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Effects of ovarian hormones on the emission of 50-kHz ultrasonic vocalizations during distributed clitoral stimulation in the rat

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ABSTRACT

Fifty-kHz ultrasonic vocalizations (USVs) are emitted by adult rats during appetitive phases of behavior in response to stimuli thought to be associated with a positive affective state. In particular, 50-kHz USVs with rapid frequency oscillations, known as trills and flat-trills, in which these oscillations are flanked by a monotonic portion, are together positively correlated with appetitive behaviors such as rough and tumble play, drug and natural reward, and mating. Female rats produce 50-kHz USVs during a variety of sexual contexts, yet data are still vague as female sexual behavior is seldom studied on its own. Distributed clitoral stimulation (CLS) offers a unique approach to investigating female 50-kHz USVs as it mimics stimulation received during mating. Although CLS induces a sexual reward state, it is unknown whether CLS elicits trills and flat-trills. We addressed this question using eight ovariectomized rats, we investigated whether ovarian hormones augmented these call subtypes in response to CLS. The combined and separate effects of estradiol benzoate (EB) and progesterone (P), and oil vehicle were assessed through comparison of these call subtypes between CLS and inter-CLS interval. We found that CLS with EB + P significantly increased call duration and rate, lowered peak frequency, and widened the bandwidth of trills. Flat-trills showed a similar pattern except for call duration. Call distribution during the CLS and inter-CLS interval suggest that trill and flat-trills may be indicative of both anticipatory and sexual reward.

1. Introduction

Adult rats emit ultrasonic vocalizations (USVs) in aversive and rewarding situations. These vocalizations can be divided into two main categories: 22-kHz and 50-kHz calls. Twenty-two-kHz vocalizations (~20–30-kHz) are emitted during aversive situations such as fighting (Kaltwasser, 1990; Sales, 1972a), drug withdrawal (Covington III and Miczek, 2003; Vivian et al., 1994), and fear conditioning (Wöhr et al., 2005; Yee et al., 2012) and during states of sexual satiety such as ejaculation (Barfield and Geyer, 1972; Bialy et al., 2016). Fifty-kHz vocalizations (~32–92-kHz) are emitted in rewarding situations in which appetitive or consummatory behaviors are displayed, or during a combination of those behaviors, such as rough and tumble play (Knutson et al., 1998; Webber et al., 2012), receipt of natural and drug rewards (Burgdorf et al., 2001; Thompson et al., 2006; Wright et al., 2010), and sexual interaction (Burgdorf et al., 2008; Sales, 1972b; Thomas and Barfield, 1985).

Vocalizations within the 50-kHz range have been classified into 14 distinct call categories based on temporal continuity, fundamental frequency, and structure (Wright et al., 2010), and certain categories are preferentially associated with specific behaviors (Assini et al., 2013; Laplagne and Elias Costa, 2016; Sirotin et al., 2014). Of the 14 distinct call categories, the trill and flat-trill call subtypes have been positively correlated with reward states, including conditioned approach latency (Burgdorf et al., 2008), CPP (Burgdorf et al., 2008), self-administered playback (Willadsen, Seffer et al., 2014; Wöhr and Schwarting, 2013), and self-administered sucrose and drug reward (Barker et al., 2010; Browning et al., 2011; Ma et al., 2010; Meyer et al., 2012).

Given the general association of 22- and 50-kHz USVs with aversive and reinforcing contexts, respectively, a subset of these calls has been posited as an unconditioned measure of affect (Seffer et al., 2014). Due to the association of these vocalizations with unconditioned affect, both main categories of USVs are suggested to serve distinct roles in socio-affective communication in rats (Seffer et al., 2014). For instance, 50-kHz USVs emission should elicit social approach while 22-kHz USV emission should elicit social withdrawal (Wöhr and Schwarting, 2013). Studies utilizing USV playback and approach tasks have however reported mixed evidence concerning whether the emission of 50-kHz USV

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by male and female rats during sexual interaction is a form of socio-affective communication that facilitates copulation (Agmo and Snoeren, 2015; Barfield et al., 1979; Barfield and Thomas, 1986; McGinnis and Vakulenko, 2003; McIntosh and Barfield, 1980; Seffer et al., 2014; Snoeren and Agmo, 2013; Thomas et al., 1982; White and Barfield, 1987; Willadsen et al., 2014).

Three distinct hypotheses exist based on the data of these studies. One posits that male (but not female) 50-kHz USVs are prosocial and elicit approach behaviors in the female sex partner, which in turn permits the regulation of copulatory behaviors (Barfield and Thomas 1986; Snoeren and Agmo 2013; Willadsen et al., 2014). Another suggests that USVs during copulation are not prosocial because they do not increase approach behavior towards, or influence sexual attractiveness of, a potential mating partner (Agmo and Snoeren 2015; Chu et al. 2017; Snoeren and Agmo 2013; Thomas et al. 1982). The third hypothesis suggests that 50-kHz USVs are indicative of affective and reward states experienced by the individual, but do not necessarily serve as social communication (Knutson et al. 2002). Although variations in the presentation of the conspecific (e.g., free access, behind a wire mesh or removed after minute exposure) may have contributed to mixed reports between these studies, we note that it has yet to be determined whether 50-kHz USVs during sexual behavior indicate arousal, desire, and/or the anticipation of reward.

Although these three hypotheses suggest that female USVs are not prosocial during sexual interaction (i.e., do not influence copulatory events), the measurement of female rat sexual behavior is often contingent on the interaction with the male sex partner and is rarely examined on its own. The ability to examine female rat sexual arousal and reward outside the copulatory context in response to sexual stimulation would offer the ability to evaluate whether female USVs are non-prosocial and are indicative of positive affect. The use of manually applied clitoral stimulation (CLS) that is distributed in time would allow for such assessment. As shown previously, CLS in five-second intervals produces conditioned place preference (CPP) and conditioned approach behavior (Parada et al. 2010; Parada et al. 2011), and increases solicitation frequency and fertility (Cibrian-Llanderal et al. 2010). This technique mimics some aspects of the stimulation received during a sexual encounter with a male conspecific (Pacheco et al. 1989; Pfaff et al. 1977).

In a preliminary study, we established that CLS induces USVs (Pfaus et al. 2016), but we did not examine whether spectrotemporal parameters (e.g., duration, peak frequency, and bandwidth) and temporal properties (e.g., rate and distribution) of USVs were altered, nor did we examine the effect of gonadal hormones such as estradiol and/or progesterone on the type of call stimulated by the CLS. Female vocalization rates increase during peak periods of sexual receptivity in rats (e.g., during proestrus and early estrus), and decrease as estrogen levels decline (Matochik et al. 1992). Cyclic fluctuations in estradiol and progesterone throughout the estrous cycle may, therefore, influence the rate of call emission and potentially other spectrotemporal parameters. The present study examined this question using ovariectomized (OVX) females primed at different times with estradiol benzoate (EB), progesterone (P), or their combination, using a counterbalanced, within-subjects design. It was hypothesized that full hormone priming with EB + P would facilitate the induction of full calls (trills and flat-trills), alter spectrotemporal parameters and increase emission rates during, and or in anticipation of CLS, whereas EB alone would do this to a significantly lesser extent, relative to administration of P alone or the oil vehicle, which should not differ from one another.

2. Methods

2.1. Animals

Eight adult female Long-Evans rats (3–5 months, 250–400 g, Charles River, St- Constant, QC, Canada) were used. The female rats were sexually and drug-naïve but had prior CLS experience. Animals were pair-housed in a temperature and humidity-controlled colony room with a 12:12 h light/dark cycle (lights on at 20:00) with access to standard laboratory chow (Charles River #5075, Montreal, Canada) and water available ad libitum. All experimental procedures were approved by the Concordia University Animal Research Ethics Committee in Montreal, Canada and conformed to the Canadian Council on Animal Care guidelines.

2.2. Ovariectomy

All females were ovariectomized (OVX) under general anesthesia, which was induced with 4:3 mixture of ketamine hydrochloride (50 mg/mL; Ketaset©, Wyeth Canada) and xylazine hydrochloride (4 mg/mL; Rompum©, Bayer Healthcare), which was injected intraperitoneally with a final volume of 1 mL/kg per body weight. When females were unresponsive to a foot pinch, ocular ointment (Naturel Tears©, Alcon) was applied and bilateral ovariectomy (OVX) was performed via lumbar incision as described in Steele and Bennett (2011). Postoperative care consisted of the following subcutaneous (SC) injections, given 4 and 24 h after surgery: 0.2 mL penicillin G (Pen G, antibiotic), 2.5 mg/kg of body weight/mL flunixin meglumine (Banamine©, an anti-inflammatory, analgesic, and antipyretic), 0.02 mL ketoprofen (Anafen©, an anti-inflammatory and analgesic) and 2 mL of saline. Additional injections of PenG, Anafen, and saline were administered the following day after surgery to prevent infection and to manage pain and hydration. Rats were allowed to recover for one week before hormone priming and testing began.

2.3. Ovarian hormones

Estradiol benzoate (EB;10 μg) and progesterone (P; 500 μg) were dissolved in reagent-grade sesame oil (SigmaAldrich, Canada, Lot # MKBR2026V), to yield a concentration of 10 μg of EB per 0.1 mL of solution, and of 500 μg progesterone per 0.1 mL of solution. Hormones were injected subcutaneously in a constant volume of 0.1 mL per animal. The control vehicle consisted of sesame oil of an equal volume. Dosages were based on previous studies conducted in our lab that reliably induced female sexual receptivity (Jones et al., 2013; Parada et al. 2010; Parada et al. 2011). Steroid hormones, EB and P, were purchased from Steraloids INC (Newport, RI USA, Batch: B0281).

2.4. Apparatus and clitoral stimulation

Clitoral stimulation recordings took place in a transparent open-topped Plexiglas chamber (38 × 60 × 38 cm) lined with a bottom steel wire grid and beta chips. Two openings (13.5 × 13.5 cm) on either side of the front wall of the Plexiglas chamber allowed for experimenter access for CLS application. Experimenter-delivered CLS consisted of lifting the base of the tail and then lightly brushing the clitoris using a DeSerres number 4 synthetic fiber paintbrush dabbed with K-Y® Jelly, a water-soluble and non-toxic lubricant. Use of K-Y® Jelly was to enhance CLS and minimizing potential discomfort during CLS application. Stimulation was applied as quick three down strokes approximately every 5 s, during a one-minute period. This method and stimulation frequency has previously been shown within our laboratory to induce a CPP (Parada et al. 2010) and conditioned partner preference (Parada et al. 2011).

2.5. Experimental procedure

Eight female rats with previous CLS experience (3 stimulation session in total, each lasting 5 min with 4 days between each session) were tested in a fully counterbalanced within-subject design. As a control for carry over effects, treatment order was counterbalanced using a Williams design. Females received either EB + P, EB alone, P alone, and

oil vehicle. To mimic rises in plasma hormonal levels that occur during the estrous cycle, females receiving EB + P were injected subcutaneously with EB + P, 48 h and 4 h before testing, respectively (Albert et al. 1991; Boling and Blandau 1939; Hardy and DeBold 1972; Whalen 1974). Females receiving EB alone were injected 48 h prior to testing whereas females receiving P alone were injected 4 h prior. Finally, when tested in the oil condition females received oil 48 h and 4 h before testing. Each test day was separated by a 9-day washout period to eliminate potential carry-over effects of previous treatments (Kow and Pfaff, 1973). Recording sessions consisted of a 4-minute period where the rat was left in the chamber without experimenter manipulations (i.e., inter-CLS interval), followed by 1 min of CLS, and this was repeated for 7 cycles for total session duration of 35 min. The length of the Inter-CLS interval was to ensure that female rats would adequately return to baseline level of sexual excitability (i.e., heightened locomotor activity in the anticipation to sexual stimulation; Pfaus et al. 2001).

2.6. Analysis and classification of USVs

A condenser ultrasound microphone (CM16/CMPA, Avisoft Bioacoustic, Berlin, Germany) was manually secured in the center of the long wall of the chamber with a microphone holder above the cage. The microphone was positioned 15–30 cm away from rats during recording. The positioning of the microphone was tested before recording utilizing a Batty Ultrasound Generator (Goffin, 2012), a simple circuit that emits ultrasonic chirps, to ensure that vocalizations would be captured from all angles. Signals from the microphone were fed into an UltraSoundGate 416H data acquisition device (Avisoft Bioacoustics) and recorded with a sampling rate of 250-kHz and a 16-bit resolution.

Acoustical analysis of rat USVs was performed using Avisoft SASLab Pro (version 4.2, Avisoft Bioacoustics). A fast Fourier transform length of 512 points with an overlap of 75% (FlatTop window, 100% frame size) was used to generate the spectrograms, which had a frequency resolution of 490 Hz and a time resolution of 0.5 ms. An investigator, who was blind to the hormonal treatments of the subjects, manually selected and labeled calls from these spectrograms for classification purposes. Each call had to meet several spectrographic criteria: temporal continuity (i.e., maximal intra-call interruption of 17 ms), fundamental frequency (i.e., 20- to 90-kHz), and intensity (i.e., distinct from background noise). The classification of identified 50-kHz calls was based on the syllabic composition of the trills and flat-trill combination categories (Wright et al. 2010).

2.7. Call parameter measurements

Acoustic properties of duration, bandwidth, and peak frequency of each trill and flat-trill calls were measured by an automatic feature of the Avisoft SASLab Pro software. The accuracy of these automatic measurements was improved by setting a threshold of −50 dB ("Reject if peak amplitude < ") and by manually erasing background noise that overlapped with sound elements from each spectrogram. Sound elements that were overlaid by background noise were excluded from parameter analysis.

Bandwidth was calculated as the difference between the maximum and minimum spectrum of the entire element whereas peak frequency was the average of these elements provide by this automatic feature. Call rate was calculated by dividing the total number of calls for each subtype per recording block by overall duration of each recording block in minutes. For CLS recording blocks, call distribution was calculated by transforming start and end times of each call to a value between 0 to 60 s to correspond to the duration of the CLS recording block. For inter-CLS, each call start and end time was transformed to 0 to 240 s to correspond to the duration of the inter-CLS interval. Time across CLS and inter-CLS intervals were made into 50-time bins.

2.8. Statistical analysis

Spectrotemporal data were analyzed using R software version 3.4.4 (R Development Core Team, 2018) through RStudio: Integrated Development Environment for R (RStudio Team, 2016, version 1.1.383). All missing data cases were omitted from subsequent analyses. To remedy distributive skew in the call parameters of duration, peak frequency, and bandwidth, these parameters underwent log10 transformations. Although significance tests using the mixed linear models were conducted on log transformed data, we refer to the estimated means in raw units throughout the results section.

The same model structure was used to test for the interaction effect of Hormonal Condition and Recording Block on the parameters of interest for trill and flat-trill calls. We specified our model in the following manner: 1) the interaction of Hormonal Condition and Recording Block as a fixed factor. 2) as a random effect we specified the crossed effects of hormonal condition across subjects with uncorrelated random intercepts and slopes. This was to consider variability in response to hormonal treatment. Recording block was not entered into the model as a random effect, as it has been suggested that the inclusion of a factor with two levels in the random effects' structure results in an overfitting of the model (Scheipl and Bolker 2016). Variance components and maximum likelihood were implemented in the linear mixed model as repeated covariance structure and parameter estimation. The mixed linear model was fitted using Analysis of Factorial Experiments (afex) package (Singmann et al. 2018). The mixed linear model fit was calculated using Maximum Likelihood Estimation and null hypothesis significance testing of the model was conducted with a modified F-test using Satterthwaite's approximation. The data and R-code for all analyses will be available at osf.io.

To examine the effects of the previously mentioned factors on trill and flat-trill call rates, two repeated measures ANOVA was used, with hormonal treatment and CLS recording blocks, and their interaction as within-subject factors. The dependent variable in this analysis was calls made per minute of recording block (call rate). The within-subject ANOVAs were conducted using the afex package (Singmann et al. 2018). Effect sizes for the repeated measure ANOVAs main effects and interactions were calculated using generalized eta squared. Generalized eta squared has been proposed to offer greater generalizability compared to partial eta squared (Olejnik and Algina 2003). The data were visualized using ggplot2: Elegant Graphics for Data Analysis (Wickham 2016).

Testing of interactions were performed with simple contrasts through statistical packages emmeans: Estimated Marginal Means, aka Least-Squares Means (Lenth et al. 2018). Estimated marginal means for simple contrasts were calculated using an asymptotic correction for degrees of freedom (Singmann and Kellen 2017). To control for type 1 errors, multiple comparisons were adjusted for using the Holm adjustment (Aickin and Gensler 1996). We conducted eight simple contrasts of interest, which can be found in Table 4. Effect sizes of these contrasts (Hedge's G_{avg}) were calculated using the supplementary material of Lakens (2013).

3. Results

3.1. Trills

For the trills, the combination of EB + P and distributed CLS increased the duration and rate, lowered the peak frequency, and widened the bandwidth of the call subtype. The distribution of the number of trills across individual CLS recording blocks was high and constant. This was based on comparisons to the inter-CLS interval alone and/or in combination with other hormonal treatments, which did not significantly influence spectrotemporal parameters and temporal properties of trills. During individual inter-CLS intervals, the number of trills decreased post-CLS recording block followed by an increased

Table 1
Estimated marginal means for the spectrotemporal parameters and call rates of trill and flat-trill calls.

Call subtype	Recording block	Hormonal treatment	Duration (ms)	Peak frequency (kHz)	Bandwidth (kHz)	Call rate
			EMM	*EMM*	*EMM*	*EMM*
Trill	CLS					
		EB + P	59.94	56.48	24.97	158.29
		EB alone	53.49	55.76	17.52	62.29
		P alone	47.85	57.72	17.88	34.43
		Oil vehicle	45.63	55.43	12.41	35.86
	Inter-CLS interval					
		EB + P	42.26	58.36	17.95	125.43
		EB alone	42.99	56.65	12.11	53.29
		P alone	40.6	57.63	13.58	44.71
		Oil vehicle	36.41	55.24	4.85	41.57
Flat-trill	CLS					
		EB + P	101.36	55.1	32.38	122.25
		EB alone	103.15	55.32	25.66	44
		P alone	95.27	57.62	27	11.25
		Oil vehicle	79.45	55.82	22.54	10.25
	Inter-CLS Interval					
		EB + P	60.59	56.03	24.34	31.88
		EB alone	64.62	54.78	19.91	16.88
		P alone	59.56	53.44	19.49	8.13
		Oil vehicle	56.67	55.34	15.67	3

number of trills before the next CLS record block.

3.1.1. Overall effects

There was a significant interaction of Hormonal Condition and Recording Block on trill duration (F (3, 1870) = 6.97, p = 0.0001), trill peak frequency (F (3, 310) = 4.62, p = 0.004), and trill bandwidth (F (3, 3024) = 5.46, p = 0.001), as shown in Table 2. Estimated marginal means (EMM) of trill spectrotemporal parameters and call rate are summarized in Table 1. Simple contrasts were conducted on the estimated marginal means of the Hormonal Condition x Recording block groups for each spectrotemporal parameter.

For the call rate of trill calls, a repeated measures ANOVA revealed a large and significant main effect of Hormonal Condition, F (1.86, 11.17) = 4.81, p = 0.03, $\eta^2_{Generalized}$ = 0.26. The assumption of sphericity as indicated by the Mauchly's Test of Sphericity was violated for hormonal treatment, Ɛ (3) = 0.889, $p < 0.018$, thereby a Greenhouse-Geiser correction was used.

3.1.2. Duration

As shown in Fig. 1A, when treated with EB + P females emitted significantly longer trills during the CLS recording block (*EMM* = 59.944, *SE* = 1.907), compared to the inter-CLS interval (*EMM* = 42.261, *SE* = 2.025), p = 0.01, g = 0.79. Trills were significantly longer during the CLS recording block when females were treated with EB + P, compared to treatment with P (*EMM* = 47.853, *SE* = 3.017), p = 0.0001, g = 0.61, and Oil (*EMM* = 45.634, *SE* = 3.173), p = 0.0035, g = 0.86. There were no significant differences in the mean trill duration during the inter-CLS interval when comparing EB + P to the EB, P, and Oil treatments.

3.1.3. Peak frequency

When females were treated with EB + P, trills were lower in peak frequency during the CLS recording block (*EMM* = 56.48, SE = 2.618) when compared to those emitted during the inter-CLS interval (*EMM* = 58.36, SE = 2.622), $p < 0.0001$, g = 0.2, as shown in Fig. 1C. Mean trill peak frequency did not significantly differ between CLS and Inter-CLS Intervals when females received EB + P compared to other hormonal treatments.

3.1.4. Bandwidth

Mean trill bandwidth, as shown in Fig. 1E, was wider during the CLS recording block (*EMM* = 24.947, SE = 5.479) than during the Inter-CLS interval (*EMM* = 17.947, SE = 5.485) when females were treated with EB + P, $p < 0.0001$, g = 0.84. Mean trill bandwidth was also wider during the CLS recording block (*EMM* = 17.525, *SE* = 0.969) compared to the inter-CLS interval (*EMM* = 12.113, *SE* = 0.9811) when females received EB alone, $p < 0.0001$, g = 1.73.

3.1.5. Call rate

The call rate of trill subtype, as shown in Fig. 2A, significantly increased during the CLS recording block when females were treated with EB + P (*EMM* = 158.286, *SE* = 28.886) compared to P alone (*EMM* = 34.428, *SE* = 28.886, p = 0.009, g = 1.34) and oil treatment (*EMM* = 35.857, SE = 28.886, p = 0.009, g = 1.33), but not EB alone (*EMM* = 62.286, SE = 28.886, p = 0.051). Trill call rate did not significantly differ between hormonal treatments during the inter-CLS intervals. Trill call rate did not significantly differ between the CLS and inter-CLS intervals.

3.1.6. Call distribution

As shown in Fig. 2D, when females were treated with EB + P the number of trill calls remained high and constant across the duration of the CLS recording block. Over the CLS recording block, when females received EB alone, trills decreased from approximately 60 to 10 calls. From the start of the inter-CLS interval until the 75 s, as shown in Fig. 2C, calls decreased from approximately 80 to 15 calls with EB + P treatment then moderately increased to 40 calls 125 s and 50 s prior to the next CLS recording block. Decreases in trill calls post CLS with increase calling prior to CLS was also demonstrated with EB alone treatment but with a small number of calls emitted. Trill calls were low and constant, 20 to 10, across CLS and inter-CLS interval with P alone and oil vehicle treatment.

3.2. Flat-trills

For flat-trills, results for spectrotemporal parameter and temporal properties resemble those found with trills except for call duration. Call duration of flat-trills was found to be influenced by distributed CLS alone rather than the combination of EB + P treatment and distributed CLS as demonstrated by trills.

3.2.1. Overall effects

There was a significant interaction effect of Hormonal Condition and Recording Block on flat-trill subtype calls. As shown in Table 2, the

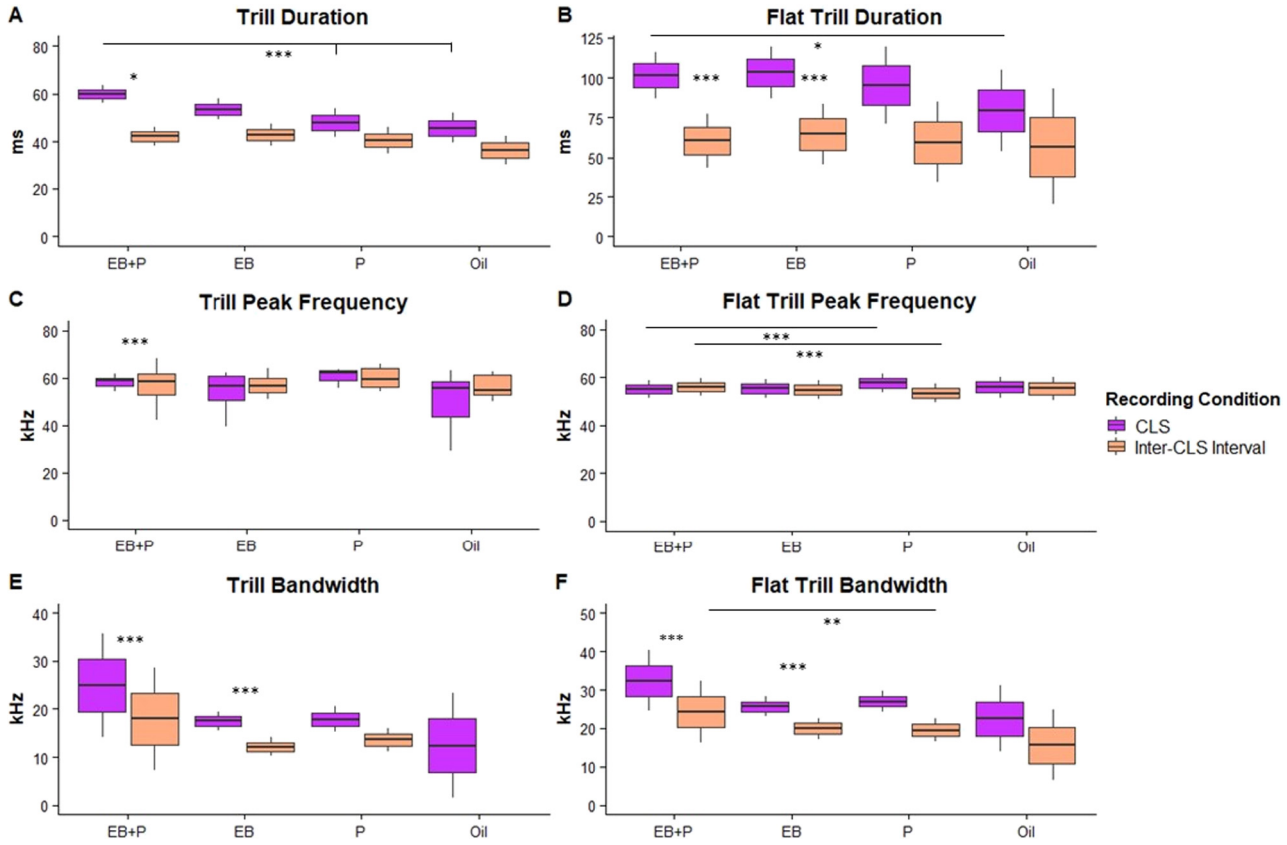

Fig. 1. Boxplots with the midline representing the estimated marginal means from the mixed linear models. The boxes represent ± 1 SEM, and the whiskers the 95% CIs of the estimated means. * < 0.05, ** < 0.01, *** < 0.005.

mixed linear model for flat- trill subtype calls revealed significant interaction effect on trill peak frequency (F (3, 1864) = 9.12, $p < 0.0001$), and flat-trill bandwidth (F(3, 1067) = 5.48, $p = 0.001$), but not on flat-trill duration (F (3, 803) = 0.30, $p = 0.83$). The mixed linear model revealed a significant main effect of Recording Block on flat-trill duration, (F (1, 1368) = 54.77, $p < 0.0001$). Estimated marginal means of trill spectrotemporal parameters and call rate are also summarized in Table 1. Complex contrasts were also conducted on the estimated marginal means of the Hormonal Condition × Recording block groups for each spectrotemporal parameter of flat-trill subtype calls (Table 3).

A repeated measures ANOVA for flat-trill call rate revealed a significant interaction effects of Hormonal Condition and Recording Block, F (1.19, 8.30) = 8.13, $p < 0.02$, $\eta^2_{Genaralized} = 0.12$. Again, the assumption of sphericity as indicated by the Mauchly's Test of Sphericity was violated for Recording Block x Hormonal Condition, Ɛ (3) = 0.395, $p = 0.018$, thereby a Greenhouse-Geiser correction was used.

3.2.2. Duration

When females were treated with EB + P, flat-trill duration was significantly longer during the CLS recording block (EMM = 101.361, SE = 7.499) compared to the inter-CLS interval (EMM = 60.594, SE = 8.657), $p < 0.0001$, $g = 1.90$, as shown in Fig. 1B. Flat-trill duration was significantly longer during the CLS recording (EMM = 103.148, SE = 8.316) than the inter-CLS interval (EMM = 64.623, SE = 9.672) when females received EB alone, $p < 0.0001$, $g = 1.62$. During CLS block recording, flat-trill duration was significantly longer when females received EB + P compared to oil treatment (EMM = 79.453, SE = 12.894, $p = 0.025$, $g = 1$), but not EB alone and P alone. Flat-trill duration did not significantly differ during the inter-CLS interval when comparing EB + P to EB alone and P alone treatments.

3.2.3. Peak frequency

As shown in Fig. 1D, when females were treated EB + P (EMM = 55.104, SE = 1.920), mean flat-trill was significantly lower during the CLS recording block than when females received P alone treatment, (EMM = 57.619, SE = 1.973), $p = 0.005$, $g = 0.32$. Mean flat-trill peak frequency was significantly higher during the inter-CLS interval when females received EB + P (EMM = 56.026, SE = 1.943) compared to P alone treatment, (EMM = 53.442, SE = 1.997), $p = 0.009$, $g = 0.43$. When compared to other hormonal treatments, females treated with EB + P in the both CLS and inter-CLS intervals, peak frequency did not significantly differ to other hormonal treatment groups.

3.2.4. Bandwidth

The mean bandwidth was significantly wider during the CLS recording block (EMM = 32.375, SE = 4.041) than during the inter-CLS interval (EMM = 24.342, SE = 4.086) when females were treated with EB + P, $p < 0.0001$, $g = 1.14$. When females received EB alone, the bandwidth of flat-trills was wider during the CLS recording block (EMM = 25.653, SE = 1.285) compared to the inter-CLS interval, (EMM = 19.908, SE = 1.424), $p < 0.0001$, $g = 0.8$. During the inter-CLS interval, the bandwidth of flat-trills was wider when females who received EB + P compared to P alone treatment (EMM = 19.49, SE = 1.521), $p = 0.009$, $g = 1.4$, but not EB alone and oil. There were no significant differences in flat-trill bandwidth during the CLS recording block when comparing EB + P to the EB alone and Oil treatments.

3.2.5. Call rate

Call rate was significantly higher in females who receiving EB + P during the CLS recording block (EMM = 122.25, SE = 17.95) compared to the inter-CLS interval (EMM = 31.88, SE = 17.95), $p = 0.0003$,

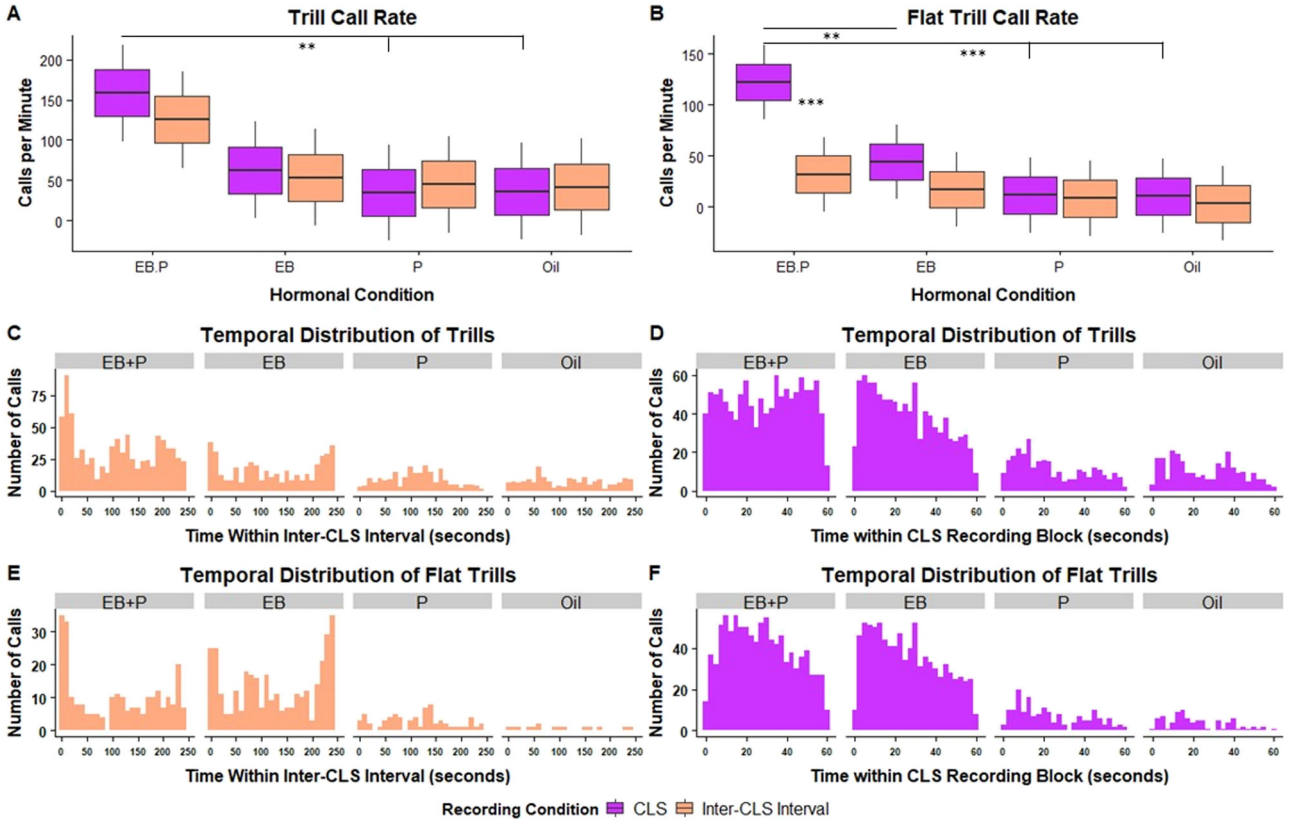

Fig. 2. Figs. A–B midlines in boxplot show the estimated marginal mean, boxes demonstrate ± 1 SEM, and the whiskers display the 95% Cis around the mean. A) demonstrates the trill call rate with no interaction between Hormonal Condition and recording block B) flat trill call rate is highest in females treated with EB + P during the CLS recording block. Figs. C–F show the temporal distribution of calls during recording block times. C) and E) shows the temporal distribution of trills and flat-trills during the inter-CLS interval. Call rate is highest during the first 60s the start of the Inter-CLS interval which is the first minute post-CLS stimulation, particularly in the EB + P and EB groups. D) and F) show the temporal distribution of trills and flat-trills during the CLS recording blocks. The frequency of calls appears to be equally distributed across the blocks, regardless of hormonal condition, compared to the Inter-CLS intervals. * < 0.05, ** < 0.01, *** < 0.005.

$g = 1.58$. Flat-trill call rate was significantly higher during the CLS recording block in females treated with EB + P compared to females treated with EB (*EMM* = 44, *SE* = 17.95), $p = 0.003$, $g = 1.37$. The call rate during the CLS recording block was also significantly higher than both P treatment (*EMM* = 11.25, *SE* = 17.95), $p < 0.0001$, $g = 1.94$, and Oil treatment (*EMM* = 10.25, *SE* = 10.25), $p < 0.0001$, $g = 1.96$.

3.2.6. Call distribution

Flat-trill calls were high and constant throughout the CLS recording block with EB + P treatment, as shown in Fig. 2F. When females were treated with EB alone, flat-trill calls decreased from approximately 55 to 10 calls over the duration of the CLS recording block. As shown in Fig. 2E, when females were treated with EB + P, flat-trill calls from the

Table 2
Model statistics for spectrotemporal parameters of trill subtype calls.

Model statistics	Trills												
	Duration			Peak frequency			Bandwidth			Call rate			
	F	df_{error}	*p*-Value	F	df_{error}	*p*-Value	F	df_{error}	*p*-Value	F	df_{error}	*p*-Value	$\eta^2_{Generalized}$
Recording block	88.92	2618.8	< 0.0001	7.09	219.24	0.008	251.67	3378.84	< 0.0001	0.3	6	0.61	0.002
Hormonal treatment	6.44	2.55	0.1	4.56	2	0.18	1.7	6.26	0.26	4.81	11.17	0.03	0.26
Hormonal treatment × recording block	6.97	1868.92	0.0001	4.62	310.12	0.004	5.46	3023.5	0.001	1.91	10.07	0.2	0.01

Fixed effects	Trills								
	Estimate	*t*-Value	*p*-Value	β	*t*-Value	*p*-Value	β	*t*-Value	*p*-Value
Intercept	1.563	39.29	< 0.0001	1.748	120.88	120.88	1.0320	42.02	< 0.0001
Recording block (CLS vs. no CLS)	0.042	9.43	< 0.0001	−0.003	−2.66	0.008	0.1071	15.865	< 0.0001
EB + P vs. EB alone	0.035	3.71	0.08	0.008	0.49	0.64	0.1117	1.37	0.21
EB + P vs. P alone	0.023	2.61	0.11	−0.009	−1.13	0.44	−0.0064	−0.3	0.75
EB + P vs. Oil vehicle	−0.005	−0.27	0.79	0.008	3.5	0.54	0.0536	1.73	0.14
EB alone × recording block	0.026	4.53	< 0.0001	−0.005	−3.64	0.0003	0.0113	1.29	0.2
P alone × recording block	−0.004	−0.55	0.58	−0.001	−36	0.72	−0.0197	−1.77	0.08
Oil vehicle × recording block	−0.017	−1.96	0.049	0.003	1.52	0.13	−0.0346	−2.58	0.01

Table 3
Model statistics for spectrotemporal parameters of flat-trill subtype calls.

Model statistics	Flat trills												
	Duration			Peak frequency			Bandwidth			Call rate			
	F	df_{error}	*p*-Value	F	df_{error}	*p*-Value	F	df_{error}	*p*-Value	F	df_{error}	*p*-Value	$\eta^2_{Generalized}$
Recording block	54.72	1367.98	< 0.0001	6.9	1921.64	0.009	118.97	1349.76	< 0.0001	6.27	7	0.04	0.1
Hormonal treatment	3	8.94	0.09	0.11	10.69	0.95	2.21	10.11	0.15	6.51	8.42	0.03	0.26
Hormonal treatment × recording block	0.3	803.33	0.83	9.12	1863.99	< 0.0001	5.48	1067.16	0.001	8.13	8.3	0.02	0.12

Fixed effects	Flat trills								
	β	*t*-Value	*p*-Value	β	*t*-Value	*p*-Value	β	*t*-Value	*p*-Value
Intercept	1.796	67.53	< 0.0001	1.740	112.498	< 0.0001	1.301	98.95	< 0.0001
Recording block (CLS vs. no CLS)	0.075	7.4	< 0.0001	0.005	2.62	0.008	0.088	10.91	< 0.0001
EB + P vs. EB alone	0.038	2.77	0.006	0.001	0.296	0.78	0.094	2.426	0.051
EB + P vs. P alone	0.035	1.93	0.09	−0.003	−0.46	0.66	−0.027	−0.65	0.53
EB + P vs. Oil vehicle	−0.012	−0.4	0.71	0.000	0.03	0.97	−0.017	−0.49	0.64
EB alone × recording block	0.001	0.64	0.52	−0.008	−4.08	< 0.0001	−0.019	−1.95	0.051
P alone × recording block	0.001	0.05	0.96	−0.002	−0.95	0.34	−0.034	−3.16	0.002
Oil vehicle × recording block	0.010	0.52	0.61	0.013	3.96	< 0.0001	0.053	3.29	0.001

Table 4
Post Hoc comparisons of hormonal condition × recording block groups. *p*-Values shown have undergone the holm adjustment.

	Duration	Peak frequency	Bandwidth	Call rate
Trills				
EB + P: CLS vs inter-CLS interval	0.012	< 0.0001	< 0.0001	0.14
EB: CLS vs inter-CLS interval	0.37	0.61	< 0.0001	0.6
CLS block: EB + P vs EB	0.08	1	0.41	0.051
CLS block: EB + P vs P	< 0.0001	1	0.74	0.009
CLS block: EB + P vs oil	0.0035	1	0.6	0.009
Inter-CLS interval: EB + P vs EB	0.37	1	0.74	0.12
Inter-CLS interval: EB + P vs P	0.36	1	0.89	0.1
Inter-CLS interval: EB + P vs oil	0.08	1	0.41	0.1
Flat trills				
EB + P: CLS vs inter-CLS interval	< 0.0001	0.08	< 0.0001	0.0003
EB: CLS vs inter-CLS interval	< 0.0001	1	< 0.0001	0.56
CLS block: EB + P vs EB	1	1	0.07	0.003
CLS block: EB + P vs P	1	0.0048	0.47	< 0.0001
CLS block: EB + P vs oil	0.03	1	0.37	< 0.0001
Inter-CLS interval: EB + P vs EB	1	0.98	0.28	0.56
Inter-CLS interval: EB + P vs P	1	0.02	0.009	0.56
Inter-CLS interval: EB + P vs oil	1	1	0.31	0.56

start of the inter-CLS interval until 85 s, decreased from approximately 55 to 15. Flat-trills also demonstrated with EB alone treatment post CLS decreases followed by pre CLS increase with a smaller number of emitted calls. Across the CLS and inter-CLS interval, flat-trill calls were low and constant when females received P alone and oil vehicle treatment.

4. Discussion

The present study examined whether distributed CLS could influence spectrotemporal parameters and rate of 50-kHz emission, and whether CLS-specific alterations were altered by different steroid hormone priming. Trills and flat-trills were the focus of our analysis because these calls are suggested to be indicative of a reward state (see introduction). Comparison of the CLS and the Inter-CLS interval showed that distributed CLS altered the spectrotemporal parameters and increased the call rate of both call subtypes. The influence of distributed CLS was also hormonally dependent, as EB + P treatment significantly increased USV emission during CLS recording blocks. When females were administered EB + P, distributed CLS increased both trill duration and call rate, and widened the bandwidth, but did not influence peak frequency. Flat-trill calls showed a similar pattern as trills except for duration, which had a main effect of CLS block alone. Unlike spectrotemporal parameters, call rates of both call subtypes increased in the EB + P relative to the oil vehicle condition during the CLS block only. The call distribution of trills and flat-trills across individual inter-CLS intervals showed that after the CLS recording block trills and flat-trills decreased steadily but increased before the next CLS block (Fig. 2C and D). Compared to inter-CLS intervals, call distribution of trills and flat-trills in the CLS recording block appeared constant across time (Fig. 2E and F). We speculate that differences in call distribution patterns across time in each recording block suggest that calls during the Inter-CLS interval are anticipatory, whereas calls during the CLS recording block are reflective of consummatory sexual reward.

Prior studies have shown 50-kHz USVs to be hormonally dependent in natural cycling and OVX female rats. Cyclic fluctuations of ovarian hormones influence the rate of 50-kHz vocalization (Matochik et al., 1992), and sufficient hormone priming is necessary to increase 50-kHz vocalizations during copulation with devocalized males (Thomas and Barfield 1985). In the latter study, female vocalizations during mating with a devocalized male partner consisted of flat, trill, flat trill, and composite call category subtypes, which varied widely in their frequency patterns and duration. Although our experiment did not include flat and composite call subtypes, and utilized a paintbrush rather than a

devocalized sexually vigorous male partner, distributed CLS elicited trills and flat-trill calls that varied in duration and rate of call emission in OVX female rats primed with EB + P. Specifically, trills and flat-trill calls were significantly longer and more frequent when females were primed fully. The present experiment is therefore consistent with previous studies showing that 50-kHz vocalizations of females during paced copulation are dependent on ovarian hormones. Furthermore, distributed stimulation of the external clitoral glans applied in a manner that mimics the downward pelvic thrusts of males during mounts with or without intromission (Pfaff et al. 1977) induces a sexual reward state similar to that induced by paced copulation. The size and hemodynamic function of the vagina and clitoris decrease after OVX, and increase with subsequent estradiol treatment (Comeglio et al. 2016; Korenchevsky and Hall 1937). If clitoral sensitivity is altered accordingly, then it follows that brain activation by CLS (e.g., Marson 1995; Parada et al. 2010) should be altered following different hormone priming regimens or across the estrous cycle. However, it is not yet known if a similar pattern of CLS-specific vocalizations occurs in gonadally-intact females during proestrus. Because OVX females experienced CLS during peak sexual receptivity via EB + P, it is possible that CLS-specific vocalizations vary across the estrous cycle and are influenced by the natural hormonal state the female is in when she first experiences CLS.

The present results were also in align with previous findings of our group, who reported that distributed CLS is rewarding for female rats based on the induction of conditioned place and partner preference (Parada et al. 2010; Parada et al. 2011). In the present study, distributed CLS elicited call subtypes that are associated with rewarding contexts, and indeed most of these calls were more frequent within CLS recording blocks. Parada et al. (2013) showed previously that recall of the CLS-induced reward state did not require priming with EB + P, as rats treated with the oil vehicle on the final test day showed a significant CPP. However, in that study, partial extinction of CPP (induced subsequently in conditioned females by placing them into the preferred compartment of the CPP box but without prior CLS) occurred only in females primed with EB + P, suggesting that the full hormone priming condition also augmented the expectation for CLS as a reward. In the present study, call parameters were modulated the most by CLS when females were treated with EB + P. It is not surprising that in response to a sexual stimulus this effect is consistent with the hormonal activation required for the full expression of female appetitive and consummatory behaviors. Thus, we suggest that the calls observed in the present study are a useful index of a sexual reward state in the female rat. Currently, conditioned place preference and sexually conditioned partner preference are used to assess sexual reward states. These paradigms are dependent on the memory of prior experiences of the animal and can be labor and time intensive as numerous learning trials are required. If trills and flat trills related to distribute CLS are indeed indicative of sexual reward, then CLS-specific USVs offer a real-time in-vivo method of assessing sexual reward in the female rat from the first sexual experience onward.

Gonadal hormones and their metabolites also influence the spectrotemporal parameters and call rate of courtship USVs in gonadally intact male rats (Chen et al. 2017; Fernandez-Vargas 2017; Floody et al. 1979; Parrott 1976). Administration of T alone, EB alone, or quinestrol (the active, 3-cyclopentyl ether of ethinyl estradiol) to gonadally intact male rats decreases the secretion of gonadotropin releasing hormone via negative feedback and decrease the duration of courtship USVs but not bandwidth or peak frequency (Chen et al. 2017). Call duration decreases in response to EB alone treatment of male rats was also reported by Fernandez-Vargas (2017). In contrast, EB treatment to OVX rats in the present experiment increased the duration and the call rate of USVs. Although this could suggest a potential sex difference in the effect of gonadal hormones on sexual USVs, we believe it is more likely that, in OVX rats, EB does not induce negative feedback on gonadotropin secretion but rather stimulates mechanisms of sexual arousal and desire at both central and peripheral levels. However, comparable to the findings of Fernandez-Vargas (2017) and Chen et al. (2017), hormonal condition alone also did not significantly influence bandwidth and peak frequency in our study. It remains to be determined whether CLS-specific vocalizations in females are comparable to courtship-related USVs, or whether they represent only an expectation to CLS reward. This would require a better understanding of call profiles (i.e., changes in the proportion of call subtypes) associated with CLS both across the estrous cycle and as a function of sexual experience.

5. Conclusion

The present results show that distributed CLS induces vocalization subtypes that have been associated with reward. Although distributed CLS was shown previously to induce reward states and, based on our results, elicit reward call subtypes, it is too early to determine which USVs specific to distributed CLS are indicative of sexual reward. If future evidence supports such an association between CLS specific USVs and sexual reward states, these calls may be utilized as a subjective measure of female sexual reward. The results of this experiment are consistent with the overall finding that gonadal hormones play an important role in modulating temporal properties of USVs, and it is also the first study to demonstrate without the context of a courtship procedure that gonadal hormones modulate 50-kHz USVs in OVX female rats. The technique of distributed CLS thus offers a unique method for investigating female sexual reward outside of copulatory interaction.

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