Ultrasonic vocalization production and playback predicts intra-pair and extra-pair social behaviour in a monogamous mouse

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ABSTRACT

Most studies examining rodent ultrasonic vocalizations (USVs) have investigated pericopulatory vocal behaviour in polygynous rodents, while vocalizations related to pair bond maintenance in monogamous rodents remain unexplored. In the monogamous California mouse (*Peromyscus californicus*) we used ultrasonic playbacks and post-playback social interactions to assess possible functions of USVs. We demonstrated that females respond with approach toward USVs of an unfamiliar male (bonded male from another pair) compared to a noise control, but displayed no difference in response to calls of their partner versus noise. Responsiveness to unfamiliar males does not appear to reflect an interest in extra-pair copulations because during post-playback social interactions, females displayed more agonistic and fewer affiliative behaviours toward unfamiliar, sexually naïve “stranger” males than toward their partners. We speculate that approach to unfamiliar male USVs instead may be related to territorial defense. We further explored associations within the data set. Interestingly, female affiliation with her partner was predicted by USV output, particularly a higher number and proportion of complex call types, produced during the male partner USV elicitation phase. Female approach towards USVs was related to syllable duration of one call type in partner USVs (not in unfamiliar USVs) but no other features, and sufficient variation exists in syllable duration to allow females to theoretically distinguish between individuals based on this measure. Similarly, while pregnancy state did not influence female social behaviour, it decreased approach to playback of partner, but not unfamiliar, USVs. Overall, our results illuminate concepts about vocal communication in monogamous rodent species with strong pair bonds and suggest that functions of USVs in rodents can extend beyond mate choice.

INTRODUCTION

Among socially monogamous species, intersexual vocal communication plays a key role in pair bond maintenance among mates in a variety of taxa, most notably birds (Hall 2009), although examples in mammalian species have emerged (Geissman & Orgeldinger 2000, Rukstalis & French, 2005; Ham, Hedwig, Lappan & Choe, 2016). In socially monogamous birds, vocal signals may maintain pair bonds by sustaining contact when physically separated (Silcox & Evans 1982, Robertson 1996) and quality of vocal communication in response to an intruder can be positively related to paternity assurance (Baldassarre, Greig, & Webster, 2016). Individual recognition of pair-mate vocalizations also occurs in monogamous birds (Robertson 1996; Blumenrath, Dabelsteen, & Pedersen, 2007). However, there is a surprising dearth of studies on the role of acoustic communication in pair bonding and maintaining social bonds in rodents.

Ultrasonic vocalizations (USVs) of adult rodents are high-frequency, quickly-attenuating sonic signals studied primarily in mice and rats in the context of sexual motivation (Holy & Guo, 2005; Portfors, 2007; Brudzynski, 2009; Matsumoto & Okanoya, 2016). Males produce USVs in mating contexts (Sales, 1972) and in response to ovulatory chemosignals in female urine (Whitney & Nyby 1979; Nyby, Bigelow, Kerchner, & Barbehenn, 1983; White, Colona, & Barfield, 1991), and male USVs facilitate proximity association and subsequent female mating behaviour (White & Barfield, 1989; Pomerantz, Nunez, & Bean, 1983). Ultrasonic playbacks have added significantly to this knowledge base (*Rattus norvegicus*; Willadsen, Seffer, Schwarting, & Wöhr, 2014; Pultorak, Kelm-Nelson, et al., 2015; *Mus musculus*; Hammerschmidt, Radyushkin, Ehrenreich, & Fischer, 2009; Musolf, Hoffmann, & Penn, 2010). Acoustic communication in monogamous rodent

species likely has additional functions related to challenges associated with long-term social bonds such as pair bond maintenance (Tomaszycki & Adkins-Regan 2005) and mate coordination related to territory defense and rearing offspring (Kleiman 1977), but this remains to be tested. To help fill this gap, we examine female approach behaviour during playback of USVs in a monogamous species, the California mouse (*Peromyscus californicus*).

The monogamous (Ribble, 1991; Gubernick & Nordby, 1993), biparental (Cantoni & Brown, 1997; Bester-Meredith & Marler, 2003), territorial, and aggressive (Marler, Bester-Meredith, & Trainor, 2003; Trainor, Bird, & Marler, 2004; Davis & Marler, 2004; Bester-Meredith & Marler, 2007) California mouse is ideal for studying pair-mate social behaviour (Gleason & Marler, 2010; Gleason, Holschbach, & Marler 2012), and vocal behaviour. Both members of a pair maintain overlapping territories (Ribble & Salvioni, 1990) and produce several distinct USV types in field and laboratory settings (Kalcounis-Rueppell, Metheny, & Vonhof, 2006, Kalcounis-Rueppell et al., 2010; Briggs & Kalcounis-Rueppell, 2011), and the functions of calls continue to be identified. When presented with unfamiliar females, but without physical contact, sexually naïve males produce “simple sweep” USVs at higher rates than do paired males, and these rates are rapidly modulated by pulses of testosterone (Pultorak, Fuxjager, Kalcounis-Rueppell, & Marler, 2015), suggesting that sweeps are used in courtship. Moreover, field research indicates that temporal features of lower frequency, further traveling “SV” type USVs are altered based on whether mates are present (Briggs & Kalcounis-Rueppell, 2011). Here we examine aspects of USV function by assessing whether females differentially respond to their mates’ (henceforth “partner”) USVs compared to noise versus the USVs of an unfamiliar bonded male and noise, and exploring how aspects of USVs relate to approach to playback as well as intra-pair and extra-pair behaviour.

We considered predictions for response to playback arising from two alternative hypotheses: First, if vocalizations in this context function in pair bond maintenance, analogous to observations in some socially monogamous birds (Robertson 1996; Blumenrath et al., 2007), we would predict that females would approach their partner's USVs more compared to unfamiliar male USVs. Alternatively, if females investigate USVs for seeking extra pair mating opportunities, as suggested by findings in polygynous house mice (Asaba et al., 2014; Musolf et al., 2010) or to contribute to the combined territorial defense exhibited in this monogamous species (Ribble & Salvioni, 1990), we would predict that females would show comparatively greater approach toward unfamiliar male USVs. Following playbacks, we predicted that if their interest in unfamiliar bonded male USVs is a result of seeking extra-pair copulations, females would show affiliative and not aggressive behaviour towards unfamiliar, sexually naïve "stranger" males, but the reverse result if females are expressing territorial defense. Due to the dearth of studies of USVs in a monogamous species we also explored several relationships between USVs and behavioural responses: 1) whether male partner USV production during elicitation (reunion after a period of pair separation) was predictive of subsequent behaviour during the social interaction, 2) whether females could theoretically distinguish males based on SV syllable duration since this call characteristic emerged as an important feature in playback, and 3) whether pregnancy influenced approach to playback or social behaviour since approximately half of the females were pregnant at the time of the trial. Overall, we used variation in male vocal production, female approach to playback stimuli, and female behavioural responses during a post-playback social interaction to examine possible roles of ultrasonic communication in California mouse pairs.

METHODS

Animals

We used 68 adult female and 111 adult male California mice (mean age =183 days, s.d. =40.9 days) reared in our laboratory colony at the University of Wisconsin-Madison. Animals were maintained in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC) approved animal treatment and research protocols (IACUC number L0054470-A01). Mice were housed in standard cages (48.3 cm x 26.7 cm x 15.6 cm) with 1-3 same-sex conspecifics after weaning (PND 30) and given water and food (Purina 5015™ mouse chow) *ad libitum*. Colony rooms and testing rooms were maintained at 20-23 °C under a 14:10 light/dark cycle. All behavioural testing was conducted under red light within 3 h of the onset of the dark cycle. Animals used in male-female dyads did not share common ancestry for a minimum of two prior generations.

USV Elicitation for Playback

Because pilot data show that vocalizations are most reliably produced upon reunion after mate separation, we paired male-female dyads on Day 0 for 12 days, separated pairs for 24 h on Day 12, and made recordings upon reintroduction on Day 13. Specifically, on Day 12 the male partner was removed from the cage and was housed in a separate room in a Plexiglas “elicitation arena” (90.0 cm X 30.0 cm X 30.0 cm), with food, water and bedding. The arena was divided into two chambers separated by a translucent wall containing two

139 symmetrically located circular openings (3.8 cm diameter, center of opening 7 cm from the
 140 side wall) covered by mesh to allow for auditory and olfactory communication but not
 141 physical contact. Two microphones sensitive to ultrasound (Emkay/Knowles FG series,
 142 frequency range 10-120 kHz, Avisoft Bioacoustics, Berlin, Germany) were positioned in
 143 opposite corners of the arena, 90 cm apart and 30 cm from the arena floor, with one
 144 corresponding to each chamber. The microphones were calibrated to equal gain (-60dB
 145 noise floor) and recordings were collected at a 250 kHz sampling rate with 16 bit
 146 resolution using RECORDER software (Avisoft Bioacoustics). On Day 13 the female was
 147 removed from her home cage and placed into the chamber opposite the male. The
 148 recording was initiated immediately after the first male vocalization was observed on the
 149 real-time spectrogram (produced using a 512 Fast Fourier Transform, 50% frame overlap,
 150 high pass filter at 10 kHz) for the channel pertaining to the male's chamber. A trained
 151 observer watched the real-time spectrogram and noted female vocalizations during the
 152 elicitation session (scored as a "yes"/"no"), as indicated by any vocalization uniquely
 153 present in the channel pertaining to the female chamber or any vocalization with higher
 154 amplitude (i.e. darker) than that same vocalization in the channel pertaining to the male
 155 chamber. After 2-min, the recording was stopped and the female was removed. If no
 156 vocalizations were identified within 5-min, no recording was initiated and the session was
 157 stopped. We determined whether each female had mated by the time of the elicitation trial
 158 using birth latency data (number of days from pairing to birth of offspring), based on a
 159 gestation period of approximately 32 days (Gleason & Marler, 2010).

160
 161 *USV Playback Setup and Approach Behaviour*

162 After the elicitation session, the male was returned to the home cage and the female was
163 housed in a separate room in a different Plexiglas “playback arena”. The playback arena
164 was composed of three equally-sized chambers (30.5 cm X 30.5 cm X 30.5 cm) separated by
165 translucent walls, with each side chamber containing centrally located openings (11.5 cm
166 wide X 11.5 cm tall) covered by a sliding wire mesh gate. The outer wall of the left chamber
167 (henceforth “playback chamber”) contained a centrally located, floor-level 7.6 cm diameter
168 hole covered with mesh, with the playback speaker (Vifa Dynamic Ultrasound; 1-120kHz
169 frequency range, Avisoft Bioacoustics) placed 3cm beyond. The female was housed in the
170 middle chamber for 24 h with food, water and bedding. On Day 14, the female in the
171 playback arena was brought into the experimental room and habituated for 10 min prior to
172 playback. To match the naturally occurring amplitude of vocalizations using a
173 representative “SV” vocalization (see below), we recorded a male in the central chamber of
174 the playback arena with the microphone at a distance of 30 cm above the mouse. After
175 laterally placing a microphone 30 cm from the playback speaker, output gain/volume was
176 adjusted until the amplitude of the playback of the sample vocalization (as measured from
177 30 cm) matched the amplitude of the original sample vocalization. Once established, the
178 output gain setting was maintained across all playbacks. A video camera (Panasonic SDR-
179 SW20, Panasonic Corporation, Kadoma, Osaka, Japan) was level with the floor of and aimed
180 at the side of the arena. A visit was defined as the mouse orienting itself toward the gate or
181 speaker with its nose within a head’s length (approximately 3 cm) of the mesh and its hind
182 limbs stationary. Since there is no *a priori* reason to assume that frequency or duration of
183 visits better reflects the motivation of a female approaching a male under natural situations
184 in the field, we scored both the total number and duration of visits to the mesh for each

period. Among blind scorers the inter-rater reliability was measured (assessed for 5% of trials) using the intra-class correlation coefficient which was greater than 0.95 for both visit number and duration).

Playback Tracks and USV Composition

Each female was randomly assigned to one of three playback conditions: presentation of a stimulus track of her partner's USVs, an unfamiliar male's USVs, or control (no USVs). "Unfamiliar male USVs" tracks referred to randomly selected elicitation recordings of another paired male (to limit number of animals used) in the experiment (partner of a different female) with whom the female had no prior experience. By chance, 8 of 46 available recordings were used as tracks more than once (2-3 times). Regardless of condition, all females were also presented with a background noise playback track ("Noise track") as a control stimulus for within-subject comparisons. The Noise track contained background noises (e.g. ambient room noise and noise from the male movements in the elicitation arena; Figure 1) observed in USV stimuli tracks, but no USVs, similar to previous acoustic playback studies in rodents (Brudzynski & Chiu, 1995; Seffer et al., 2014; Sadananda, Wöhr, & Schwarting, 2008). Females in the control condition (never presented with USVs) were presented with a second background noise playback track ("Noise track 2") similar to the Noise track but differed in temporal location and amplitude of background noise, in lieu of a USV track to control for changing-stimulus novelty and duration of the playback session. The Noise track and Noise track 2 were also 2-min in length.

USV playback tracks were composed of the 2-min recordings collected during the elicitation phase and contained different types of vocalizations (a minimum of 20 total USVs). There were no significant differences in any measure of USV composition, including total number of USVs, or number or proportion of any USV type, between tracks used as partner USVs and tracks used as unfamiliar USVs (all P values ≥ 0.33 ; Table 1). Vocalizations were limited to previously described USV categories adapted from recent laboratory (Pultorak, Fuxjager, et al., 2015) and field (Kalcounis-Rueppell et al., 2010; Briggs & Kalcounis-Rueppell, 2011) studies for this species. Briefly, these included “simple sweeps”, “complex sweeps”, and 1- to 11- syllable vocalizations “SVs” (shown and described in Figure 1). USVs were counted in Avisoft SASlab (Avisoft Bioacoustics, Berlin, Germany). SVs were identified such that syllable durations (start and end times) could be cataloged and exported for temporal analysis. We used a clustering program (“group.pl”) that we developed in PERL programming language that automatically clustered SV syllables into phrases of different sizes. Adapted from the approach of Chabout, Sarkar, Dunson, & Jarvis (2015), we identified a peak in the distribution (supplemental materials Figure A) of inter-syllable intervals (ISIs) at 150 ms and set the maximum ISI at twice the value of this peak (300 ms) for phase clustering. We included the total number of USVs (simple sweeps + complex sweeps + SV syllables), the number and proportion of each call type, mean duration of SV syllables, and mean SV phrase size for each recording/track in analyses that assessed the relationship between USV features with approach to playback or with social behaviour.

Composition Variation in USV Recordings

Of the 68 males that completed the elicitation session, 46 (68%) produced USVs and 34 (50%) produced SV calls (min=1, max=157, mean=42.2). We compared SV syllable duration across males exhibiting at least 20 SV syllables (to obtain a sufficient sample) and found significant differences (ANOVA, $F_{15, 1218}=35.54$, $p<0.0001$), such that each male differed from at least 3 other males (and up to 15 other males) as revealed through significant post-hoc pairwise comparisons after Bonferroni correction. Though our spectral analysis was limited to this measure, it is consistent with previous work demonstrating signatures of individuality are revealed, in large part, through call duration (Hoffmann, Musolf, & Penn, 2012; Liu, Miller, Merzenich & Schreiner, 2003).

Twenty-seven of 68 females (40%) produced USVs during the male elicitation (see *USV Elicitation for Playback* above). Although confined to a separate chamber, we cannot exclude the possibility that female USVs were picked up by the male's microphone and present on recordings randomly selected for use as playback tracks ($N=12$ out of 42 tracks). As such, we conservatively include additional analyses corroborating the same patterns of approach to playback after excluding these trials (reduced sample: Total $N=30$; $N=13$, partner USV playback trials; $N=17$, unfamiliar USV playback trials) in the supplemental materials.

Playback Regimen

Approach behaviour was videotaped during two, 6-min periods: (1) the "Gate" period during which a female was confined to the middle chamber of the playback arena to examine approach behaviour toward the gate adjacent to the playback chamber, and (2) the "Speaker" period, during which the gate was lifted and the female explored the

chamber adjacent to the playback speaker (as in Pultorak, Kelm-Nelson, et al., 2015). We therefore assessed behavioural responses to playback in both a restricted “home” and exploratory “novel” context (middle chamber and playback chamber, respectively). For each period, the female was presented with an identical playback regimen of 2-min of silence followed by two separate 2-min acoustic stimuli composed of background noise and/or USVs (based on condition), totaling 6-min. Behaviour was videotaped over 12-min (Gate period: 6-min; Speaker period: 6-min). During the Gate period, presentation began with 2-min of silence (to assess baseline approach behaviour), then the first 2-min stimulus (noise or USVs), followed by a second 2-min stimulus (noise or USVs). Order of presentation was counterbalanced, with half of the females presented with USVs (partner or unfamiliar male) as the first stimulus and Noise track second, and half of the females presented with Noise track as the first stimulus and USVs second. Control females were similarly presented with two stimuli (Noise track and Noise track 2) in the same counterbalanced fashion. Immediately after presentation of the second stimulus, the Gate period ended and the Speaker period began. The gate was lifted and the identical playback regimen (2-min of silence, 2-min of the first acoustic stimulus, 2-min of the second acoustic stimulus) began.

Post-playback Social Interaction Trial

Immediately following the playbacks, the gate separating the middle and playback chambers was replaced, with the female restricted to the middle chamber. The male (either the partner or a male “stranger”, an unfamiliar sexually naïve male distinct from “unfamiliar” bonded males described in relation to playback) was transferred to the room

in his home cage and placed in the previously unused far right chamber, separated from the female by a mesh divider gate. We videotaped female's time in contact with the gate ("divider gate contact time") for 1-min, and then the divider gate was lifted and the mice were allowed to physically interact for 4-min. An observer, blind to conditions, scored measures of social behaviour (Gleason & Marler, 2010), including latency to contact, sniffs (approach and investigation of the other stationary animal in nose-to-nose or nose-to-anogenital contact), follows (continual pursuit of the other ambulatory animal at a slow consistent pace), chases (movement at a rapid pace towards the other, often resulting in biting or wrestling), and wrestles (aggression develops into a tumble in which biting can occur). Intra-rater reliability was assessed for 10% of the trials and ICC > 0.92 for all measures.

Since we made predictions about female extra-pair behaviour in the case of approach to unfamiliar USVs, we obtained a larger sample of social interaction trials with a male stranger for adequate power, whereas we obtained a smaller but sufficient sample of social interaction trials with the partner for comparisons across the two social conditions. Trials from each of the three playback conditions were balanced across the social conditions (partner $N = 21$ ($N_{\text{partnerUSVs}} = 7$, $N_{\text{unfamiliarUSVs}} = 8$, $N_{\text{control}} = 6$), stranger $N = 42$ ($N_{\text{partnerUSVs}} = 14$, $N_{\text{unfamiliarUSVs}} = 13$, $N_{\text{control}} = 15$)). It was logistically impossible to use the same bonded males that were used for unfamiliar USV stimuli as strangers for each female, but we considered naïve males to be adequate to test female aggression toward male intruders and also assess the potential for extra pair copulations using the same females and similar laboratory conditions to those used in the playbacks.

Statistical Analysis

Data were analyzed using SPSS (Version 23, SPSS Inc, Chicago IL) and graphs produced using Graphpad Prism 5 (La Jolla, CA, USA) and Free Statistics Software (Wessa, 2016). Due to technical (audio and/or video) failures, the sample size was decreased from 68 to a 63 (final sample sizes for each group: partner USVs $N = 21$, unfamiliar male USVs $N = 21$, control $N = 21$; social interaction conditions: partner $N = 21$, stranger male $N = 42$). Owing to non-normality, approach behaviour in response to playback stimuli was assessed using non-parametric repeated measures Friedman tests (χ^2_2) and post-hoc pairwise Wilcoxon signed rank test (Z) comparisons within each animal (i.e. approach during presentation of USVs versus silence, USVs versus noise, and noise versus silence). In order to minimize Type 1 error due to multiple comparisons, post-hoc test P values for approach behaviour were adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) procedure (Benjamini & Hochberg, 1995; Thissen, Steinberg, & Kuang, 2002). There were three social interaction trials in which animals never made contact, and a score of the maximum 240 s was assigned for the “latency to contact” measure. Between-subjects comparisons were made using Student’s t -tests and one-way analysis of variance (ANOVA), or if data were not normally distributed (based on the Shapiro-Wilk test of normality), the non-parametric equivalents, Mann-Whitney U and Kruskal-Wallis H tests. Bivariate relationships were assessed with Pearson’s Product-Moment correlation, or Spearman Rank correlation if normality could not be assumed for one or both variables. 2X2 chi-square tests were used to assess relationships between two categorical (binary) variables. For all analyses, we used two-tailed tests with a significance level of $\alpha = 0.05$.

Consolidation of “Gate” and “Speaker” Playback Approach Periods

Since our question, “Does the stimulus induce approach behaviour?”, was identical across the Gate and Speaker periods, we assessed whether the two periods could be consolidated. Approach behaviours across both periods were positively correlated during silence ($N = 63$; visits, $r_s = 0.495$, $P < 0.001$; duration, $r_s = 0.592$, $P < 0.001$) and presentation of the Noise track ($N = 63$; visits, $r_s = 0.462$, $P = 0.001$; duration, $r_s = 0.55$, $P < 0.001$). Similarly, for animals receiving presentation of a USV stimulus, visits to the gate during presentation of the USV stimulus were positively correlated with visits to the speaker during the same USV stimulus presentation ($r_s = 0.41$, $N = 42$, $P = 0.008$), as were the total durations spent at the gate and speaker ($r_s = 0.33$, $N = 42$, $P = 0.034$). We created additive composite variables for the two approach behaviour scores (henceforth “visits” and “duration”) in response to each independent stimulus (silence, Noise tracks, USVs). A minority of females (28%) did not enter the chamber adjacent to the playback speaker (i.e. they continued to explore and/or groom themselves within the central chamber), and were equally spread across the playback treatments ($N = 6$, $N = 5$, and $N = 7$ for the partner USVs, unfamiliar USVs, and background noise control, respectively; $\chi^2 = 0.47$, $df = 2$, $P = 0.79$). We additionally provide the approach data for each period separately as well as the strong correlation between visits and duration measures in the supplemental materials (Tables A and B).

RESULTS

Approach to Partner USVs Playback

Among females presented with their partner's USVs, approach differed across playback stimuli (silence, noise track, and partner's USVs) as measured by duration (Friedman test, $\chi^2_2 = 6$, $N = 21$, $P = 0.047$) but not visits ($\chi^2_2 = 5$, $N = 21$, $P = 0.11$). However, post-hoc tests revealed that this difference was due to females visiting longer during presentation of the Noise track than during silence (Wilcoxon signed rank test, $Z = 2.14$, $N = 21$, $P = 0.048$); there were no significant differences in duration between presentation of partner's USVs and silence ($Z = 2.17$, $N = 21$, $P = 0.09$) or partner's USVs and the Noise track ($Z = 0.46$, $N = 21$, $P = 0.64$; Figure 2). Thus, there was no direct evidence of approach to partner USVs compared to control stimuli.

Approach to Unfamiliar USVs Playback

Females presented with unfamiliar USVs varied their approach across playback stimuli as measured by both visits (Friedman test, $\chi^2_2 = 7$, $N = 21$, $P = 0.025$) and duration ($\chi^2_2 = 10$, $N = 21$, $P = 0.004$), and in contrast, these differences were due to greater approach during presentation of USVs (Figure 2); Females made more visits during presentation of USVs than during silence (Wilcoxon signed rank test, $Z = 2.79$, $N = 21$, $P = 0.015$) and the Noise track ($Z = 2.45$, $N = 21$, $P = 0.017$), and visited for longer in response to USVs compared to silence ($Z = 3.29$, $N = 21$, $P = 0.003$) but not the Noise track, although there was a non-significant trend toward visiting longer during USV presentation ($Z = 1.76$, $N = 21$, $P = 0.078$). These females also showed greater approach in response to the Noise track than silence, as measured by duration ($Z = 2.25$, $N = 21$, $P = 0.036$) but not visits ($Z = 1.78$, $N = 21$, $P = 0.075$). Thus, our behavioural paradigm provided evidence for female approach to unfamiliar USVs as compared to control stimuli.

Assessing Noise Controls and Carry-Over Effects on Approach to Playback

Control females (Noise tracks) showed no differences in approach (Figure 2) across playbacks of silence or noise track (Noise track, Noise track 2) as measured by both visits (Friedman test, $\chi^2_2 = 1$, $N = 21$, $P = 0.71$) and duration ($\chi^2_2 = 1$, $N = 21$, $P = 0.67$). However, because (1) females presented with unfamiliar or partner USVs showed greater approach duration (but not visits) during the Noise track as compared to silence (described earlier) and (2) presentation of the Noise track and USVs was counterbalanced, we tested for carry-over effects (Diaz-Uriarte, 2002) (full analyses in supplemental materials, Table C). Briefly, for unfamiliar USVs, when USVs preceded the Noise track, approach duration during presentation of USVs was greater than during silence (Wilcoxon signed rank test, $Z = 2.7$, $N = 12$, $P = 0.01$) and the Noise track ($Z = 2.8$, $N = 12$, $P = 0.02$). However, when the Noise track preceded unfamiliar USVs, approach duration did not differ significantly across any playback stimuli (Friedman test: $\chi^2_2 = 5$, $N = 9$, $P = 0.07$). For partner USV approach, duration did not differ across playback stimuli, regardless of whether USVs preceded the Noise track ($\chi^2_2 = 4$, $N = 10$, $P = 0.14$) or vice versa ($\chi^2_2 = 5$, $N = 11$, $P = 0.07$). Taken together with the result that females did not approach the Noise track (control), these findings suggest that females were more likely to approach the Noise track when it was associated with USVs. Most importantly, females differentially responded to partner USVs and unfamiliar USVs when USVs were presented prior to noise controls, statistically consistent with the original findings above (i.e. when considering all trials, regardless of presentation order).

Approach to Partner USV Playback Predicted by SV Syllable Duration

We assessed whether USV features predicted approach response during playback. Despite no direct evidence of approach to partner USVs compared to control stimuli, female approach duration (but not visits) during partner USV presentation was positively correlated with track SV mean syllable duration ($r_s = 53$, $N = 17$, $P = 0.03$), but no other USV features (number or proportion of call type, or SV mean phrase size; $P \geq 0.11$). No USV features correlated with approach behaviour during unfamiliar USV presentation (visits, $p \geq 0.38$; duration, $p \geq 0.07$). Thus SV syllable duration emerged as a potentially important call characteristic for vocal interaction between partners.

Female Social Behaviour with Partners and Male Strangers

As a control, we tested whether playback condition influenced female social behaviour during the post-playback interaction. Playback condition did not influence social behaviours regardless of whether social conditions (interaction with partner and interaction with stranger) were or were not collapsed (Kruskal-Wallis H tests, $P \geq 0.14$). This was also true within each individual playback presentation order subgroup (all P values > 0.05).

We compared female behaviour during post-playback interactions with their partners versus during interactions with strangers (sexually naïve males) (Figure 3). Females exposed to their partner showed a higher divider gate contact time prior to physical interaction (Mann-Whitney U test, $U = 285.5$, $N_1 = 21$, $N_2 = 42$, $P = 0.020$), and fewer chases (Mann-Whitney U test, $U = 293.5$, $N_1 = 21$, $N_2 = 42$, $P = 0.013$) and wrestles (Mann-Whitney U test, $N_1 = 21$, $N_2 = 42$, $U = 268$, $P = 0.005$) as compared to females exposed

to a stranger. There was a non-significant trend toward more follows (Mann-Whitney U test, $U=327.5$, $N_1=21$, $N_2=42$, $P=0.083$) by females interacting with their partner, but no differences in female sniffs (Mann-Whitney U test, $U=417$, $N_1=21$, $N_2=42$, $P=0.726$) or latency to contact (Mann-Whitney U test, $U=338$, $N_1=21$, $N_2=42$, $P=0.132$). As predicted, these results indicate more affiliative and fewer agonistic behaviours by females toward their partners than toward male strangers.

Partner's Vocal Output Predicts Intra-pair Female Social Behaviour

Given the variation in male vocal output in response to female partners during the elicitation, we explored whether this variation was predictive of the females' intra-pair or extra-pair behaviours during the post-playback social interaction. Because 32% (20 of 63 trials) of males did not vocalize, we were able to first compare behaviour during the post-playback interaction across trials for females paired with "vocal" partners versus those paired with "non-vocal" partners. During interactions with a male stranger ($N=42$), female behaviour did not differ between females paired with vocal partners ($N=29$) vs. those with paired with non-vocal partners ($N=13$; Mann-Whitney U tests, $P \geq 0.204$). However, during interaction with their partner ($N=21$), females with vocal partners ($N=14$) showed a higher divider gate contact time (Mann-Whitney U test, $U=12$, $N_1=14$, $N_2=7$, $P=0.004$), and more follows (Mann-Whitney U test, $U=17$, $N_1=14$, $N_2=7$, $P=0.016$) and sniffs (Mann-Whitney U test, $U = 19$, $N_1 = 14$, $N_2 = 7$, $P = 0.025$) than females with non-vocal partners ($N=7$), whereas latency to contact and number of chases and wrestles did not differ (Mann-Whitney U tests, $P \geq 0.255$). The results indicate a link between female affiliation and partner vocal behaviour.

Given the exploratory results above for male partner vocal behaviour during elicitation predicting female affiliative response to their partners during the post-playback social interaction, we further explored whether specific USV types or features predicted these female behaviours within the subset of trials for which females interacted with partners who vocalized during elicitation ($N=14$). Female divider gate contact time was positively correlated with the number of SVs ($r_s=0.561$, $N=14$, $P=0.037$) and proportion of SVs ($r_s=0.554$, $N=14$, $P=0.040$). Female follows were positively correlated with the proportion of complex sweeps ($r_s=0.571$, $N=14$, $P=0.033$). Similarly, female sniffs were positively correlated with the number ($r_s=0.599$, $N=14$, $P=0.024$) and proportion ($r_s=0.638$, $N=14$, $P=0.014$) of complex sweeps, and accordingly, negatively correlated with the proportion of simple sweeps ($r_s=-0.667$, $N=14$, $P=0.009$). No other associations between partner USVs (number/proportion of any call type, SV mean phrase size or SV mean syllable duration) and female social behaviour with her partner were found ($P \geq 0.06$). To conclude, complex sweeps and SVs produced during elicitation were positively associated with later female affiliative behaviour with her partner.

Effect of Female Pregnancy on Approach to Playback of Partner USVs

Thirty-four of 63 females (54%) were likely pregnant by the time of the elicitation trial based on estimations from birth latency data (see methods). No associations were found between pregnancy state (not pregnant “np” vs. pregnant “p”; used as subscripts) and vocal behaviour (i.e. vocalizations vs. no vocalizations) of females (Chi square test of independence, $\chi^2_1 = 1.68$, $P = 0.20$) or males ($\chi^2_1 = 1.86$, $P = 0.67$) during the elicitation phase. Among vocal males ($N = 43$ of 63), USV features (number or proportion of each USV type,

SV mean phrase size, or SV mean syllable duration) did not differ based on female pregnancy state ($P \geq 0.37$) with the notable exception of a non-significant trend toward a decreased proportion of simple sweeps produced by males when exposed to pregnant female partners (Mann-Whitney U test, $U=149$, $N_{np}=19$, $N_p=24$, $P=0.053$). Pregnant females made fewer visits during presentation of partner USVs than did non-pregnant females ($U=24.5$, $N_{np}=11$, $N_p=10$, $P=0.029$) and trended toward a lesser approach duration ($U=29.5$, $N_{np}=11$, $N_p=10$, $P=0.072$), whereas female approach behaviour during unfamiliar USVs playbacks did not differ based on pregnancy status (visits, $U = 34.5$, $N_{np} = 6$, $N_p = 15$, $P = 0.42$; duration, $U = 34.0$, $N_{np} = 6$, $N_p = 15$, $P = 0.42$). No differences in female social behaviour were found based on pregnancy state during the post-playback social interaction with either the female's partner ($P \geq 0.30$) or the stranger ($P \geq 0.10$).

DISCUSSION

Overall our results reveal several interesting findings about ultrasonic communication in a monogamous species, the California mouse. First, females showed elevated investigation of USVs from unfamiliar bonded males but not their own partners when compared to a background noise control. This approach to unfamiliar male USVs is likely linked to territorial defense and not indicative of attraction to extra-pair males, as might occur in a polygynous species, because interactions of females exposed to unfamiliar unmated (stranger) males after the playback were characterized by aggressive behaviour used in territorial behaviour and not the affiliative behaviour observed when females were reunited with their partner. Exploratory analyses revealed positive associations between partner vocal behaviour (presence / absence of vocalizations, and USV complexity) and

female affiliation. We speculate that this reflects a tighter bond between females and their partner. Finally, female approach during playback of partner's USV, but not unfamiliar USVs, was positively linked to syllable duration of "SV" (Figure 1) calls in the playback track and negatively linked to pregnancy state. SV syllable duration also significantly varied among males. Our design did not involve a direct choice test, but these findings are consistent with the concept of female individual discrimination between male USVs (see below). Overall, we have evidence for some relatively sophisticated functions for USVs in California mice indicating that the role of these calls extends beyond sexual motivation.

Functions of Female Approach to Unfamiliar versus Partner USVs

In the present study, females' approach toward USVs of unfamiliar partnered males but not their partners' USVs (Figure 2) and their subsequent behaviour during social interactions (Figure 3) supports the hypothesis that bonded females respond to the challenge of an intruding male with approach behaviour, but not for potential extra-pair copulations. This evidence for behavioural fidelity is consistent with the strongly monogamous behaviour found in this species in both field and laboratory studies (Ribble, 1991; Gubernick & Nordby, 1993). The aggressive behaviour by females towards intruding males is also consistent with female *P. californicus* territorial behaviour (Ribble & Salvioni, 1990). Past studies have found high levels of female-female aggression in this species (Davis & Marler, 2004; Bester-Meredith & Marler, 2007) but our results suggest that females may defend against naïve male intruders as well (i.e. "strangers" in the present study); the influence of male bonding or dominance status on female aggression has yet to be tested. The aggressive response toward opposite-sex individuals also suggests an opportunity for the

pair to cooperate in territorial defense. In Javan gibbons (*Hylobates moloch*), another monogamous and territorial species, both males and females display elevated defense responses to playbacks of unfamiliar vocalizations (Ham, Hedwig, Lappan, & Choe, 2016).

The lack of response to the male partner's USVs as compared to noise may be a result of low interest in, or rapid habituation to (Hammerschmidt, et al., 2009) familiar calls. However, a study in house mice found that co-housing actually reinforced female preference for male cage mate USVs (Shepard & Liu, 2011). Our null result may also be a function of the specific behavioural paradigm or a Type II error, since females preferentially approached noise controls over silence when partner USVs preceded noise, and approach toward USVs was positively correlated with partner SV syllable duration and negatively associated with pregnancy state, suggesting some investigation of their partner's calls. Taken together, these exploratory findings suggest that females are cuing into partner male calls and future studies should elucidate how female response to partner male calls may be used as indicators of pair bond quality.

Female Affiliative Behaviour Linked with Male Partner Vocal Behaviour

Pair bond quality may also be revealed through vocal behaviour during pair reunion. We found that pre-playback measures of male partner vocal behaviour predicted female affiliative behaviour towards her partner after the playback. First, "vocal" male partners elicited greater interest from the female (Figure 4) than "non-vocal" partners, and second, female affiliation measures were associated with specific call types such that the time that females spent at the divider gate separating her from her partner (Blocker & Ophir, 2015) and the number of follows after the gate was lifted were positively correlated with the

number of partner SV syllables and complex (but not simple) sweeps (Figure 1), respectively. The fact that female affiliation was associated with these call types, which are comparatively rare (Table 1) and syntactically complex, is consistent with studies indicating that complex calls may be more attractive in house mice (Chabout et al., 2015) and other vertebrate taxa (Marshall, Leisler, Catchpole, & Schwabl, 2005; Kime, Rand, Kapfer, & Ryan, 1998; Ryan & Rand, 1990). Interpretations should be met with caution since the vocal measurements were made 24 h prior to the social interaction (after a period of separation) and not during the post-playback social interactions. We also cannot rule out that some vocalizations counted in the male's channel during elicitation may have originated from the female. Nonetheless, these results are interesting and suggest that communication between pairs can predict pair affiliation.

Individual Variation and Discrimination of Male Vocalizations

Although our design did not involve choice tests, we have evidence consistent with the idea that individual discrimination of calls may be occurring. First we found preliminary evidence of individual variation among male mean SV syllable duration (see "*Composition Variation in USV Recordings*" in Methods), and this variation predicted female approach, but only in response to partner USVs, not unfamiliar USVs. Second, pregnancy reduced female approach to playback of partner USVs, but had no effect on approach to playbacks of unfamiliar USVs, suggesting that females can recognize calls of their mate since female state is expected to act in an identical fashion across both USV stimuli treatments if females could not distinguish between calls. This effect was revealed through "visits" and would

not have been revealed if duration were the sole approach measure, as in many playback studies (see Seffer et al., 2014).

The concept of discrimination between calls of individuals is dependent on individual variation of USVs. Recent evidence suggests subspecies differences in quantity and quality of USVs across wild mouse populations (von Merten, Hoier, Pfeifle, & Tautz, 2014) and playback studies in house mice reveal discrimination between USVs based on species (Musolf, Meindl, Larsen & Kalcounis-Reuppell, 2015) and relatedness (Asaba et al., 2014; Musolf et al., 2010), but the acoustic features used for discrimination are only beginning to be understood. The present study primarily used the number and proportion of each call type to quantify variation in vocal behaviour, but females may discriminate based on other features. Our preliminary evidence of individual differences in SV syllable duration is consistent with previous studies in house mice that indicate signatures of individual variation may be rooted in spectral (primarily frequency and call duration) and temporal (e.g. periodicity and clustering of repeated phrases) features (Hoffman et al., 2012; Liu et al., 2003). Future playback studies should consider the incorporation of syllable duration as an important factor influencing behavioural responses, among other possible features not yet examined in this species.

Female Pregnancy and Male Partner Vocal Behaviour

Since estrous was not assessed in the present study, we cannot exclude the possibility that male vocal behaviour was influenced by the estrous state of the female as suggested by research in house mice (Hanson & Hurley, 2012). However, while estrous state was found to influence male social behaviour in closely related polygynous white-footed mice (*P.*

leucopus), no association was found in monogamous California mice (Karelina et al., 2010), suggesting a decreased salience of female estrous in this species. Future studies might assess possible impacts of estrous state on male vocal behaviour during both intra-pair and extra-pair interactions in this species. We were, however, able to assess possible effects of reproductive status by estimating pregnancy in the current study. No significant associations between pregnancy state and male vocal behaviour were found, but there was notably a non-significant trend ($p=0.053$) toward a decreased proportion of simple sweeps produced by males when exposed to pregnant, as compared to non-pregnant, female partners. Given that testosterone increases the proportion of simple sweeps among non-bonded males toward females (Pultorak, Fuxjager, et al., 2015), this might indicate a decrease in courtship-like behaviour, possibly accompanied by an increase in pair bond maintenance. These results hint at the possibility of changes in call types across the pair bonding period.

Noise Controls and Carry-Over Effects for Playback Approach

While our design was similar to previous rodent USV playback studies because it assessed approach behaviour to USV stimuli relative to a noise control stimulus and/or silence (Wöhr & Schwarting, 2007; Sadananda et al., 2008; Burgdorf et al., 2008; Musolf et al., 2010; Willadsen et al., 2014; Pultorak, Kelm-Nelson, et al., 2015), our results indicate a carry-over effect. Controlling for presentation order by counterbalancing and analyzing each group independently suggested that background noise might be considered a negative stimulus that obscured approach to USVs when presented prior to USVs. However, when USVs preceded noise, animals showed approach to the background noise stimulus (as

compared to silence), likely indicating that animals associated noise with (and thus anticipated) USVs. The argument for anticipation is strengthened by the finding that animals in the control condition (i.e. presented with noise controls but never USVs) did not show approach to noise over silence. This is potentially useful knowledge for future playback designs involving vocal communication in rodents.

Conclusions

The present study extends our understanding of the functions of USVs beyond the facilitation of copulation (Pomerantz et al., 1983; White & Barfield 1989) and conveyance of affective state (Burgdorf et al., 2008, Lahvis, Alleva, & Scattoni, 2011). Our results indicate a more sophisticated role for USVs in a monogamous rodent species that needs to maintain pair bonds and cooperate in territorial defense and offspring rearing. During playbacks, females displayed a significant response to unfamiliar male USVs versus noise but not USVs of their own mate versus noise. Approach to unfamiliar USVs was likely a territorial response given the aggression to strangers following playback. Nonetheless, other analyses suggest that females are cuing in on their partner USVs; partner vocal behaviour predicted female affiliation, and approach toward partner USVs was positively associated with SV syllable duration and negatively associated with pregnancy state. Our findings highlight the fact that ultrasonic communication between partners differs from that observed between extra-pair individuals and during courtship (Pultorak, Fuxjager, et al., 2015), and may play a key role in pair bond maintenance. Overall, our results suggest that USV communication in California mice may be involved in complex behavioural interactions and that further exploration of call functions is warranted in both the

maintenance of pair bonds and the communication necessary for cooperative defense behaviour that might occur in a territorial, biparental, and monogamous species.

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Figure1

Figure2

Figure3

Figure4