partial overlaps with 'un' USVs, likely because these calls have ambiguous boundaries in the spectrograms. No temporal overlaps between adjacent USVs were observed in the recordings at the 4-LC stage when mice vocalized at their highest rates. BBVs have been suggested to be produced mainly by females (Grimsley et al., 2013; Lupanova & Egorova, 2015), although definitive analyses have not been conducted to our knowledge. We found many overlaps between USVs and BBVs just prior to female mounting, indicating that both sexes vocalize during this behaviour (see more below). MFVs have not yet been thoroughly investigated during sexual interactions in mice. However, there are several reasons to suspect

that MFVs are emitted by males: MFV and USV emissions were highly correlated, as previously reported (Zala et al., 2020), although only before the pairs' contact (1-PC stage); male sexual priming increases MFV and USV emissions (Zala et al., 2020); and temporal overlaps between USVs and MFVs were rare in our study. Further studies are needed to determine which sex emits MFVs, BBVs and USVs, not only during initial interactions but at all stages of courtship and mating.

Vocal Dynamics During the Introduction

The mice emitted specific USVs and sonic calls during the first two phases of their introduction (Figs. 3b, 4 and 5). Initially, while they were still separated by the perforated divider (1-PC phase), the mice mainly emitted 'up', 's' and 'un' USVs, and then, upon direct contact (2-FC), they increased USV numbers and syllable types. There was a major shift in the USV composition with an increased emission of all five simple types, particularly 'up' and two complex ('c' and 'h') types, while the number of 'un' USVs declined. These results support several findings from previous studies on the initial sexual interactions in mice. Wild male mice emit high proportions of 's' and 'un' USVs before contacting a female or her urine and when they are not sexually primed. In response to these sexual stimuli, they reduce 's' and 'un' emission and increase numbers of 'up' and other simple USVs (Marconi et al., 2020; Nicolakis et al., 2020; Zala et al., 2020). Similar patterns have been found in laboratory mice: 'un' calls are produced especially by low vocalizers (Scattoni et al., 2011) and singly housed mice (Grimsley et al., 2016). During the first minute of sexual encounters, laboratory mice also mainly produce 'up' and 's' calls (<60 ms in duration) and then begin to emit longer USVs over time (Matsumoto & Okanoya, 2016, 2018). After the female is removed from the male's cage, males produce more 's' calls (Yang et al., 2013) and decrease the duration of other USVs (Hanson & Hurley, 2012). The functions of shifting from emitting 's' and 'un' calls to more 'up' and other simple and longer USVs are unclear, but these changes may signal a male's intention to mate (Zala et al., 2020).

When the mice began to interact directly, their USVs also changed in spectrotemporal features, showing an overall increase in length and a decrease in frequency (Hz), including the emission of fewer ultra-high calls (Fig. 9). Male laboratory mice emit USVs at lower frequencies during direct male–female interactions than upon exposure to a female scent (Lupanova & Egorova, 2015), and they increase their dominant frequency after the female is removed (Hanson & Hurley, 2012). It is unclear why mice reduced the frequency of most vocalizations upon coming into close contact, especially because lower sound frequencies travel further distances. In the present study, many of the broadband 'un' USVs initially emitted by the mice reached the sonic range, suggesting that the mice also used a wider spectrum of frequencies while separated, whereas once they made direct physical contact, they greatly reduced the emission of these calls (they reduced 'un' calls overall during 2-FC and especially those extending into very low frequencies).

Females' initial oestrous stage did not influence USV emission during the first stages of courtship, although we previously observed that males produced a larger USV repertoire and higher mean frequencies when presented with a nonoestrous versus an oestrous female (Nicolakis et al., 2020). Several studies on laboratory mice found no effects of female oestrous state on male USV emission (Gaub et al., 2016; Keesom & Hurley, 2016; Kim et al., 2016; Pomerantz et al., 1983), although one reported higher USV rates among males paired with nonoestrous versus oestrous females (Barthelemy et al., 2004), whereas another reported that USVs were lower in their dominant frequency and highest in duration and bandwidth when females were in pro-oestrus compared with other stages (Hanson & Hurley, 2012). Previous findings are inconsistent, but only physiological oestrus (which is not equivalent to sexual receptivity) has been investigated. Further studies are needed to determine whether female behaviour (sexual receptivity, such as approach and lordosis) influences male vocalizations and courtship behaviour, as well as vice versa (sexual interaction hypothesis; Rodríguez et al., 2012; Sullivan-Beckers & Hebets, 2014). There is recent evidence that female vocalization influences male courtship behaviour and vocalization (Finton et al., 2017; Hood et al., 2023), as we address next.

The mice in our study altered the emission of sonic calls during introduction, and MFVs and BBVs exhibited the opposite patterns. During 1-PC, they emitted many MFVs, and then during 2-FC, they reduced the MFVs and increased the emission of BBVs, particularly those with spectral nonlinearities (Fig. 5a and b). Interestingly, sonic call emission, unlike USVs, depends on the female's oestrous stage: pairs with oestrous females emitted fewer MFVs during the first 20 min of introduction and fewer BBVs before direct contact with the male (Fig. 5c and d). BBVs are suspected to be emitted mainly by females, and it has been assumed that they help repel unwanted mounting attempts by courting males (Barthelemy et al., 2004; Finton et al., 2017; Sugimoto et al., 2011). Therefore, we expected oestrous females to show fewer defensive behaviours and BBVs during sexual investigations by males. Because it was previously shown that nonoestrous females show a higher interest in USV playbacks (Beck et al., 2023), another possible explanation could be that oestrous females spent less time directly at the divider, thereby eliciting fewer male vocalizations and producing fewer BBVs (which at this stage are usually emitted when the male is inspecting the female through the perforated divider). Although female oestrous state has not been found to affect male rejection, such as kicking the male or squeaking (Keesom & Hurley, 2016), it has been shown that females in the oestrous stage produce BBVs with a longer duration of nonlinear segments than nonoestrous females during sexual interactions (Finton et al., 2017). In our study, once the divider was removed, pairs switched from primarily emitting pure to producing more and mainly nonlinear BBVs, but neither class of BBVs was affected by the female oestrous stage when the sexes could freely interact.

These results show that the vocalizations emitted while the mice were still separated (1-PC) were very distinctive compared to other phases of courtship (see next section and Fig. A1b), and they were more similar to the USVs emitted in response to female urinary scent (Marconi et al., 2020). These initial vocalizations might still be classified as 'courtship calls' because we found that they were influenced by female sexual receptivity (MFVs) and predicted later copulation ('s', ultra-high USVs, see more below).

Vocal Dynamics Before, During and After Copulation and Ejaculation

We compared the vocalizations of mice emitted during the early courtship period before any mountings (3-EC phase), the period just before and during ejaculation (4-LCM) and following ejaculation (5-PE), and focused on the 14/36 (39%) males that copulated until ejaculation. The oestrous state of the female during the introduction phase did not predict copulations or latency to copulate later in the recordings, which is not surprising because the mice did not copulate until a mean of 15 h later (and we did not evaluate oestrous state at this time).

Once the mice began mounting and copulating, they increased their USV emission, in particular all complex USVs ('c', 'c2', 'c3' and 'h'), and showed a highly diverse repertoire (11/14 pairs produced the complete repertoire; Fig. 6). This coincided with changes in several USV spectrotemporal features, i.e. length, mean amplitude

and frequency bandwidth increased, mean and minimum frequencies decreased, and ISIs became shorter (Fig. 7). They also increased the emission of BBVs, while the numbers of MFVs were negligible during all stages. After the male ejaculated, the mice briefly paused (mean 5 min) and then resumed vocalizing, but they returned to producing mainly simple USVs, as they had during the early phases of courtship, and they emitted fewer BBVs. All pairs eventually started copulating again (mean 30 min after ejaculation), and some males ejaculated a second time, whereas sexual behaviour was generally absent during the period immediately following ejaculation. Hence, there was a potential shift in the courtship function of the USVs emitted during this refractory period, and the observed lack of BBVs suggests a stronger association of this vocalization type with male sexual behaviour than we found for USVs.

To our knowledge, no studies have ever recorded or classified the types of vocalizations of wild house mice emitted during mating, and the few studies on laboratory mice reported similar but also disparate results (which could be due to differences in the behaviour of the mice, methods or both). An early study found that USVs (detected at 70 kHz) were emitted throughout courtship and mating, and then after ejaculation, the mice ceased vocalizing until minutes before the start of the next copulation (Nyby, 1983). The mice in our study also paused vocalizing after ejaculation, but then began calling again without showing any sexual behaviour. More recent studies found that laboratory mice shifted towards increased emission of 'h' and long USVs containing multiple jumps when males began mounting the female (Gaub et al., 2016; Hanson & Hurley, 2012; Matsumoto & Okanoya, 2016), and also produced more 'h' syllables over time when the mice were in contact (Keesom & Hurley, 2016). During male mounting, mice emitted USVs with longer durations, higher amplitudes and lower fundamental and minimum frequencies (Gaub et al., 2016; Matsumoto & Okanoya, 2016, 2018), and USVs occurred at shorter intervals during sniffing and mounting of females than during other sociosexual behaviours (Gaub et al., 2016). Note that male laboratory mice in these studies, unlike wild mice, quickly engaged in female mounting within brief periods of sexual contact (<10 min, especially if prescreened for showing sexual behaviour or hormonally manipulated), and their calls appear to share many similarities with vocalizations associated with copulatory behaviour of wild mice. However, the wild mice in our study increased their usage of all complex syllable types during the mating sequence (versus only one type, 'h' syllables, in laboratory mice), indicative of an overall more distinctive change in their syllable type composition. Moreover, the only study that investigated changes in USV spectrotemporal features in relation to male ejaculation in laboratory mice (a hybrid strain) found no such evidence (White et al., 1998), suggesting that wild house mice also produce a richer repertoire or spectral complexity in their calls during mating than previously described in domesticated strains. BBV emission in laboratory mice was found to increase over time during opposite-sex interactions, and especially when males attempt (Grimsley et al., 2013; Sales, 1972) or begin mounting the female (Finton et al., 2017), as with wild mice; however, studies on BBVs during late courtship and mating are lacking in laboratory mice.

In summary, we found that during the early 'precopulatory period' of courtship (Dewsbury, 1988; Pomerantz & Clemens, 1981), the mice began emitting USVs upon detecting a female, and when the sexes made direct contact, they increased the number of all simple types of USVs, replicating our previous results (Nicolakis et al., 2020). The initial early courtship or appetitive phase of sexual behaviour involves sniffing and investigating a potential sexual partner (Wei et al., 2021). Then, during the consummatory or 'copulatory period', when mounting was common, the mice shifted their vocal repertoire and emitted all types of complex USVs and more BBVs. We also found tight synchrony or duetting in vocal emission just before mounting attempts and copulation, as we address below. Following ejaculation ('postejaculatory period'), marking the end of the consummatory phase, the mice produced USVs that were similar in rates, composition and spectral features to those in the early stages of courtship. Of all the analysed spectral features, USV length (duration) was the most highly modulated; the duration of USVs not only showed prominent changes with each courtship phase, but it was also rapidly adjusted as the mice performed sexual behaviour (see below). Our findings here and in the next section are consistent with the hypothesis that courtship vocalizations provide a reliable index signal of male sexual arousal and affective state in house mice (Zala et al., 2020) and other rodents (Fernández-Vargas, 2018).

Rapid Changes in Vocalizations During Male Sexual Behaviour

We conducted additional analyses of male sexual behaviours (mating attempts, such as chasing females and attempting mounts, and actual copulations) and vocal emission at a finer temporal scale (second-by-second) during the late stage of courtship (4-LC). Males increased the rate of sexual behaviours over time, and although all males attempted to mount the female, most attempts were unsuccessful, regardless of whether the pairs eventually copulated and ejaculated or not (mounting attempts were never successful among noncopulating pairs at this stage, and they were only successful in 54% of attempts of copulating pairs). USVs were emitted during nearly all sexual interactions, and the rates of male sexual behaviours and USVs were positively correlated, particularly among pairs that copulated until ejaculation. We found an extremely close temporal association between male sexual behaviour and the emission of USVs and BBVs (see more below).

During male sexual behaviours, they modulated their vocalizations very rapidly and dynamically shifted the usage of certain syllable types and spectral features before approaching and mounting a female. USV sequences preceding male sexual behaviour contained high proportions of complex syllables, and the emission of the 'h' type was in particular associated with copulations (intromissions with or without ejaculation; Fig. 11b and c). Approximately 26% of the USVs emitted during the seconds before copulations were 'h' calls, which is comparable to laboratory mice (up to 30% were associated with male mountings; Gaub et al., 2016; Hanson & Hurley, 2012). Once males had begun a copulatory mount, we observed a shift from 'h' to 's' and one frequency jump ('c2') USVs. Mice also emitted USVs with unusually high spectral complexity (increased length, amplitude, frequency bandwidth and maximum frequency) and shortened intervals between USVs immediately preceding an approach to mount (Fig. 12). Before copulations, the males' USVs were found to be significantly longer (ms), broader (Hz) and lower in minimum frequencies (Hz) compared with when the attempt failed. As soon as the male achieved a copulatory mount, most of these spectral changes reversed, and their USVs again became shorter and decreased in amplitude and frequency range. Mice also altered the temporal pattern of USV emission, with particularly long ISIs during copulation.

Mice have been described to exhibit characteristic vocal repertoires in association with particular social behaviours (Sangiamo et al., 2020), and laboratory rats, *Rattus norvegicus*, were found to emit more complex USVs in the 5 s before intromissions than for mounts (Ågmo & Snoeren, 2015). Reports on laboratory strains suggest that mice emit mostly simple calls, such as flat syllables, in the seconds before female mounting (Wang et al., 2008), and then switch to emitting longer and more complex calls with frequency

steps and harmonics during the copulatory mount (Barthelemy et al., 2004; Sales, 1972; Wang et al., 2008). However, except for the more detailed descriptions by Sales (1972), these reports were mostly anecdotal. We observed that the 'h' syllables, which were emitted at high numbers before copulations, had several distinctive spectral features: the vast majority contained jumps in frequency (and 89% had two jumps), they were emitted at the highest amplitude and lowest mean and minimum frequency among all syllables, and they were longer in duration than most other syllables. Our results are consistent with descriptions of 'h' syllables associated with male mounting in laboratory mice, which had the longest duration and lowest fundamental frequency among all USV types (Matsumoto & Okanoya, 2016). Harmonic elements are generally more common among louder (dB) USVs, such as those emitted during male mounting or attempts, and the minimum frequency of USVs is lower in the presence of frequency jumps, as previously reported (Gaub et al., 2016). The 'h' and multijump USVs of long duration have been shown to share many structural similarities and to correlate with changes over distinct phases of courtship (Matsumoto & Okanoya, 2016, 2018). Hence, our findings are consistent with experimental evidence showing that USVs facilitate male mounting success compared with devocalized males (Nomoto et al., 2018). Furthermore, they extend previous observations of a close temporal association between some syllable types and parameters with certain aspects of male sexual behaviour (Hanson & Hurley, 2012; Matsumoto & Okanoya, 2016). Moreover, we found novel evidence that short-term adjustments in the complexity of call features in the seconds before mounting are associated with subsequent copulatory success in males. Notably, we showed that the particular vocal parameters proposed to increase female attraction towards males in previous studies (i.e. complex, and especially 'h', syllables, and calls of long duration) were exhibited directly before males successfully mounted the female and copulated. Calls emitted just before mice initiate copulations provide the best candidates for persuasive signals influencing male mating outcomes. Nevertheless, these results are observational, and future studies are needed to experimentally test the effects of these candidates on male copulatory success.

We conducted further detailed analyses of the precise timing of the vocalizations emitted in the seconds directly before the males attempted to mount. We found that the mice invariably increased USV rates in the seconds just prior to any sexual behaviour, and USV sequences or bouts began earlier when a copulation was initiated (with or without ejaculation) compared to unsuccessful mounting attempts (Fig. 13a). Recently developed methods that enable the identification of the vocalizing individual within a pair or group of mice found that USV emission is closely associated with males chasing females, and USVs are predominantly emitted when a male is positioned behind a female or with its snout directed towards the female's anogenital region (Neunuebel et al., 2015; Oliveira-Stahl et al., 2023). Male laboratory rats also increase USV emission before an intromission or lordosis (Ågmo & Snoeren, 2015) and emit more vocalizations before an intromission than before a mount (Barfield et al., 1979). It is unclear why males increase USV emission just seconds before attempting a mount, but perhaps it provides a signal to telegraph their next behaviour. Male USVs may function as appeasement signals of male sexual motivation to females, which prevent or de-escalate female aggressive responses towards males (Musolf & Penn, 2012). Such appeasement may also help to coordinate male and female mating behaviour and induce female lordosis (Barfield et al., 1979). In rats, displays of female solicitation and lordosis behaviour increase in the presence of USVs, and female responses closely follow fluctuations in the periodic emissions of these vocalizations (McIntosh et al., 1978).

We found that BBV emission was closely associated with male sexual behaviour and their rates were positively correlated, although this trend was not statistically significant ($P = 0.06$). Unexpectedly, the number of these calls did not correlate with male-directed rejection behaviour. Successful and unsuccessful attempts to mate were accompanied by many BBVs; therefore, these vocalizations did not appear to deter male sexual behaviour. Females in our study also initiated many interactions by first approaching males, and surprisingly, the number of active female approaches were positively correlated with BBVs. In rodents, female approach is a type of proceptive behaviour, as during the mating sequence females repeatedly approach and retreat from the male, thereby driving the timing of intromissions (Garey et al., 2002; D.P., personal observation). Female mice are also more likely to exhibit lordosis when they actively approach the male prior to mating (Tomihara & Makino, 1991), whereas males mount unsuccessfully more frequently if females are prevented from controlling copulation by escaping from the male (Johansen et al., 2008). It has been suggested that in small cages, females use BBVs to repel males in the absence of opportunities to retreat from the male (Finton et al., 2017). A closer inspection of the 5 s period directly preceding the males' mating behaviour in our study revealed that females first emitted more BBVs before copulations, but then the emission declined and they produced more BBVs in the final second when the attempt failed (Fig. 13b). This suggests that differences in the timing of BBVs (and USVs) in the seconds before male behaviour could influence the outcome. For example, if a male intensifies USV emission in response to female BBVs, this might enhance the chance of a successful mount by inhibiting female withdrawal or inducing more female solicitation behaviour in response. However, the signalling function of BBVs may also completely change depending upon the context and association with other behaviours (e.g. female BBVs emitted with a kick towards the male could signal rejection to a male, whereas in another context or behaviour (lordosis), females may use the same vocal cues to direct the males' actions and establish a successful mount).

During the 5 s preceding mating behaviour, USV and BBV emissions increased and became synchronized (Fig. 14), as observed in duetting songbirds. A previous study found that USVs and BBVs of laboratory mice were closely timed before mounting, suggesting that the USVs were produced by males and BBVs by females (Finton et al., 2017). We found that USVs usually preceded BBVs in time (60% of temporal overlaps), indicating that females called in response to male vocalizations rather than vice versa. The functions of duetting are generally unclear, although several hypotheses have been proposed for birds (Hall, 2004) and nonhuman primates (De Gregorio et al., 2024). Duetting requires close attention to the vocal behaviour of a mate, and its functions may include signalling willingness to cooperate, stimulating and synchronizing copulatory behaviour between the sexes. However, we found that vocal synchrony occurred during copulations and failed mating attempts; thus, its function is unclear.

Once males had mounted the female and began copulating, the mice continued to vocalize, but USV rates decreased during the copulation and always ceased before dismounting (Fig. 13c). Similarly, USVs in laboratory mice have been described as becoming less frequent as the mice continued to copulate (Barthelemy et al., 2004; White et al., 1998). We found that BBVs declined with the onset of copulation but increased again towards the end of mating. Potentially driven by these differences in emission patterns, there were reduced overlaps in the timing of the two vocalization types when males mounted on the female. It is also possible that this change in synchrony functions to coordinate the termination of mating.

Vocalizations of Copulating Versus Noncopulating Mice

We found several differences between the vocalizations of the pairs that successfully copulated (with male ejaculation) and noncopulating mice, and some types of USVs emitted during the early stages of courtship predicted whether mice would copulate or not in the next few days. (1) Copulating mice produced more 's' USVs during the first 10 min of their introduction in cage 1, before direct contact of the sexes (1-PC phase; Fig. 8a). A study on laboratory mice reported that the usage of a wider range of different USV durations in the first minute of a male–female encounter predicted whether mounting occurred later in the 10 min recording (Matsumoto & Okanoya, 2016). (2) Copulating mice also emitted more ultra-high frequency USVs (many of which were of short duration), and their USVs overall had higher frequencies (Hz) at the 1-PC stage (Fig. 9d–f). Nicolakis et al. (2020) also found that opposite-sex pairs of wild house mice that emit higher frequency USVs while still being separated by a divider later had shorter latencies to produce their first litter. Wild male mice emit USVs at higher frequencies during opposite-sex interactions compared with same-sex interactions (Zala et al., 2017a), and BALB/c males produce USVs that reach higher frequencies when exposed to females than when exposed to male soiled bedding (Gourbal et al., 2004). A specific type of USV characterized by high frequency and short duration has been described as occurring more often in the vocal repertoire of testosterone-treated male mice than in controls (Kikusui et al., 2021). (3) Copulating mice emitted USVs spaced out with longer intervals within bouts (ISI) than noncopulating pairs (Fig. 9e). ISIs (< 300 ms) decreased in all pairs over time and courtship phases, whereas the intervals between bouts (> 300 ms) remained unchanged. This result indicates that the timing of USV emission within, rather than between, bouts reflects the males' motivational state or sexual arousal. Our results are consistent with evidence that mice modulate the break duration between USVs in connection with certain behaviours (Gaub et al., 2016) and that females preferentially approach recordings of male courtship USVs with intact temporal regularity over those with artificially interrupted pauses between USVs (Perrodin et al., 2023). (4) USV emission correlated with male sexual behaviours, and this relationship was stronger for pairs that successfully copulated than for noncopulating pairs (Fig. 11a). This suggests that higher rates of overall USV emission by themselves are not sufficient to influence mating success, but that copulatory success is enhanced by an increased USV emission along with sexual behaviours. (5) Pairs that successfully copulated emitted more purely harmonic BBVs overall (Fig. 10). The presence or absence of spectral nonlinearities in vocalizations potentially provides a cue or signal for a female's motivational or emotional state. Studies on a variety of species suggest that nonlinear structures indicate arousal, and although they are associated with positive as well as negative emotional states, they appear to be perceived by receivers as aversive (Anikin et al., 2020; reviewed in Briefer, 2012). Our findings are consistent with experimental evidence (Nomoto et al., 2018), but like all correlations, inferences about causation should be treated with caution.

Several vocalizations have been hypothesized to facilitate male copulation, but we did not detect large effects or significant correlations for these candidates, including the following. (1) Pairs that successfully copulated appeared to emit more USVs than the other pairs during the first stages of courtship (Fig. A2a); however, these differences were not significant. (2) Copulating mice consistently had a larger repertoire of USV syllable types and more complex types than noncopulating pairs, as expected (Chabout et al., 2015; Hanson & Hurley, 2012; Matsumoto & Okanoya, 2016; Nicolakis et al., 2020); however, the results were marginally nonsignificant ($P = 0.052$ and $P = 0.059$, respectively). (3) Although the mice significantly altered their USV emission and composition over different phases of courtship, these changes were largely similar between pairs with successful copulations and unsuccessful pairs (e.g. our multivariate analyses detected no interactions with courtship phases). The mice generally increased USV diversity and their spectral complexity, displaying more USVs with rapid changes in frequencies, frequency jumps, harmonic elements or sidebands (overtones), over time, and these changes coincided with male sexual behaviours, regardless of whether the pair eventually copulated with ejaculation or not. (4) USV amplitudes did not differ (dB) overall, and on the contrary, noncopulating mice emitted USVs at higher amplitudes during the 4-LC stage. (5) The mice engaged in vocal synchrony, just before male mounting; however, vocal synchrony or duetting had no effect on whether males achieved copulations or not. (6) BBV emission did not appear to inhibit male courtship, mating attempts or copulatory success, contrary to what we expected (Hood et al., 2023). (7) We expected sexually receptive females to produce fewer BBVs, as low rates of BBVs emitted early during sexual interactions predicted male mounting in laboratory mice (Finton et al., 2017). Such discrepancies between our study and previous studies might be explained by the more rapidly initiated courtship by laboratory mice when encountering an unfamiliar female (McGill, 1962) than by wild house mice, as mentioned above. Wild males rarely engaged in mounting behaviour during the first hours of exposure to the female; thus, the context in which they emit vocalizations during the early stages of courtship may differ between laboratory and wild mice.

We would not rule out the influence of these candidate vocalizations on male mating success for several reasons, and we emphasize the following caveats and limitations with our study. (1) We may have failed to detect significant differences for vocal parameters that have only a small influence on mating success, and further studies are needed with larger sample sizes and analysis over a longer period of sexual interactions (>40 min). (2) Our results are overly conservative because we selected segments of recordings of noncopulating males to analyse during the time that they were most sexually active (at the 4-LC stage), and more differences might have been detected if we had used an unbiased sampling design. This conservative bias may explain why some of the apparent differences that we observed between the groups early in courtship reduced or disappeared in late courtship (e.g. number of 's', complex and overall USVs and USV repertoire). (3) We did not manipulate female oestrus (or sexual receptivity), and males may increase their courtship effort when females are not sexually receptive or resistant to mating, which could mask actual effects or generate correlations opposite to those that are expected (e.g. amplitude). (4) Male USVs may influence mating in ways that we might have missed (such as keeping females in proximity) because our study was conducted in cages, which are only a small fraction of the space that wild house mice utilize during courtship and mating in seminatural conditions (D.P., personal observation). Additionally, unreceptive females could not escape the male in cages, and the act of not fleeing may send a misleading signal to males, which then continue courting and emitting vocalizations that they would not produce in more natural contexts. (5) Vocalizations may not influence copulatory success in the laboratory if their functions in the wild include species recognition (Musolf et al., 2015), social recognition (Hoffman et al., 2012b; Marconi et al., 2020; Musolf et al., 2010), assessment of male social status (D'Amato, 1991) or other aspects of quality and compatibility (Lopes & König, 2016; Nicolakis et al., 2020). (6) The influence of males' calls on their mating success (at each stage, approach, mounting, copulation, etc.) may also depend upon individual females (and their particular preferences) or the pair's interactions (Nicolakis

et al., 2020) even if factors such as familiarity and kinship are controlled. (7) Male courtship vocalizations may have other effects on female sexual behaviour and physiology, such as inducing oestrous cycling (but see Wölfl et al., 2023) and long-term pair bonding (Pultorak et al., 2018), which we did not measure.

Conclusions and Future Directions

Our results show that the vocalizations emitted by wild house mice during courtship and mating are more complex and dynamic than has been assumed, and we found several vocalizations that correlated with male copulatory success. To our knowledge, this is the first study on the courtship vocalizations and mating success in house mice, wild or domesticated. We did not correct for multiple testing because we used multivariate tests for our main questions, we focused on candidate calls that were proposed to influence mating success in previous studies, and although we conducted exploratory analyses, many of our main results were highly significant. The differences in vocalizations and their timing between copulating and noncopulating pairs provide candidates that need to be tested in the future, such as in no-choice and free-mate choice experiments (Nomoto et al., 2018). Future studies are needed to identify which sex produces each vocalization during courtship and mating, and several methods now make it possible to identify callers in a group, and some allow pin-pointing the source of the signal when mice are within 5 mm of each other (Heckman et al., 2017; Sterling et al., 2023; Warren et al., 2018). Studies are also needed to manipulate the behaviour of both sexes (male courtship behaviour and female sexual receptivity) to determine whether any effects of male courtship vocalizations on mating success are due to a simple unidirectional influence on the female (Asaba et al., 2017) or whether they arise from more complex, bidirectional sexual interactions (e.g. males may adjust their courtship and vocalizations in response to behavioural and vocal cues from the female, and females' receptivity may depend on males' adjustments). Such experiments would make it possible to determine the functions of duetting in house mice, and several hypotheses have been proposed for other species (De Gregorio et al., 2024; Hall, 2004). Finally, studies are needed to record wild mice during courtship and mating in larger and more natural social contexts (e.g. see Fig. S1 in Luzynski et al., 2021), which poses a major technical challenge, especially for identifying individual vocalizers.

Author Contributions

Bettina Wernisch: Visualization, Methodology, Investigation. **Dustin J. Penn:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Funding acquisition, Conceptualization. **Sarah M. Zala:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Teresa Klaus:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Data Availability

The data sets generated and analysed in the current study are available through the Phaidra repository of the University of Veterinary Medicine Vienna (https://phaidra.vetmeduni.ac.at/detail/o:2807).

Declaration of Interest

The authors declare there are no conflicts of interest.

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Appendix: Age of Mice

We found no effects of male or female age on the number of USVs or BBVs during any courtship stage or behavioural context. The effects of female age on MFV emission were found over the two initial courtship phases, and MFVs were emitted less often among pairs with older females (GLMM: female age: $\chi^2_1 = 4.82$, $P = 0.028$). We found no effects of male or female age on the probability of copulating within the approximately 40 h of observation; both age classes of males and females were present in 14 pairs that successfully copulated with ejaculation (older males $N = 7$ and older females $N = 3$) and 22 pairs that did not copulate (older males $N = 15$ and older females $N = 3$). Age effects on the USV repertoire, USV frequency and ISI (<300 ms) were present in the cage 2 recordings (but not during the pairs' introduction in cage 1), as described in more detail below.

Vocal Dynamics Before, During and After Copulation and Ejaculation

Younger males had a larger USV repertoire than older males (GLMM: $\chi^2_1 = 4.15$, $P = 0.042$). The partners' age affected the maximum frequency of USVs, which was higher in pairs with younger males and females (GLMM: male age: $\chi^2_1 = 5.29$, $P = 0.021$; female age: $\chi^2_1 = 4.65$, $P = 0.031$). The USVs frequency bandwidth was larger in pairs with younger males (GLMM: $\chi^2_1 = 4.83$, $P = 0.028$). ISIs were significantly shorter in pairs with younger male than in pairs with older males (GLMM: $\chi^2_1 = 5.36$, $P = 0.021$).

Vocalizations of Copulating and Noncopulating Pairs

USVs with significantly larger frequency bandwidths were found in pairs with younger males and females than in older pairs. Pairs with younger males emitted calls at shorter ISIs than other pairs (Table A2).

Vocal Dynamics During Sexual Behaviour

The mean and maximum frequencies of USVs were influenced by male age and were higher in younger males. ISIs were significantly shorter in pairs with younger versus older males (Table A3).

Table A1
Results of generalized linear mixed-effects models investigating the effects of group (copulating and noncopulating pairs) and courtship phase on vocalization numbers

Factor	$\chi 2$	*df*	*P*	Pairwise comparison of courtship phases or groups	Estimate	SE	z	*P*
USV numbers								
Group	0.78	1	0.377	1-PC vs 2-FC	−0.52	0.23	−2.21	0.121
Phase	19.34	3	**<0.001**	1-PC vs 3-EC	−0.02	0.23	−0.09	1.000
Male age class	0.02	1	0.882	1-PC vs 4-LC	−0.91	0.24	−3.75	**0.001**
Female age class	0.53	1	0.468	2-FC vs 3-EC	0.49	0.23	2.14	0.140
				2-FC vs 4-LC	−0.39	0.24	−1.60	0.377
				3-EC vs 4-LC	−0.88	0.24	−3.62	**0.002**
USV repertoire								
Group	3.78	1	0.052	1-PC vs 2-FC	−0.19	0.08	−2.44	0.070
Phase	51.07	3	**<0.001**	1-PC vs 3-EC	−0.13	0.08	−1.63	0.363
Male age class	0.06	1	0.799	1-PC vs 4-LC	−0.48	0.07	−6.59	**<0.001**
Female age class	1.56	1	0.212	2-FC vs 3-EC	0.06	0.07	0.81	0.849
				2-FC vs 4-LC	−0.29	0.07	−4.27	**<0.001**
				3-EC vs 4-LC	−0.35	0.07	−5.06	**<0.001**
Short syllables[1]								
Group	0.26	1	0.609	1-PC*CP vs 1-PC*NCP	1.14	0.31	3.64	**0.007**
Phase	37.84	3	**<0.001**	2-FC*CP vs 2-FC*NCP	0.51	0.31	1.63	0.732
Male age class	0.13	1	0.720	3-EC*CP vs 3-EC*NCP	−0.13	0.33	−0.38	1.000
Female age class	1.06	1	0.304	4-LC*CP vs 4-LC*NCP	0.17	0.32	0.51	1.000
Group*phase	10.85	3	**0.013**					
Short syllables								
Group	4.87	1	**0.027**	1-PC vs 2-FC	0.50	0.20	2.45	0.069
Phase	30.61	3	**<0.001**	1-PC vs 3-EC	1.08	0.21	5.17	**<0.001**
Male age class	0.06	1	0.809	1-PC vs 4-LC	0.86	0.21	4.10	**<0.001**
Female age class	0.59	1	0.443	2-FC vs 3-EC	0.59	0.21	2.84	**0.023**
				2-FC vs 4-LC	0.36	0.21	1.73	0.308
				3-EC vs 4-LC	−0.23	0.21	−1.09	0.698
Simple syllables								
Group	0.68	1	0.410	1-PC vs 2-FC	−0.90	0.22	−4.07	**<0.001**
Phase	36.58	3	**<0.001**	1-PC vs 3-EC	−0.66	0.23	−2.92	**0.019**
Male age class	0.03	1	0.865	1-PC vs 4-LC	−1.25	0.21	−5.91	**<0.001**
Female age class	1.05	1	0.306	2-FC vs 3-EC	0.25	0.20	1.22	0.611
				2-FC vs 4-LC	−0.35	0.19	−1.84	0.256
				3-EC vs 4-LC	−0.60	0.19	−3.08	**0.011**
Complex syllables								
Group	3.56	1	0.059	1-PC vs 2-FC	−0.40	0.28	−1.41	0.492
Phase	112.10	3	**<0.001**	1-PC vs 3-EC	−0.38	0.28	−1.37	0.515
Male age class	3.65	1	0.056	1-PC vs 4-LC	−2.07	0.25	−8.33	**<0.001**
Female age class	2.39	1	0.122	2-FC vs 3-EC	0.02	0.26	0.06	1.000
				2-FC vs 4-LC	−1.67	0.23	−7.33	**<0.001**
				3-EC vs 4-LC	−1.69	0.22	−7.54	**<0.001**
Unstructured syllables								
Group	1.14	1	0.285	1-PC vs 2-FC	0.82	0.20	4.11	**<0.001**
Phase	273.04	3	**<0.001**	1-PC vs 3-EC	3.06	0.24	12.79	**<0.001**
Male age class	1.27	1	0.260	1-PC vs 4-LC	3.16	0.24	13.39	**<0.001**
Female age class	1.92	1	0.166	2-FC vs 3-EC	2.23	0.24	9.39	**<0.001**
				2-FC vs 4-LC	2.33	0.24	9.84	**<0.001**
				3-EC vs 4-LC	0.10	0.27	0.37	0.982
Ultra-high USVs								
Group	6.89	1	**0.009**	1-PC vs 2-FC	2.20	0.36	6.09	**<0.001**
Phase	54.59	3	**<0.001**	1-PC vs 3-EC	2.30	0.37	6.24	**<0.001**
Male age class	0.10	1	0.755	1-PC vs 4-LC	1.89	0.38	5.02	**<0.001**
Female age class	0.63	1	0.428	2-FC vs 3-EC	0.10	0.38	0.26	0.994
				2-FC vs 4-LC	−0.32	0.39	−0.81	0.848
				3-EC vs 4-LC	−0.42	0.39	−1.06	0.714
USVs with sidebands								
Group	0.31	1	0.575	1-PC vs 2-FC	−0.42	0.65	−0.65	0.912
Phase	17.38	3	**<0.001**	1-PC vs 3-EC	1.49	1.12	1.34	0.522
Male age class	3.39	1	0.065	1-PC vs 4-LC	−1.62	0.57	−2.85	**0.021**
Female age class	3.33	1	0.068	2-FC vs 3-EC	1.91	1.08	1.77	0.273
				2-FC vs 4-LC	−1.21	0.49	−2.47	0.059
				3-EC vs 4-LC	−3.12	1.04	−3.00	**0.013**
MFV numbers								
Group	0.75	1	0.388	1-PC vs 2-FC	2.15	0.18	11.75	**<0.001**
Phase	303.71	3	**<0.001**	1-PC vs 3-EC	2.87	0.21	13.99	**<0.001**
Male age class	0.83	1	0.361	1-PC vs 4-LC	2.59	0.19	13.31	**<0.001**
Female age class	1.12	1	0.289	2-FC vs 3-EC	0.72	0.22	3.28	**0.006**
				2-FC vs 4-LC	0.44	0.21	2.09	0.156
				3-EC vs 4-LC	−0.28	0.23	−1.20	0.625
Nonlinear BBV numbers								
Group	0.22	1	0.637	1-PC vs 2-FC	−3.39	0.28	−12.09	**<0.001**
Phase	212.28	3	**<0.001**	1-PC vs 3-EC	−3.27	0.28	−11.73	**<0.001**
Male age class	0.97	1	0.324	1-PC vs 4-LC	−3.96	0.28	−14.11	**<0.001**
Female age class	1.02	1	0.313	2-FC vs 3-EC	0.11	0.22	0.53	0.950

(*continued on next page*)

Table A1 (*continued*)

Factor	χ2	*df*	*P*	Pairwise comparison of courtship phases or groups	Estimate	SE	z	*P*
				2-FC vs 4-LC	−0.57	0.23	−2.53	0.055
				3-EC vs 4-LC	−0.68	0.21	−3.18	**0.008**
Pure BBV numbers								
Group	3.89	1	**0.049**	1-PC vs 2-FC	−1.35	0.22	−6.10	**<0.001**
Phase	67.10	3	**<0.001**	1-PC vs 3-EC	−1.29	0.22	−5.88	**<0.001**
Male age class	0.12	1	0.728	1-PC vs 4-LC	−1.75	0.22	−7.98	**<0.001**
Female age class	0.00	1	0.959	2-FC vs 3-EC	0.05	0.20	0.27	0.993
				2-FC vs 4-LC	−0.40	0.20	−2.03	0.177
				3-EC vs 4-LC	−0.45	0.20	−2.28	0.101

Post hoc analyses (Tukey's HSD) were performed using the package 'multcomp' or 'emmeans'. Significant differences ($P < 0.05$) are highlighted in bold.
[1] Group by phase interaction model. 1-PC: precontact; 2-FC: first contact; 3-EC: early courtship; 4-LC: late courtship; CP: copulating pairs; NCP: noncopulating pairs.

Table A2
Results of generalized linear mixed-effects models investigating the effects of group (copulating and noncopulating pairs) and courtship phase on ultrasonic vocalization spectrotemporal features

Factor	χ2	*df*	*P*	Pairwise comparison of courtship phases or groups	Estimate	SE	z	*P*
Length								
Group	1.11	1	0.291	1-PC vs 2-FC	−0.35	0.08	−4.53	**<0.001**
Phase	258.94	3	**<0.001**	1-PC vs 3-EC	−0.50	0.07	−7.31	**<0.001**
Male age class	0.88	1	0.349	1-PC vs 4-LC	−1.09	0.08	−13.67	**<0.001**
Female age class	0.33	1	0.568	2-FC vs 3-EC	−0.15	0.04	−3.75	**<0.001**
				2-FC vs 4-LC	−0.74	0.06	−12.80	**<0.001**
				3-EC vs 4-LC	−0.59	0.04	−13.37	**<0.001**
Mean amplitude								
Group	1.09	1	0.296	1-PC vs 2-FC	0.00	0.03	−0.04	1.000
Phase	112.19	3	**<0.001**	1-PC vs 3-EC	−0.09	0.03	−3.12	**0.009**
Male age class	0.44	1	0.505	1-PC vs 4-LC	−0.32	0.03	−9.28	**<0.001**
Female age class	1.32	1	0.251	2-FC vs 3-EC	−0.09	0.03	−3.07	**0.011**
				2-FC vs 4-LC	−0.32	0.04	−8.73	**<0.001**
				3-EC vs 4-LC	−0.23	0.03	−9.16	**<0.001**
Mean frequency[1]								
Group	5.37	1	**0.021**	1-PC*CP vs 1-PC*NCP	0.26	0.08	3.21	**0.029**
Phase	6.79	3	0.079	2-FC*CP vs 2-FC*NCP	0.00	0.04	−0.08	1.000
Male age class	0.20	1	0.652	3-EC*CP vs 3-EC*NCP	0.14	0.06	2.32	0.284
Female age class	0.42	1	0.519	4-LC*CP vs 4-LC*NCP	0.01	0.03	0.22	1.000
Group*phase	9.91	3	**0.019**					
Mean frequency								
Group	3.66	1	0.056	1-PC vs 2-FC	−0.15	0.05	−3.27	**0.005**
Phase	26.35	3	**<0.001**	1-PC vs 3-EC	−0.19	0.04	−4.85	**<0.001**
Male age class	0.20	1	0.653	1-PC vs 4-LC	−0.13	0.05	−2.83	**0.021**
Female age class	0.49	1	0.485	2-FC vs 3-EC	−0.04	0.03	−1.21	0.594
				2-FC vs 4-LC	0.02	0.01	1.64	0.327
				3-EC vs 4-LC	0.06	0.03	1.99	0.172
Minimum frequency[1]								
Group	1.97	1	0.160	1-PC*CP vs 1-PC*NCP	0.26	0.09	3.08	**0.044**
Phase	11.71	3	**0.008**	2-FC*CP vs 2-FC*NCP	−0.01	0.04	−0.17	1.000
Male age class	0.04	1	0.847	3-EC*CP vs 3-EC*NCP	0.06	0.04	1.40	0.856
Female age class	0.38	1	0.540	4-LC*CP vs 4-LC*NCP	0.00	0.03	0.03	1.000
Group*phase	9.21	3	**0.027**					
Minimum frequency								
Group	1.00	1	0.318	1-PC vs 2-FC	−0.16	0.05	−3.31	**0.005**
Phase	38.18	3	**<0.001**	1-PC vs 3-EC	−0.17	0.04	−4.05	**< 0.001**
Male age class	0.03	1	0.874	1-PC vs 4-LC	−0.09	0.05	−1.96	0.184
Female age class	0.46	1	0.497	2-FC vs 3-EC	−0.01	0.02	−0.52	0.948
				2-FC vs 4-LC	0.07	0.02	4.26	**< 0.001**
				3-EC vs 4-LC	0.08	0.02	3.50	**0.002**
Maximum frequency								
Group	5.04	1	**0.025**	1-PC vs 2-FC	−0.12	0.04	−2.83	**0.020**
Phase	14.02	3	**0.003**	1-PC vs 3-EC	−0.14	0.04	−3.62	**0.001**
Male age class	2.62	1	0.105	1-PC vs 4-LC	−0.13	0.04	−3.20	**0.006**
Female age class	1.20	1	0.274	2-FC vs 3-EC	−0.02	0.02	−0.63	0.915
				2-FC vs 4-LC	−0.01	0.01	−0.67	0.898
				3-EC vs 4-LC	0.01	0.02	0.29	0.990
Frequency bandwidth								
Group	0.01	1	0.923	1-PC vs 2-FC	0.01	0.08	0.07	1.000
Phase	68.69	3	**<0.001**	1-PC vs 3-EC	0.00	0.08	−0.03	1.000
Male age class	7.22	1	**0.007**	1-PC vs 4-LC	−0.34	0.08	−4.21	**<0.001**
Female age class	5.09	1	**0.024**	2-FC vs 3-EC	−0.01	0.06	−0.13	0.999
				2-FC vs 4-LC	−0.35	0.05	−6.70	**<0.001**
				3-EC vs 4-LC	−0.34	0.05	−7.09	**<0.001**

Table A2 (*continued*)

Factor	χ2	*df*	*P*	Pairwise comparison of courtship phases or groups	Estimate	SE	z	*P*
Intersyllable interval (ISI)								
Group	9.20	1	**0.002**	1-PC vs 2-FC	−0.06	0.02	−2.58	**0.047**
Phase	118.69	3	**<0.001**	1-PC vs 3-EC	−0.02	0.02	−0.93	0.785
Male age class	12.73	1	**<0.001**	1-PC vs 4-LC	0.16	0.02	8.51	**<0.001**
Female age class	3.13	1	0.077	2-FC vs 3-EC	0.04	0.02	2.06	0.163
				2-FC vs 4-LC	0.22	0.02	9.49	**<0.001**
				3-EC vs 4-LC	0.18	0.02	8.11	**<0.001**
Interbout interval (IBI)								
Group	0.00	1	0.970					
Phase	6.51	3	0.090					
Male age class	0.22	1	0.642					
Female age class	0.44	1	0.507					

Post hoc analyses (Tukey's HSD) were performed using the package 'multcomp' or 'emmeans'. Significant differences ($P < 0.05$) are highlighted in bold.
[1] Group by phase interaction model. 1-PC: precontact; 2-FC: first contact; 3-EC: early courtship; 4-LC: late courtship; CP: copulating pairs; NCP: noncopulating pairs.

Table A3
Results of generalized linear mixed-effects models investigating the effect of behavioural context and group (copulating and noncopulating pairs) on ultrasonic vocalization spectrotemporal features

Factor	χ2	*df*	*P*	Pairwise comparison of behavioural contexts	Estimate	SE	z	*P*
Length								
Context	126.70	4	**<0.001**	NON vs PRIOR_MA	−0.27	0.04	−7.58	**<0.001**
Group	0.06	1	0.810	NON vs DURING_MA	−0.16	0.04	−3.71	**0.002**
Male age class	0.00	1	0.952	NON vs PRIOR_C	−0.53	0.06	−9.04	**<0.001**
Female age class	0.31	1	0.579	NON vs DURING_C	0.18	0.08	2.42	0.093
				PRIOR_MA vs DURING_MA	0.11	0.04	3.06	**0.016**
				PRIOR_MA vs PRIOR_C	−0.26	0.06	−3.99	**<0.001**
				PRIOR_MA vs DURING_C	0.46	0.09	5.31	**<0.001**
				DURING_MA vs PRIOR_C	−0.37	0.07	−4.99	**<0.001**
				DURING_MA vs DURING_C	0.34	0.09	3.73	**0.002**
				PRIOR_C vs DURING_C	0.71	0.10	7.37	**<0.001**
Mean amplitude								
Context	62.44	4	**<0.001**	NON vs PRIOR_MA	−0.11	0.02	−6.61	**<0.001**
Group	7.72	1	**0.005**	NON vs DURING_MA	−0.08	0.02	−3.29	**0.008**
Male age class	1.10	1	0.294	NON vs PRIOR_C	−0.16	0.04	−4.21	**<0.001**
Female age class	0.35	1	0.552	NON vs DURING_C	0.06	0.03	1.99	0.240
				PRIOR_MA vs DURING_MA	0.03	0.02	1.47	0.540
				PRIOR_MA vs PRIOR_C	−0.05	0.04	−1.35	0.620
				PRIOR_MA vs DURING_C	0.17	0.03	4.91	**<0.001**
				DURING_MA vs PRIOR_C	−0.08	0.04	−1.83	0.320
				DURING_MA vs DURING_C	0.14	0.04	3.45	**0.004**
				PRIOR_C vs DURING_C	0.22	0.04	5.74	**<0.001**
Mean frequency								
Context	19.19	4	**<0.001**	NON vs PRIOR_MA	−0.02	0.01	−1.97	0.246
Group	0.12	1	0.725	NON vs DURING_MA	−0.01	0.01	−0.82	0.910
Male age class	6.59	1	**0.010**	NON vs PRIOR_C	0.01	0.02	0.39	0.994
Female age class	1.29	1	0.257	NON vs DURING_C	−0.06	0.02	−2.61	0.057
				PRIOR_MA vs DURING_MA	0.01	0.01	1.40	0.591
				PRIOR_MA vs PRIOR_C	0.02	0.02	1.11	0.773
				PRIOR_MA vs DURING_C	−0.04	0.02	−1.92	0.270
				DURING_MA vs PRIOR_C	0.02	0.02	0.74	0.938
				DURING_MA vs DURING_C	−0.05	0.02	−2.39	0.100
				PRIOR_C vs DURING_C	−0.06	0.02	−3.32	**0.006**
Minimum frequency								
Context	48.95	4	**<0.001**	NON vs PRIOR_MA	0.02	0.01	1.59	0.457
Group	0.04	1	0.833	NON vs DURING_MA	0.02	0.01	1.50	0.516
Male age class	1.58	1	0.209	NON vs PRIOR_C	0.08	0.02	3.69	**0.002**
Female age class	1.93	1	0.165	NON vs DURING_C	−0.05	0.03	−1.60	0.447
				PRIOR_MA vs DURING_MA	0.00	0.01	−0.22	0.999
				PRIOR_MA vs PRIOR_C	0.07	0.02	2.74	**0.039**
				PRIOR_MA vs DURING_C	−0.07	0.03	−2.07	0.197
				DURING_MA vs PRIOR_C	0.07	0.03	2.75	**0.037**
				DURING_MA vs DURING_C	−0.07	0.03	−2.02	0.220
				PRIOR_C vs DURING_C	−0.14	0.02	−5.75	**<0.001**
Maximum frequency								
Context	35.54	4	**<0.001**	NON vs PRIOR_MA	−0.04	0.01	−3.94	**<0.001**
Group	0.30	1	0.582	NON vs DURING_MA	−0.03	0.01	−2.46	0.093
Male age class	13.61	1	**<0.001**	NON vs PRIOR_C	−0.08	0.02	−4.35	**<0.001**
Female age class	2.58	1	0.108	NON vs DURING_C	−0.07	0.02	−4.57	**<0.001**
				PRIOR_MA vs DURING_MA	0.01	0.01	0.96	0.861
				PRIOR_MA vs PRIOR_C	−0.04	0.02	−2.22	0.159
				PRIOR_MA vs DURING_C	−0.03	0.02	−1.94	0.280
				DURING_MA vs PRIOR_C	−0.05	0.02	−2.5	0.083
				DURING_MA vs DURING_C	−0.04	0.02	−2.59	0.067
				PRIOR_C vs DURING_C	0.01	0.02	0.36	0.996

(*continued on next page*)

Table A3 (*continued*)

Factor	χ2	*df*	*P*	Pairwise comparison of behavioural contexts	Estimate	SE	z	*P*
Frequency bandwidth								
Context	120.76	4	**<0.001**	NON vs PRIOR_MA	−0.24	0.04	−5.7	**<0.001**
Group	0.35	1	0.556	NON vs DURING_MA	−0.17	0.05	−3.41	**0.005**
Male age class	3.12	1	0.077	NON vs PRIOR_C	−0.63	0.07	−8.7	**<0.001**
Female age class	0.36	1	0.549	NON vs DURING_C	−0.16	0.11	−1.47	0.543
				PRIOR_MA vs DURING_MA	0.07	0.05	1.39	0.597
				PRIOR_MA vs PRIOR_C	−0.39	0.08	−4.64	**<0.001**
				PRIOR_MA vs DURING_C	0.08	0.12	0.7	0.948
				DURING_MA vs PRIOR_C	−0.46	0.09	−4.99	**<0.001**
				DURING_MA vs DURING_C	0.01	0.12	0.12	1.000
				PRIOR_C vs DURING_C	0.47	0.09	5.09	**<0.001**
Intersyllable interval (ISI)								
Context	235.35	4	**<0.001**	NON vs PRIOR_MA	0.06	0.01	4.09	**<0.001**
Group	0.46	1	0.495	NON vs DURING_MA	−0.03	0.02	−1.59	0.456
Male age class	3.05	1	0.081	NON vs PRIOR_C	0.07	0.03	2.52	0.072
Female age class	0.05	1	0.820	NON vs DURING_C	−0.24	0.03	−7.38	**<0.001**
				PRIOR_MA vs DURING_MA	−0.09	0.02	−5.16	**<0.001**
				PRIOR_MA vs PRIOR_C	0.01	0.02	0.29	0.998
				PRIOR_MA vs DURING_C	−0.30	0.03	−9.21	**<0.001**
				DURING_MA vs PRIOR_C	0.09	0.03	3.03	**0.017**
				DURING_MA vs DURING_C	−0.22	0.04	−5.77	**<0.001**
				PRIOR_C vs DURING_C	−0.31	0.02	−14.82	**<0.001**

Post hoc analyses (Tukey's HSD) were performed using the package 'multcomp'. Significant *P* values (<0.05) are in bold. DURING_C: during copulation; DURING_MA: during mating attempt; NON: nonsexual; PRIOR_C: prior to copulation; PRIOR_MA: prior to mating attempt.

Table A4
Linear mixed-effects model results investigating the effects of behavioural context and group (copulating and noncopulating pairs) on the proportion of BBV/USV overlaps

Factor	χ2	*df*	*P*	Pairwise comparison of behavioural contexts	Estimate	SE	z	*P*
Context	74.35	4	**<0.001**	NON vs PRIOR_MA	−0.18	0.05	−3.45	**0.005**
Group	0.11	1	0.745	NON vs DURING_MA	−0.07	0.05	−1.39	0.628
				NON vs PRIOR_C	−0.29	0.08	−3.70	**0.002**
				NON vs DURING_C	0.36	0.08	4.80	**<0.001**
				PRIOR_MA vs DURING_MA	0.11	0.05	2.02	0.248
				PRIOR_MA vs PRIOR_C	−0.11	0.08	−1.36	0.647
				PRIOR_MA vs DURING_C	0.55	0.08	7.13	**<0.001**
				DURING_MA vs PRIOR_C	−0.22	0.08	−2.70	0.052
				DURING_MA vs DURING_C	0.44	0.08	5.64	**<0.001**
				PRIOR_C vs DURING_C	0.66	0.09	7.48	**<0.001**

Post hoc analysis (Tukey's HSD) was performed using the package 'multcomp'. Significant *P* values (<0.05) are in bold. DURING_C: during copulation; DURING_MA: during mating attempt; NON: nonsexual; PRIOR_C: prior to copulation; PRIOR_MA: prior to mating attempt.

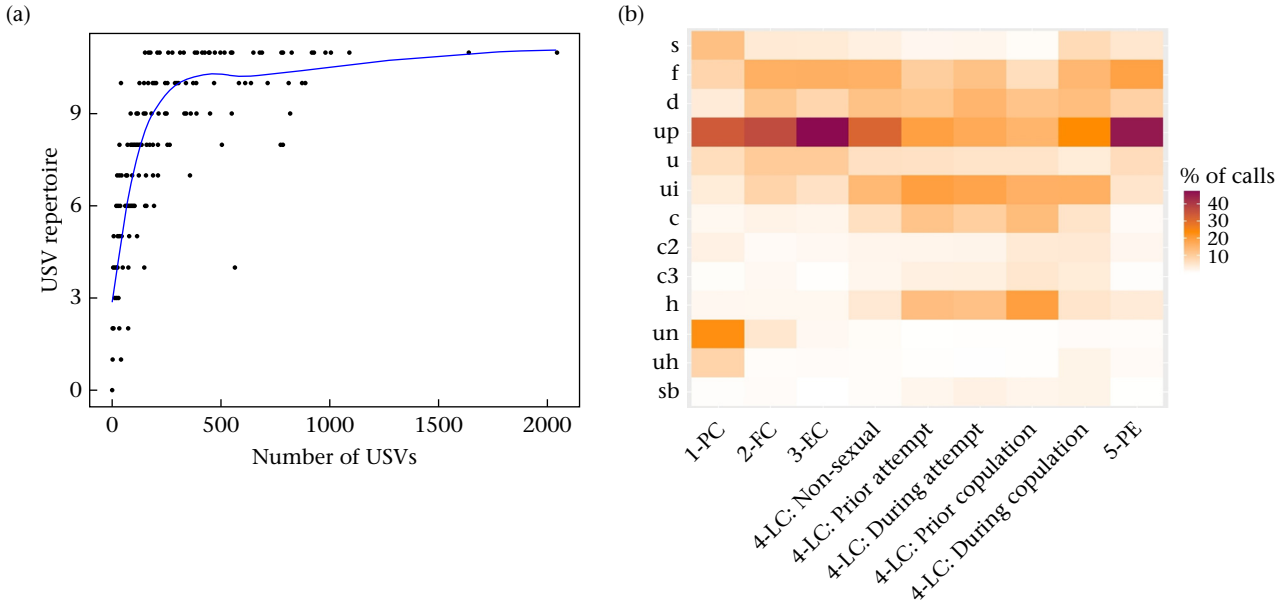

Figure A1. (a) Association between the total number of ultrasonic vocalizations (USVs) per recording and repertoire size, i.e. the occurrence of different USV types (between 0 and 11). (b) Heatmap of the proportional appearances of the 11 syllable types ('s', 'f', 'd', 'up', 'u', 'ui', 'c', 'c2', 'c3', 'h' and 'un'), ultra-high calls (uh) and calls containing sidebands (sb) for each stage of courtship. The 'late courtship' phase (4-LC) is separated into five behavioural contexts, including the periods just before or during (failed) mating attempts or copulations. 'Precontact' (1-PC, $N = 42$ pairs), 'first contact' (2-FC, $N = 42$ pairs), 'early courtship' (3-EC, $N = 36$ pairs), 'late courtship' (4-LC, $N = 36$ pairs) and 'postejaculation' (5-PE, $N = 14$ pairs).

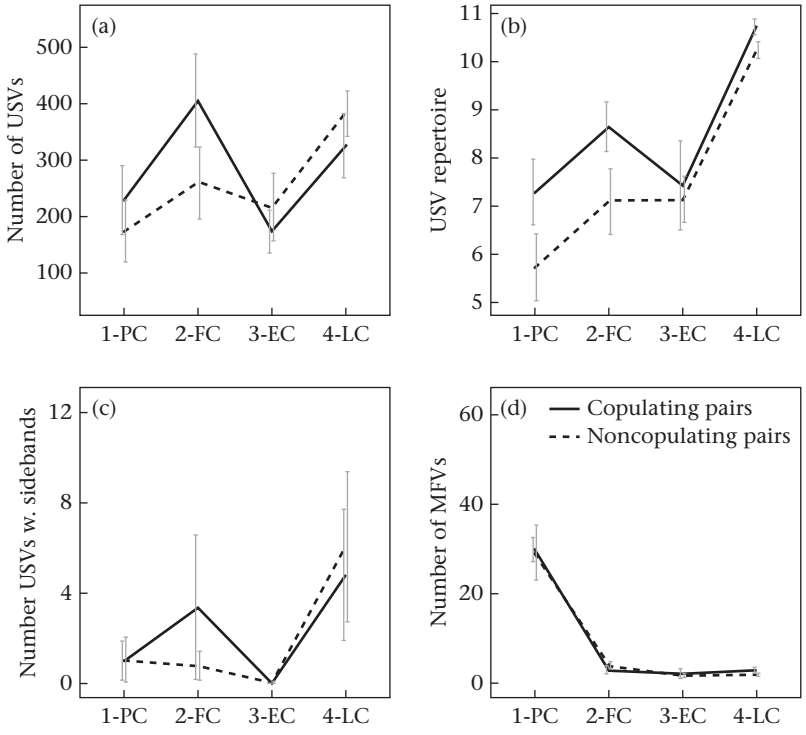

Figure A2. (a) Total number of ultrasonic vocalizations (USVs), (b) USV repertoire size, (c) number of USVs containing sidebands and (d) number of sonic mid-frequency vocalizations in pairs that copulated successfully (solid line, $N = 14$) versus noncopulating pairs (dotted line, $N = 22$) across time and different courtship stages (1-PC to 4-LC). Note that mice did not mate until the 4-LC stage. Means ± SEM are shown for each of four 10 min recordings.

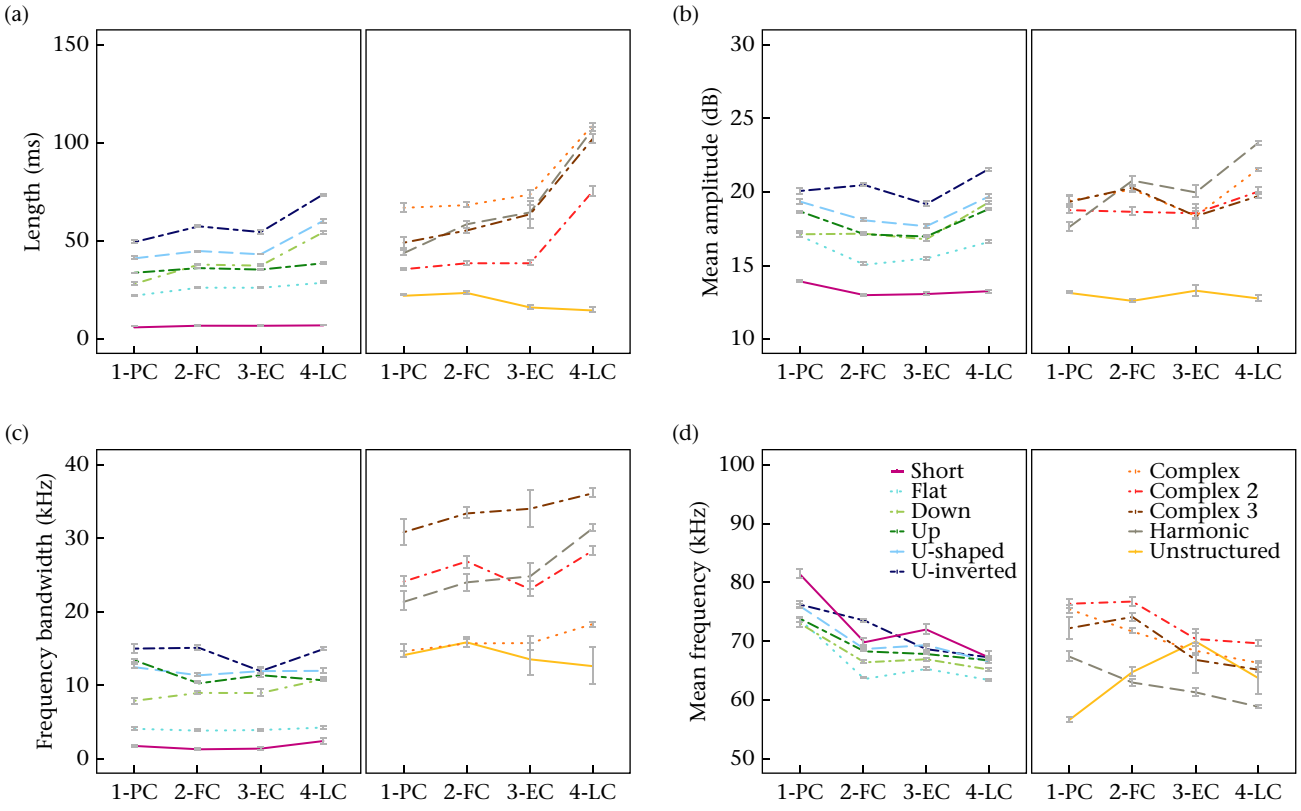

Figure A3. Ultrasonic vocalization (USV) spectrotemporal features (means ± SEM) per syllable type across four courtship stages (1-PC to 4-LC, $N = 36$ pairs): (a) syllable length, (b) mean amplitude, (c) frequency bandwidth and (d) mean frequency.

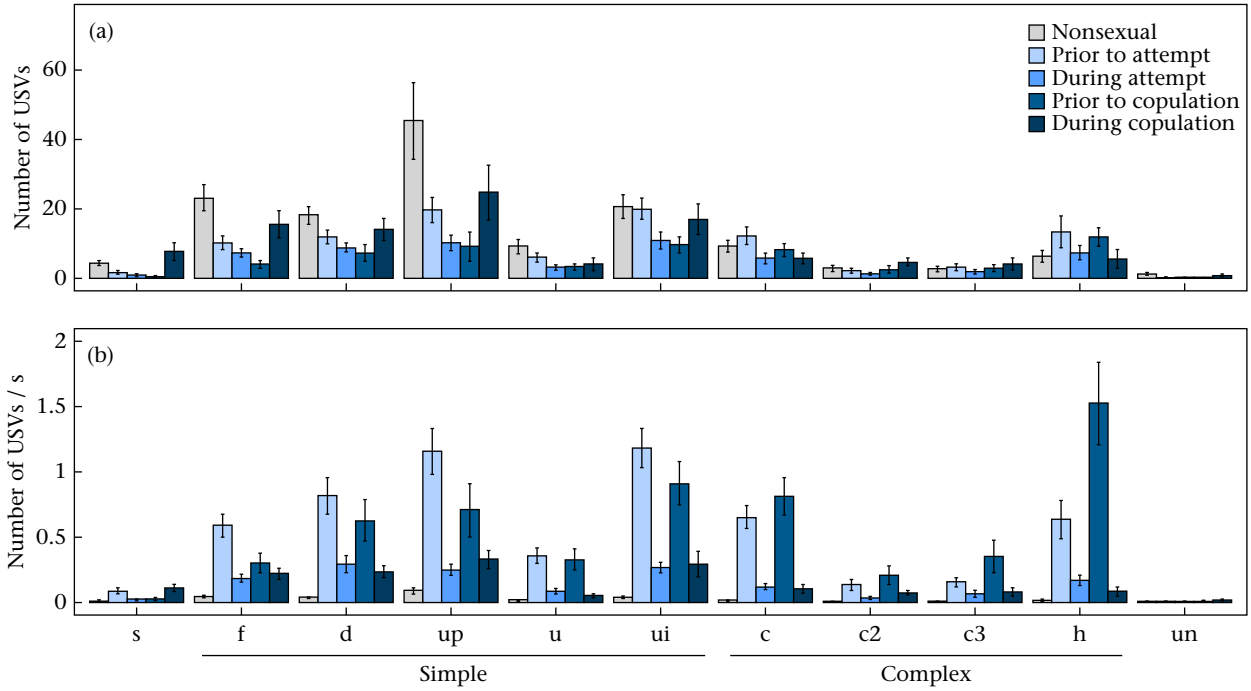

Figure A4. Dynamics of ultrasonic vocalization (USV) syllable types in the context of male sexual behaviour, showing (a) numbers of 11 syllable types and (b) rates/s (10 min recordings, $N = 36$ pairs). Nonsexual and sexual periods are shown. The latter were separated into 'prior' to and 'during' male mating attempts and copulations.

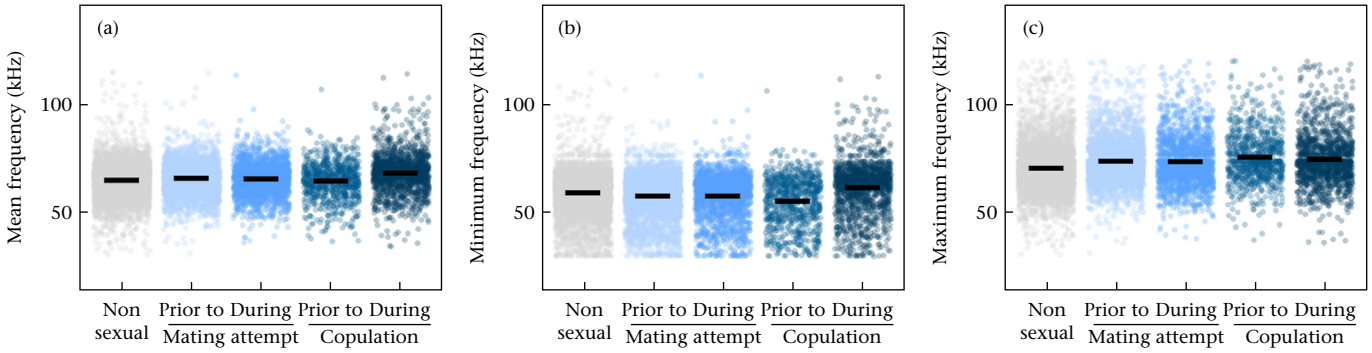

Figure A5. Ultrasonic vocalization spectrographic changes in (a) mean frequency, (b) minimum frequency and (c) maximum frequency in the behavioural context of their emission (10 min recordings, $N = 36$ pairs). Male mating attempts and copulations were separated into periods just before and during the behaviour and compared with nonsexual periods. Raw data are shown. Black lines indicate means ± SEM; because SEM are small they are not visible.